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Best therapeutic practices for the use of antibacterial agents in finfish aquaculture: a particular view on European seabass (Dicentrarchus labrax) and gilthead seabream (Sparus aurata) in Mediterranean aquaculture

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Abstract

Antibacterial therapy is still in many cases the only way to control bacterial disease outbreaks, with relevant economic issues. Nevertheless, this necessity should also be well balanced with other relevant aspects such as suitability, efficacy and refinement of the treatments but also with consumer and environmental welfare. With this aim, the literature pertaining to the use of antibacterials (i.e. oxytetracycline, oxolinic acid, flumequine and potentiated sulphonamides) in Mediterranean farmed European seabass (*Dicentrarchus labrax*) and gilthead seabream (*Sparus aurata*) was reviewed and addressed. Knowledge of drug pharmacokinetics along with the related legislation is also presented. The main criteria, technical aspects and constraints affecting the design of an appropriate antibacterial therapy are also discussed. An evaluation of available bibliography revealed the existence of considerable information on several registered antibacterials, while it is limited for others. Typically, minimum inhibitory concentrations (MIC) have been used as a reference for antibacterial selection. However, the methodologies used for MIC assessment require refinement and more sophisticated data such as epidemiological cut-off breakpoint values. Due to the characteristics of farming systems, antibacterials are mostly delivered through medicated feeds. The large number of production units and number of fish per unit, together with a limited timeframe margin for efficient therapy, makes Mediterranean gilthead seabream and European seabass, one of the best examples where the metaphylactic concept has to be considered in aquatic medicine. The information presented in this review should guide future action taken to fulfil research gaps and promote effective and prudent antibacterial practices.

Key words: European seabass, gilthead seabream, bacterial diseases, antibacterials, therapy, MIC.

Introduction

General aspects of Mediterranean aquaculture

Freshwater fish farming in the Mediterranean region began many centuries ago, while modern marine Mediterranean fish farming has only been practised effectively over the last four decades. As in several parts of the world, finfish production in the Mediterranean area has grown rapidly (Massa *et al*. 2017). Since its beginning, Mediterranean fish production has been dominated by two species, the European seabass (*Dicentrarchus labrax* Linnaeus, 1758) and the gilthead seabream (*Sparus aurata* Linnaeus, 1758), which accounted for more than 300,000 mt in 2016, representing more than 90% of Mediterranean fish production (FEAP 2017). Current farming of these two species involves facilities where large numbers of fish are kept together for a relatively long period until commercial weight is achieved. Millions of larvae and juveniles are reared for few months in hatcheries, land-based nurseries, pre-on-growing systems, open flow-through and recirculation systems, before

being transferred to floating cages mainly or other intensive land-based growing facilities. The number of fish reared in each production unit (tank/cage/pond) is particularly high (around 100,000–500,000 fish per unit) and the standard production cycle is quite long (12–15 months). These two facts exert high pressure on fish stocks, involving a wide spectrum of stressors during their life under farming conditions. At the same time, there is a risk of disease outbreaks especially in cage farming where fish biomass is relatively high. Infectious diseases in caged fish may become a substantial problem not only due to mortality, decrease of fish performance and therapeutic costs but also due to depreciation of product value and welfare issues. Among the potential disease agents, bacterial pathogens are of the most frequently diagnosed, with European seabass being more susceptible compared with gilthead seabream (Table 1).

Overview of the main bacterial diseases and conventional health management of European seabass and gilthead seabream in Mediterranean aquaculture

In Mediterranean aquaculture, the dominant bacterial pathogens in European seabass and gilthead seabream production since the 80s have been traditionally *Vibrio anguillarum* and *Photobacterium damselae* subsp. *piscicida* and later *Tenacibaculum maritimum*, *V. harveyi* and *V. alginolyticus* (Colorni & Padros 2011). More recently, the primary pathogenic potential of *Aeromonas veronii* bv. *sobria* (Smyrli *et al*. 2017) and of new extremely virulent *P. damselae* subsp. *piscicida* strains (Padrós, pers. comm.) has been noted. Commercial vaccines have been readily available for the last two decades mainly to confront *V. anguillarum* and *P. damselae* subsp. *piscicida* (Le Breton 1999), while vaccination for *T. maritimum* and *A. veronii* bv. *sobria* (autogenous products) has been based on more recent developments. Other bacteria implicated in infections and co-infections include several *Vibrio* species such as *V. harveyi*, *V. ordalii*, *V. parahemolyticus*, *V. splendidus* and *V. vulnificus* (Table 1). Disease outbreaks in European seabass and gilthead seabream have also been triggered by other rarer bacterial pathogens such as *Pseudomonas* spp., *A. hydrophila*, *Mycobacterium marinum*, *Streptococcus iniae* and *Staphylococcus epidermidis* (Table 1). Interestingly, some of the above bacteria have occasionally been considered as primary pathogens in specific cases (Colorni & Padros 2011). Rickettsiae were described as pathogenic

Table 1 Important bacterial and 'related' diseases of European seabass and gilthead seabream

Bacterial pathogens	Fish species	References
Primary		
Vibrio anguillarum	*D. labrax*, *S. aurata*	Balebona *et al*. (1998), Korun and Timur (2008), Öztürk and Altinok (2014)
Photobacterium damsela subsp. *piscicida*	*S. aurata*, *D. labrax*	Toranzo *et al*. (1991), Candan *et al*. (1996), Balebona *et al*. (1998), Essam *et al*. (2016)
Tenacibaculum maritimum	*D. labrax*, *S. aurata*	Pepin and Emery (1993), Bernardet *et al*. (1994), Balebona *et al*. (1998), Bernardet (1998), Avendaño-Herrera *et al*. (2006), Kolygas *et al*. (2012), Yardimci and Timur (2015)
Aeromonas veronii bv. sobria	*D. labrax*	Uzun and Ogut (2015), Smyrli *et al*. (2017)
V. alginolyticus	*D. labrax*, *S. aurata*	Balebona *et al*. (1998), Zorrilla *et al*. (2003), Abdel-Aziz *et al*. (2013), Öztürk and Altinok (2014)
V. harveyi	*D. labrax*, *S. aurata*	Balebona *et al*. (1998), Pujalte *et al*. (2003), Korun and Timur (2008), Haldar *et al*. (2010)
Secondary		
V. parahemolyticus	*D. labrax*, *S. aurata*	Abdel-Aziz *et al*. (2013)
P. damselae subsp. *damselae*	*D. labrax*, *S. aurata*	Öztürk and Altinok (2014), Terceti *et al*. (2016)
V. ordalii	*D. labrax*, *S. aurata*	Korun and Timur (2008), Öztürk and Altinok (2014)
V. splendidus	*S. aurata*	Balebona *et al*. (1998)
V. vulnificus	*D. labrax*, *S. aurata*	Öztürk and Altinok (2014), Uzun and Ogut (2015)
A. hydrophila	*D. labrax*	Doukas *et al*. (2008), Öztürk and Altinok (2014)
Streptococcus iniae	*D. labrax*, *S. aurata*	Zlotkin *et al*. (1998), Aamri *et al*. (2014)
Pseudomonas spp.	*S. aurata*	Zorrilla *et al*. (2003), Öztürk and Altinok (2014)
Staphylococcus epidermidis	*D. labrax*, *S. aurata*	Öztürk and Altinok (2014)
Mycobacterium marinum	*D. labrax*, *S. aurata*	Colorni (1992), Ucko *et al*. (2002), Ucko and Colorni (2005), Avsever *et al*. (2016)
Chlamydia (Intracellular bacteria)		
Epitheliocystis sp.	*D. labrax*, *S. aurata*	Crespo *et al*. (1999), Crespo *et al*. (2001), Seth-Smith *et al*. (2016)
Rickettsia		
Rickettsia-like sp.	*D. labrax*	Comps *et al*. (1996), Steiropoulos *et al*. (2002), Athanassopoulou *et al*. (2004), McCarthy *et al*. (2005)

agents in European seabass (Comps *et al.* 1996; Steiropoulos *et al.* 2002; Athanassopoulou *et al.* 2004; McCarthy *et al.* 2005). Finally, epitheliocystis is a pathological condition widely described in gilthead seabream (Crespo *et al.* 1999) but also in European seabass (Crespo *et al.* 2001), originally associated with chlamydia and recently attributed to a wide number of beta-proteobacteria and chlamydial species (Seth-Smith *et al.* 2016). The relevant bacterial diseases of European seabass and gilthead seabream are also summarized in the works of Colorni and Padros (2011) and Vendramin *et al.* (2016).

Appropriate health management practices, systematic and efficient vaccination and high standards of hygiene at the facilities (hatcheries, nurseries, on-growing sites) are the most effective preventive control methods to reduce the risks of outbreak of an infectious bacterial agent. These practices and standards are particularly relevant at hatchery and nursery level but more difficult to implement in farms using open sea cages or in large ponds or lagoons. Under these conditions, control of the quality and health of introduced juveniles and vaccination have been demonstrated to be the most effective preventive strategies, for bacterial diseases in Mediterranean farming in particular (Le Breton 2009). Commercial vaccines are still available for a limited range of pathogens (Bakopoulos *et al.* 2018), but in some cases, limited vaccine efficacy and poor implementation of systematic vaccination practices in small-sized fish are a concern. Auto-vaccines can be useful for certain bacterial diseases and emerging diseases, in particular, or for sudden changes in the profile of the strains. Nevertheless, they should be considered as emergency and temporary strategies given that they do not provide the same guarantees as commercial vaccines. In addition, the use of autogenous vaccines is strictly limited in some European Mediterranean countries. Fish vaccination is usually carried out in two stages, by bath in hatcheries and intraperitoneal injection often in floating cages (Gravningen *et al.* 2007).

However, despite the preventive measures adopted, in some cases, bacterial disease outbreaks are inevitable and require antibacterial treatments to reduce the direct impact on the affected fish populations. Registered antibacterial agents such as oxytetracycline (OTC), oxolinic acid (OA), flumequine (FLU) and potentiated sulphonamides have been widely used in Mediterranean aquaculture (Rigos & Troisi 2005). Due to its relevance at environmental and human health level, therapeutic antibacterial delivery is made according to strict regulatory frameworks. Efficacy and safety as regards consumer and environmental welfare are two of the main areas to consider for correct and sustainable use of antibacterials.

Thus, knowledge of the efficacy of these therapeutic practices in gilthead seabream and European seabass is not as well developed as in other farmed fish species such as salmonids or terrestrial vertebrates and requires substantial improvement based on scientific data. These actions require a wide and critical review of available technical and scientific information along with the acquisition of further knowledge on promising new candidate antibacterials.

It is anticipated that this review will (i) guide future research in support of good practice in the industry and (ii) provide a scientific foundation to aid the design of future therapeutic regimes in Mediterranean marine fish farming.

Antibacterials and management for fish disease control

General considerations

One of the most relevant particularities of finfish aquaculture is the large number of individuals (hundreds of thousands or even millions) reared in a single rearing unit (farm) compared with other terrestrial animal production such as poultry or pigs. If the animal husbandry technique and the prophylactic measures are insufficient or inadequate to prevent the introduction and spread of pathogens at the facilities, disease outbreaks tend to occur in a particularly fast and aggressive way. This is due to high biomass and other relevant epidemiological factors, such as pathogen transmission capacity in aquatic environments or a much more stressful environment.

Usually, mass therapy strategies are used to confront disease outbreaks caused by bacteria in aquaculture, as no other direct and efficient therapeutic alternatives are available. As antibacterial parenteral delivery (intramuscular or intracoelomic) is logistically not feasible due to the huge number of fish to treat and that bath treatments with antibacterials are discouraged due to the high volumes of water, high amounts of antibacterial, increased risk of generation of antibacterial resistances and relevant environmental and sustainability issues. For this reason, antibacterials are ideally administered orally in feed. This delivery system should be considered, for various reasons discussed below, as a mainly and purely metaphylactic treatment of the affected fish stock rather than a true curative treatment.

As in terrestrial animal veterinary medicine, to select the best therapeutic approach in aquaculture it is important to identify the responsible pathogen or pathogens and determine their antibacterial sensitivities. Antibacterial drugs are not always used and delivered in the most appropriate, rational and efficient manner. This is not due to negligent conduct of fish health veterinarians and fish health care staff but to other external factors beyond their control. These external factors include the urgency of farmers for an immediate response to an outbreak, as diseases spread particularly fast in finfish. Another special characteristic is the

particularly complex logistics associated with the time required to proceed with veterinary prescription and ordering, production, transportation and delivery of medicated fish feed; authorized feed mills are often located at a long distance from production sites. All these problems often result in ill-informed decision-making based on a rushed diagnosis followed by failure to use the most appropriate drugs and available pharmaceutical products. This can lead to suboptimal or unsuccessful therapy.

Antibacterial therapy in Mediterranean finfish farming

The aquaculture industry in Mediterranean countries, as any other aquaculture farming activities is regulated by national and international legislation. As there are many countries in the Mediterranean area, national legislation varies, and the differences are sometimes substantial. In European Union (EU) countries, the use of antibacterials is subjected to strict EU (European Medicines Agency: EMA) and national regulations. Discussions on the control of trade, development and use of veterinary medicines among EU Member States have been ongoing for almost 30 years. The establishment of the open market within the EU in the 90s further increased the importance of regulating the use of medicines throughout the EU. Consequently, the legislation of EU countries has had to bear a common regulatory environment across all member countries.

Several antibacterial substances regulated by EU legislations are currently used in Mediterranean finfish farming (Table 2). It should be noted that these regulations focus mainly on the approval of active substances and medicines and not so much on the prescription and delivery processes. These processes are usually defined according to different national regulations and in line with terrestrial veterinary medicines.

The availability of suitable pharmaceutical products for finfish aquaculture is low, while for gilthead seabream and European seabass it is extremely low. The number of medicines that can be used 'on label' is relatively low. The differences between countries (even at EU country level) regarding licensed products constitute an additional problem at Mediterranean level. If no licensed medicine for fish is available in one EU country but available in other EU countries, another legal mechanism known as important and use of veterinary medicines under exceptional circumstances can be used. In such cases, it is possible to apply to the responsible national authorities for special import authorization. Unfortunately, given the lack of specific licensed medicines for gilthead seabream and European seabass, this mechanism is common rather than exceptional and requires additional bureaucratic procedures.

The scarcity of specific 'on label' medicines, also problematic for other animal species such are the so-called 'minor species', has also been alleviated by the 'prescribing cascade' mechanism. European Union regulations (90/676, 19/6, 19/4) provide a 'prescribing cascade' to support the use of medicines with MRLs established for other food-producing animals, when no suitable compound has been licensed to treat diseases in fish. In such cases, a minimum standard withdrawal period is imposed, corresponding to 500-degree days in fish. This is to ensure consumer safety, and is enforced by an established maximum residue level (MRL), which is derived from toxicity testing data. The MRL is the maximum residue concentration tested to be without toxicological risk to human health. To ensure that no residues above the MRL exist in the edible tissues of farmed products, a withdrawal period is determined for each drug in the target fish species. Accordingly, Council Regulations (2377/90, 470/09) were established laying down a Community procedure for the establishment of the MRL of veterinary medicinal products in foodstuffs of animal origin. The list of registered antibacterials for animal farming/aquaculture in Mediterranean countries along with their MRL, recommended dosing schedules and commercial forms are given in Table 2.

Antibacterial selection, prescription and delivery: main criteria, technical aspects and constraints

The selection of the most adequate antibacterial therapy for gilthead seabream and European seabass is based on relatively similar general criteria as those for humans, terrestrial animals or other fish species. Therapeutic antibacterial management of large finfish stocks and Mediterranean finfish species such as European seabass and gilthead seabream, in particular, presents several particularities. This is the first and complex point with a number of relevant considerations. These considerations set out below.

The affected fish stock

Species

The gilthead seabream and European seabass are different species, not only because they are taxonomically different teleost fish species), but also because they present many morphological, physiological, metabolic and behavioural differences. One of the most frequent mistakes made in the past in Mediterranean aquaculture was to consider both species in a similar way (e.g. in terms of husbandry and nutrition) or extrapolate available scientific information on salmonids as regards the therapeutic schedules. However, the current available scientific and technical knowledge on these two species (Malvisi *et al.* 1996; Rigos *et al.* 1999, 2002b, 2002c, 2003a, 2004b, 2006; Castells *et al.* 2000; Intorre *et al.* 2000; della Rocca *et al.* 2004a; Di Salvo *et al.* 2013), allows the development of specific and detailed

Table 2 Registered antibacterials for animal farming/aquaculture in EU/Mediterranean countries

Antibacterial drugs	Recommended dosing schedule (kg fish biomass)	Maximum residue level, MRL (μg kg^{-1} fresh edible animal part)	Marker residue	Animal	Source	Registered in mediterranean aquaculture	Commercial forms
Tetracyclines							
Oxytetracycline	75 mg kg^{-1} 10 days	100	Sum of parent drug and its 4-epimer	Fish	EMEA/MRL/023/95 (EMEA, 1995a)	Greece, Spain, Italy, Croatia	Oxyvet Vethellas 50% OTC Aquacen Oxytetracycline Acuimix 750 (Oxytetracycline 75%) Aquaculture Anprociclina 200 Ossitetraciclina cenavisa Anprociclina 200 Kyroxy 200 premix Ossibiotic premix Oxiter Oxifarm Ossitetra 200 premix Egocin 20%
Chlortetracycline	75 mg kg^{-1} 10 days	100	Sum of parent drug and its 4-epimer	Fish	EMEA/MRL/023/95 (EMEA, 1995a)	Italy	Percrison 200 Premix Clorbiotic 200 Clortetra 200 premix Solclor 200S
Doxycycline	200 mg kg^{-1} 5 days	100	Parent drug	All food-producing species	EMEA/MRL/270/97-FINAL (EMEA, 1997a) EMA/CVMP/347870 (EMA, 2015)	Not in fish	
(Fluoro)quinolones							
Oxolinic acid	10–35 mg kg^{-1} 5–7 days	100	Parent drug	Fish	EMEA/MRL/41090/05-FINAL (EMEA, 2005)	Greece, Spain	Linacivet Inoxyl acide Oxolinique 240 salmonides (oxolinic acid 25%) Oxomid 24%
Flumequine	12 mg kg^{-1} 5 days	600	Parent drug	Fish	EMEA/MRL/823/02-FINAL (EMEA, 2002c)	Spain, Italy, Greece	Flumesya Colifarm 200 (flumequine 20%) Flumequine 50% Chinogel Flumequine 200 Naquilene 500 Colifarm Agrossed Candilidis oral.sol 10%, Vitaquin Med.pre.po 500 mg g^{-1}

Table 2 (continued)

Antibacterial drugs	Recommended dosing schedule (kg fish biomass)	Maximum residue level, MRL (μg kg^{-1} fresh edible animal part)	Marker residue	Animal	Source	Registered in mediterranean aquaculture	Commercial forms
Sarafloxacin	10 mg kg^{-1} 5 days	30	Parent drug	Fish	EMEA/MRL/349/98-FINAL (EMEA, 1998)	Not in fish	
Enrofloxacin	10 mg kg^{-1} 5 days	100	Sum of enrofloxacin and ciproxacin	All food-producing species	EMEA/MRL/820/02-FINAL (EMEA, 2002b)	Not in fish	
Danofloxacin	10 mg kg^{-1} 5 days	100	Parent drug	Fish	EMEA/MRL/818/02-FINAL (EMEA, 2002a)	Not in fish	
Sulphonamides							
Sulfadiazine Sulfamethazine	25 mg kg^{-1} 5 days	100	Parent drug	Fish	EMEA/MRL/026/95(EMEA, 1995b)	Greece, Spain, Italy	Sulfatrim Tribrissen Optiprim Doxatrim 15 PM (trimethoprim + sulfadiazine 15% Neopridimet orale Trivemet premix Doxatrim 15 PM Neopridimet
Diaminopyrimidines							
Trimethoprim	5 mg kg^{-1} 5 days	50	Parent drug	Fish	EMEA/MRL/255/97-FINAL (EMEA, 1997b)	Greece, Spain	Sulfatrim Tribrissen Optiprim Doxatrim 15 PM (trimethoprim + sulfadiazine 15%)
Penicillins-B lactam							
Amoxicillin	80 mg kg^{-1} 10 days	50	Not determined	Not determined	Undated/updated 2008 (Revision1) (EMEA, 2008)	Italy	Amoxicillin 100 Colistin 250 Gammamix(PM)
Ampicillin							
Phenicols							
Florfenicol	10–15 mg kg^{-1} 10 days	1000	Sum of parent drug and its metabolites	Fish	EMEA/MRL/822/02-FINAL (EMEA, 2002d)	Spain, Italy, Croatia, Greece	Frorocol AQUA Aquaflor 50% † (florfenicol 50%) Aquaflor 500 Floron Florocol

Table 2 (continued)

Antibacterial drugs	Recommended dosing schedule (kg fish biomass)	Maximum residue level, MRL (μg kg^{-1} fresh edible animal part)	Marker residue	Animal	Source	Registered in mediterranean aquaculture	Commercial forms
Thiamphenicol	15–40 mg kg^{-1} 5 days	50	Parent drug	Fish	EMEA/CVMP/162614/2006-FINAL (EMEA, 2006)	Not in fish	
Lincosamides							
Lincomycin	100 mg kg^{-1} 5 days	100	Parent drug	All food-producing species	EMEA/MRL/749/00-FINAL (EMEA, 2000)	Not in fish	
Aminocyclitols							
Spectinomycin	50 mg kg^{-1} 5 days	300	Parent drug	All food-producing species	EMEA/MRL/826/02-FINAL (EMEA, 2002e)	Not in fish	

[†]Registered for salmonids.

management and farming protocols for each species separately. Unfortunately, this is not the case regarding therapeutic antibacterial management.

Age/size/weight

Both species have long rearing periods (more than one year for portion-sized fish and up to 2–3 years for large-sized fish) with significant metabolic changes according to size, weight or age. Gilthead seabream and European seabass fry, juveniles or adults differ substantially and these differences may have direct implications for antibacterial treatments. However, these differences remain relatively unexplored.

Stock and rearing system

The target stocks for treatment can also differ significantly according to the different rearing scenarios. As noted above, in Mediterranean aquaculture, reared stocks tend to consist of large numbers of fish, millions in larval tanks, hundreds of thousands in the postlarval and juvenile stages and from dozens of thousands (ponds, recirculation systems) to hundreds of individuals in cages. The rearing units can also differ in Mediterranean aquaculture units. Although most of the on-growing production is based in sea cages, some gilthead seabream and European seabass farms are based in ponds or tanks, with inflow or recirculation systems, while pre-on-growing is mainly based in tanks (of different volumes). All these aspects have implications for issues such as the delivery method and the therapeutic regime, as well as environmental impacts.

Water temperature

Water temperature is not given due consideration in finfish therapeutic approaches when these approaches are directly extrapolated from homeotherm terrestrial vertebrates. As water temperature strongly regulates fish metabolism, some aspects related mainly to the pharmacokinetic (PK) evolution of the different therapeutic molecules and their derivatives in the body of fish differ in fish reared at different temperature regimes. The evolution of active molecules in the different compartments considered in PK studies (liberation, absorption, distribution, metabolism and excretion) can be influenced by water temperature (Rigos *et al.* 2002a, 2002b). For example, a marked temperature-dependent kinetic profile of OTC was apparent in European seabass (Rigos *et al.* 2002a), with faster distribution and elimination in the fish compartment, indicating possible sequential dosing schedules and longer withdrawal times of the drug mainly at low water temperatures. Similar temperature effects were evident when OA was tested in the same fish species (Rigos *et al.* 2002b).

In most cases however, PK studies on different antibacterials and drugs are performed at a single temperature and very rarely at different temperatures (Table 5). As a result,

the PK results for a specific active molecule at a specific temperature are frequently extrapolated to other temperature scenarios, even if the range between both temperatures is not relevant. Consequently, most recommended therapeutic doses in fish are fixed, with no specific consideration of the rearing temperature when a disease outbreak appears. This scarcely considered aspect probably accounts for the differences between the theoretical simple calculations made for finfish treatments and the real situation of the affected stocks.

Temperature-related feeding rates (Tort *et al.* 2004) are directly related to temperature and therapeutics in the particular case of medicated feed delivery. As feed pellets are the main carrier (vehicle) of medicine to fish, dosage calculation of medicines/premixes to be added to feed and prepare the prescribed medicated feed requires knowledge of the current and expected rearing temperature of the affected stock. Fish feeding rates are lower at lower temperatures and higher at higher temperatures, so when using a single therapeutic dose (mg of the selected active molecule/kg of fish), the same amount of medicine is added to a small amount of food (at low temperature/low feeding rates) or a high amount of food (at high temperature/high feeding rates). As a consequence, the active compounds included in medicated feeds for fish reared at low water temperatures can be much more concentrated than at high temperatures, with potential consequences on the uptake of the medicated feed by the fish, palatability and, therefore, the pharmacokinetic pattern of this treatment. In European seabass and gilthead seabream, this is important for certain, well-known, different situations such as antibacterial management in case of treatments against winter disease in gilthead seabream (Ibarz *et al.* 2010) or pasteurellosis management in European seabass outbreaks at 25°C.

Other physiologic and metabolic considerations. Most of the processes involved in PK profiles can be influenced by differences in the physiology, metabolism and other related functions. Feed content or feeding regime can also be impaired in the presence of frequently described concomitant digestive parasitic diseases such as *Enteromyxum leei* or *Enterospora nucleophila* infections and winter disease in gilthead seabream or coccidiosis in European seabass. Hepatic function is also involved in other PK processes and changes in the hepatic antibacterial metabolic capacity can be found under certain conditions when hepatic metabolism has been pushed to its limits (high-energy diets, chronic stress, fatty liver) or is affected by chronic systemic diseases. In such situations, more accurate selection of the antibacterial administration regime should be recommended. However, dietary manipulation has not been found to alter quinolone uptake in gilthead seabream (Rigos *et al.* 2012).

Stock population

Under field conditions, practical therapeutic approaches require some level of implication in order to facilitate calculations and logistics. Fish populations to be treated are generally considered a 'single entity' by total biomass estimation. This approach is frequently used for feeding calculations but sometimes does not consider certain particularities relevant to the result of the treatment. Feeding calculations are usually based on highly homogeneous fish populations in terms of the results of biometry or other biomass assessment methods. In very homogenous fish populations, medicated feed distribution is easier but in fish populations with a higher level of dispersion prediction of the real delivery rate of medicated feeds is much more difficult as the variability between individuals of the therapeutic molecule levels is increased. Moreover, it should be taken into account that dose calculations are usually made in healthy populations, with more or less predictable feeding behaviour. In real situations, the sick population is already affected by the outbreak, and virtually, all infectious diseases impair appetite. This is the reason why an accurate assessment of the evolution of the disease also helps to refine the therapeutic strategies. The presence of previous diseases in the fish stock, mainly those affecting the digestive or hepatic function may also affect the result of the therapeutic strategy.

Evolution of infections in the stock

This factor is also strongly related to the disease typology and will be specifically addressed in the following section on pathogens. Assessment of the precise timing in the evolution of the outbreak is very relevant information for decision-making, but it is not always well addressed in Mediterranean aquaculture. Typical bacterial outbreaks in gilthead seabream and European seabass are associated with *P. damselae* subsp. *piscicida*, *V. anguillarum* and other related bacterial species (Muniesa *et al.* 2020) and usually display the pattern described in Figure 1. Some examples of specific disease evolution patterns are found in gilthead seabream and European seabass production. Acute fast evolution of the disease is observed in septicaemic bacterial processes in gilthead seabream and European seabass postlarvae and fry at nursery level. In such cases, vibriosis in European seabass and pasteurellosis/photobacteriosis outbreaks in European seabass and gilthead seabream develop particularly fast and antibacterial treatments are unsuccessful if they are not applied immediately after the first signs of the disease. Juveniles are still quite sensitive to these and other bacterial problems (vibriosis by other *Vibrio* species, tenacibaculosis) as specific immuno-prophylaxis, when available, cannot be applied until fry have developed a high level of immunocompetence (around 1 to 5 g), while the development of a relevant level of protection can take several weeks after vaccination.

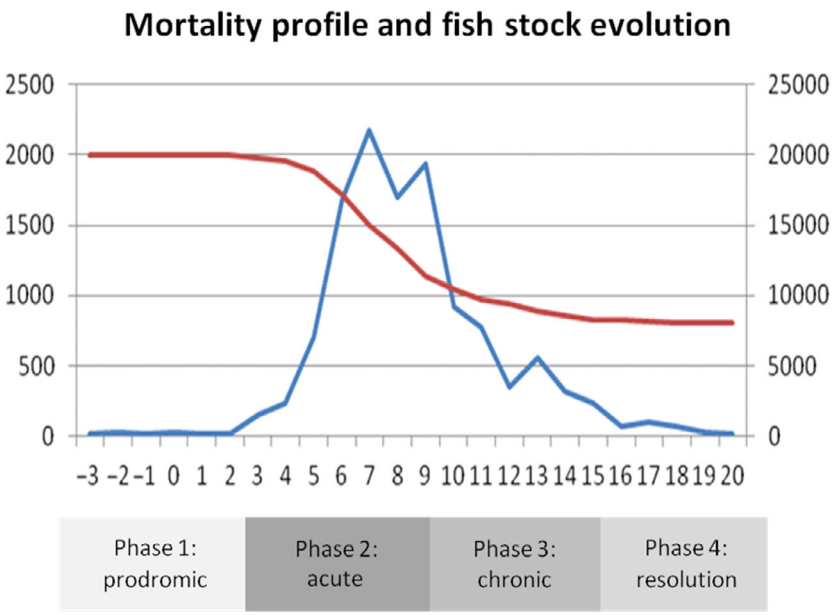


Figure 1 Simulation of the evolution of the daily mortality (scale in the left side, number of dead fish collected per day) and fish surviving population in the stock (scale in the right side, estimated number of surviving fish) in a typical severe outbreak (60% total mortality) associated to *P. damselae* subsp. *piscicida* or *V. anguillarum*. Mortality; Population.

Chronic disease is commonly seen in pasteurellosis/photobacteriosis outbreaks in Mediterranean farmed finfish species (Magarinos *et al*. 2001). Based on field observations, in particular risk situations and mainly if the outbreaks appear at the beginning of the pasteurellosis season (June–September according the different geographical and temperature regimes), prolonged antibacterial treatments are generally needed for the same stock but not if the previous antibacterial treatment failed.

The evolution of a disease modelled in four different phases, according to human epidemiology (Antia *et al*. 2003) and adjusted for aquaculture medicine (Figure 1), is described below.

Phase 1: the prodromic phase where the pathogen can be isolated from the stock but mortalities are still similar to the basal mortalities. No considerable changes are detected in the behaviour of the fish with the exception perhaps of a decrease in appetite.

Phase 2: the acute phase that is characterized by a sudden increase in mortality until it reaches a peak. The number of symptomatic fish (sick, not eating) that will die in the next 2–3 days increases and the number of affected fish increases very fast and in an exponential way. From this figure, it is possible to predict how many fish are anorectic on a certain day simply by counting daily mortalities and estimating the mortality of the next three–four days. At this point, all the fish are refractory to the treatment, and thus, the efficiency of the treatment is decreasing progressively; the number of fish with decreased appetite is also increasing.

Phase 3: the chronic phase characterized by a decreasing trend in the daily mortality rate but also a substantial decrease of the population, with the remaining survivor stock now composed of resistant or non-infected naturally resistant fish survivors, antibacterial-protected fish and chronically affected fish. In this scenario, the progress of the disease is much more difficult because the total number of fish in the stock has decreased and the total survivor fish stock is much less susceptible to the disease. As the volume of the fish stock has substantially decreased, the number of fish infected and sick for the first time during this outbreak is low. Thus, a relevant number of fish that are eating the medicated feed during this third phase corresponds to naturally resistant fish, and therefore, these fish supposedly do not require medication. In addition, antibacterial-protected fish still require a continuous supply of antibacterials. Fish that are still alive but chronically infected and still fighting the disease, also display decreased appetite and although the medicated feed is offered, it may not be ingested by fish.

Phase 4: the resolution phase achieved when mortalities are not high but still occurring. Feeding behaviour is recovering but is not completely normal. Mortality in this phase is composed mainly of chronically affected fish that finally succumb to the disease after having fought against it for a long period. This recovery phase

is characterized by a substantial reduction in the population at risk, consisting mainly of naturally survivor fish, including survivor chronic fish that are no longer susceptible to the disease (natural immunoprophylaxis) and antibacterial-protected fish.

In this four-phase scenario, the role of the potential antibacterial treatment using medicated feeds should be carefully analysed for each of the four phases. Phase 1 is the key phase when antibacterial administration makes sense and is effective; all the stock is still eating and can be protected by the medication. Antibacterial treatment efficacy decreases very fast and exponentially as phase 2 advances. Most of the disease-susceptible fish become infected in these early stages, they lose their appetite very fast and die within a few days if they are not protected by an earlier medicated feed regime. As the disease progresses in phase 2 (evaluated by an increase in mortality), the efficacy of the antibacterial treatment decreases dramatically. After reaching the plateau in phase 3 (chronic), only the susceptible but medicated fish require a continuous supply of antibacterials. In case of very early application of the treatment, the number of susceptible-medicated fish is high enough to justify prolongation of the treatment. In case of late application of the antibacterial treatment, the therapeutic value of a prolongation of the treatment is very low. In phase 4 (recovery), prolongation of the antibacterial treatment only makes sense if the treatment is applied very early (phase 1 or early phase 2 stages) and the epidemiological conditions of the fish stock or farm (temperature, high biomass, stress) indicate a relevant risk of new outbreaks. These scenarios that are based on a hypothetical bacterial outbreak are schematized in Figure 2. Such information is rather theoretical and supported by long-term farm data. However, it should be noted that it is rather unrealistic to diagnose the prodromic phase. Isolation or detection is not always connected to the initiation of an outbreak and normally, the farmers/vets wait until mortality increases before reacting, which is usually too late.

In scenario 2, a description of the recommended theoretical dosages to be used according to each phase of the evolution of the disease and to the changes in the real daily feed intake is given. It should be noted that by using this scheme and the modifications according to the mortality and adjusted feed intake, the amount of medicated feed differs substantially from the calculations made on the initial healthy stock, and without considering the changes in appetite during the different phases. These differences are shown in Table 3.

The information displayed in this simulation clearly shows that a more than 50% reduction in the total amount of medicated feed and antibacterial used is possible by simply implementing periodical corrections based on the current stock measurements and feeding rate estimates. Moreover, it should be noted that the theoretical therapeutic dose in this case is surprisingly quite stable (2.0–2.8 g kg^{-1} medicated feed) during all four phases. This simulation highlights the relevance of an accurate and precise evaluation of the outbreak follow-up, precise day-to-day stock assessment and a real feeding rate to refine the efficacy of the antibacterial treatments, that is to use the required amount of antibacterial and thus reduce the waste of medicated feeds and antibacterials and their release in the environment. These scenarios can be even more dramatic (Figures 3, 4 and 5) if we compare the differences between good delivery strategies, with the administration of antibacterials in the early stages (prodromic after efficient diagnostics) versus the later stages (when the outbreak is advanced in acute stages or even chronic stages). Using underwater camera technology for monitoring feeding response and thus, potential diet loss will aid considerably in realizing the above scenarios and adjusting medication dosing during bacterial epidemics (Parra *et al.* 2018).

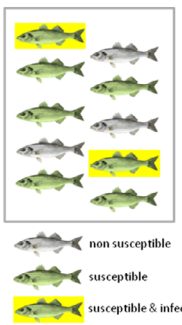
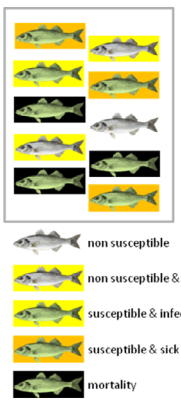
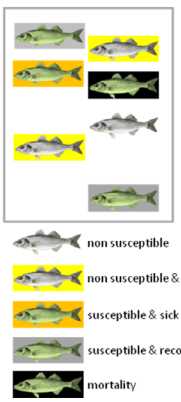
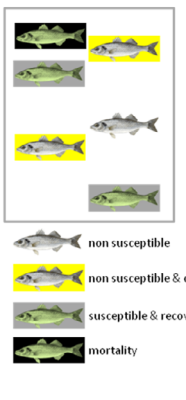
However, it should be noted that for various practical reasons, the above considerations are rarely applied in current Mediterranean farming. Generally, the initial biomass is relatively unknown and mortalities are not often estimated. Moreover, the proposed periodic corrections are difficult considering that the production and delivery of medicated feeds takes 2–4 days and that big farms require large quantities of medicated diets.

Disease and selection of the appropriate therapeutic agent

The most suitable antibacterial should be selected according to the specific characteristics of the disease and the pathogen. In finfish therapeutics, the most common criteria applied are bacterial wall characteristics (gram positive or negative) and antibacterial sensitivity of the strain. Other criteria applied, mainly in case that data on antibacterial sensitivity are not available, are historical records of the efficacy of the antibacterials for the same diseases and pathogen. The final selection is made according to these criteria and certain other external constraints such as availability of the veterinary medicine and price. A more complete and detailed description of the current methods of

Figure 2 Initial population is separated in two groups: susceptible and not susceptible, as in all outbreaks there is always a part of the population (naturally not susceptible or naturally resistant) that not become 'sick' during the process. Obviously, the percentage of susceptible fish in a population may vary according many factors (genetics, epigenetics, natural immunization, acquired immunization).

Scenario 1. European seabass stock, averaged 1 kg, without any antibacterial treatment

Prodromic phase	Acute phase	Chronic phase	Resolution phase
Stock (100%): 10,000 kg 1 kg fish seabass normal feeding rate: 1% Feeding 100% normal rate: 100 kg/day	Mortality: 3,000 kg (30%) Fish still alive: 7,000 kg (70%) Fish still "treatable and recoverable": 4,000 kg (40% of the initial stock, 57% of the current stock) Appetite decreased: Feeding 40-60% Theoretical feed to deliver: 70 kg/day Stock reduction due to mortality: 30% Real daily feed intake of the stock 28-42 kg (35 kg)	Mortality: + 1,000 kg (10%) Total mortality: 4,000 kg (40%) Fish still alive: 6,000 kg (60%) Fish still "treatable and recoverable": 5,000 kg (50% of the initial stock, 83% of the current stock) Appetite recovering: Feeding 60-70% Stock reduction due to mortality: 40% Reduced stock: theoretical feed to deliver: 60 kg Real daily feed intake of the stock 36-42 kg	Mortality: + 1,000 kg (10%) Total mortality: 5,000 kg (50%) Fish still alive: 5,000 kg (50%) Fish still "treatable and recoverable": 5,000 kg (50% of the initial stock, 100% of the current stock) Appetite nearly recovered: Feeding 90% Total stock reduction due to mortality: 50% Reduced stock: theoretical feed to deliver: 50 kg Real daily feed intake of the stock 45 kg
 non susceptible susceptible susceptible & infected	 non susceptible non susceptible & carrier susceptible & infected susceptible & sick mortality	 non susceptible non susceptible & carrier susceptible & sick susceptible & recovering mortality	 non susceptible non susceptible & carrier susceptible & recovering mortality

Scenario 2. European seabass stock, averaged 1 kg, with antibacterial treatment

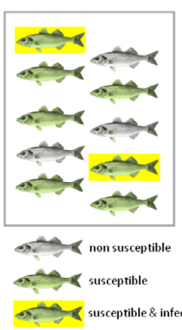
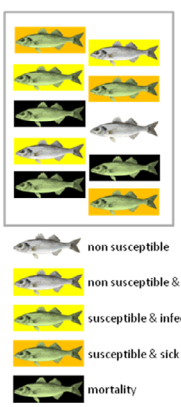
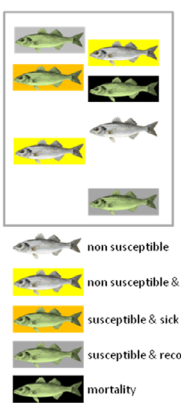
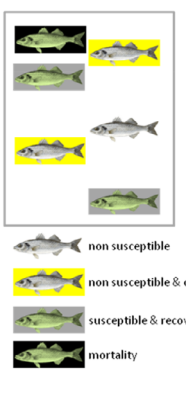
Prodromic phase	Acute phase	Chronic phase	Resolution phase
Stock (100%): 10,000 kg seabass normal feeding rate: 1% Feeding 100% normal rate: 100 kg/day Antibiotic dose: 20 mg/kg BW/day Antibiotic requirement: 200 g/day Dose: 200 g/100 kg = 2 g antibiotic per kg of feed/day	Appetite decreased: Feeding 40-60% Stock reduction due to mortality: 30% Reduced stock: theoretical feed to deliver: 70 kg/day Real daily feed intake of the stock 28-42 kg (35 kg) Fish still alive: 7,000 kg Fish still "treatable and recoverable": 4,000 kg Antibiotic dose: 20 mg/kg BW/day Theoretical amount: 200 g/day Real antibiotic requirement: 80 g Dose: 80 g/35 kg = 2.28 g antibiotic per kg of feed/day	Appetite recovering: Feeding 60-70% Stock reduction due to Reduced stock: theoretical feed to deliver: 60 kg Real daily feed intake of the stock 36-42 kg Fish still alive: 6,000 kg Fish still "treatable and recoverable": 5,000 kg Antibiotic dose: 20 mg/kg BW/day Theoretical amount: 200 g/day Real antibiotic requirement: 100 g Dose: 100 g/42 kg = 2.38 g antibiotic per kg of feed/day	Appetite nearly recovered: Feeding 90% Total stock reduction due to mortality: 50% Reduced stock: theoretical feed to deliver: 50 kg Real daily feed intake of the stock 45 kg Fish still alive: 5,000 kg Fish still "treatable and recoverable": 5,000 kg Antibiotic dose: 20 mg/kg BW/day Theoretical amount: 200 g/day Real antibiotic requirement: 100 g Dose: 100 g/45 kg = 2.22 g antibiotic per kg of feed/day
 non susceptible susceptible susceptible & infected	 non susceptible non susceptible & carrier susceptible & infected susceptible & sick mortality	 non susceptible non susceptible & carrier susceptible & sick susceptible & recovering mortality	 non susceptible non susceptible & carrier susceptible & recovering mortality

Table 3 Fish appetite changes in the different disease phases-a hypothetical scenario concerning calculations of medicated diets

	Phase 1	Phase 2	Phase 3	Phase 4
General calculations	Stock: 10,000 kg Feed: 100 kg Antibacterial dose in the medicated feed: 2 g kg^{-1} feed Total antibacterial used: 200 g day^{-1}	Stock: 10,000 kg Feed: 100 kg Antibacterial dose in the medicated feed: 2 g kg^{-1} feed Total antibacterial used: 200 g day^{-1}	Stock: 10,000 kg Feed: 100 kg Antibacterial dose in the medicated feed: 2 g kg^{-1} feed Total antibacterial used: 200 g day^{-1}	Stock: 10,000 kg Feed: 100 kg Antibacterial dose in the medicated feed: 2 g kg^{-1} feed Total antibacterial used: 200 g day^{-1}
Specific realistic calculations	Stock: 10,000 kg Feed: 100 kg Antibacterial dose in the medicated feed: 2 g kg^{-1} feed Total antibacterial used: 200 g day^{-1}	Stock: 7,000 kg Feed: 35 kg Antibacterial dose in the medicated feed: 2.8 g kg^{-1} feed Total antibacterial used: 98 g day^{-1}	Stock: 6,000 kg Feed: 42 kg Antibacterial dose in the medicated feed: 2.4 g kg^{-1} feed Total antibacterial used: 100 g day^{-1}	Stock: 5,000 kg Feed: 50 kg Antibacterial dose in the medicated feed: 2.2 g kg^{-1} feed Total antibacterial used: 110 g day^{-1}

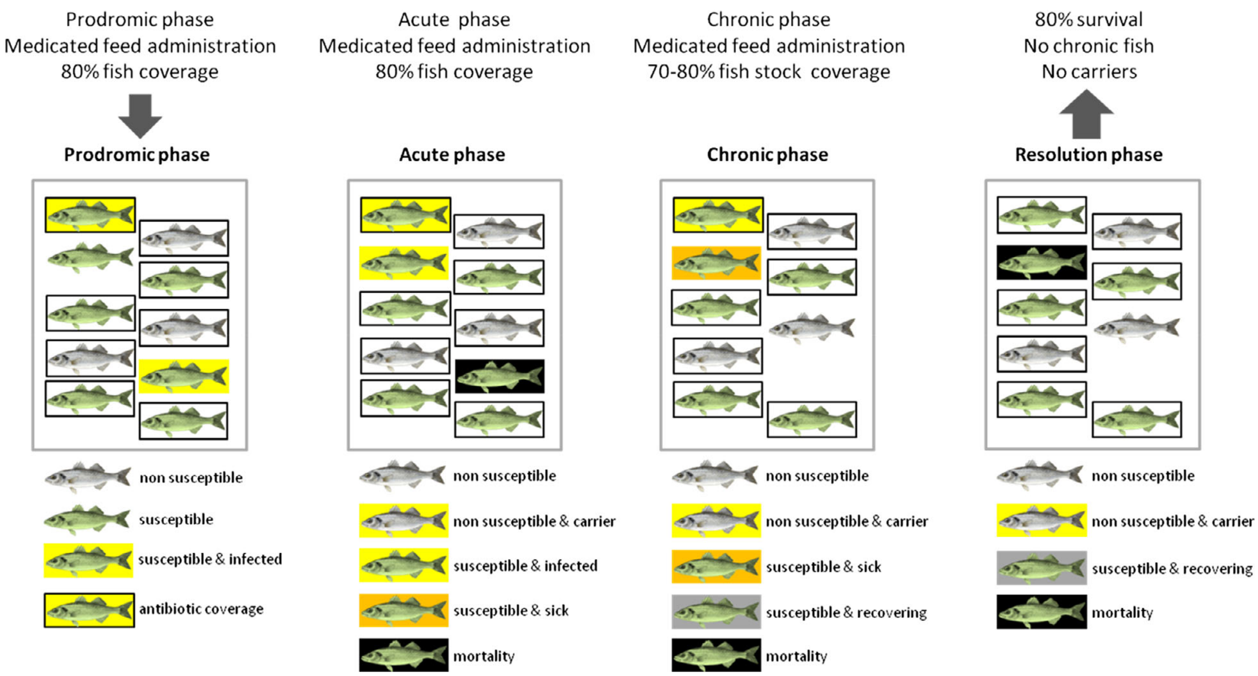


Figure 3 Early administration strategy: high antibacterial coverage is maintained during the outbreak.

antibacterial sensitivity assessment in Mediterranean aquaculture is set out in this report, in the section relating to pharmacodynamics (PD) and minimum inhibitory concentrations (MIC) evaluation.

In addition to the laboratory results and the information available on breakpoints, epidemiological cut-off breakpoints (ECOFF) (Kronvall 2010) and/or MIC are also very important; they should be taken into account based on the history or previous records of antibacterial use in the same stock and also at farm level. The efficacy results of different methods of antibacterial administration for the same pathogen, as well as data about the evolution of antibacterial sensitivity are very important as regards predictions of the potential efficacy of antibacterial treatments. Surveillance of antimicrobial resistance at farm level directly benefits the improvement of the efficacy of the treatments, besides the importance of such data for the implementation of general national, transnational, European and global antimicrobial resistance assessment programmes. As for the 3Rs principle in animal research (Aske & Waugh 2017), antibacterial treatments constitute a valuable tool. Nevertheless, they should be Replaced by more efficient and powerful preventive

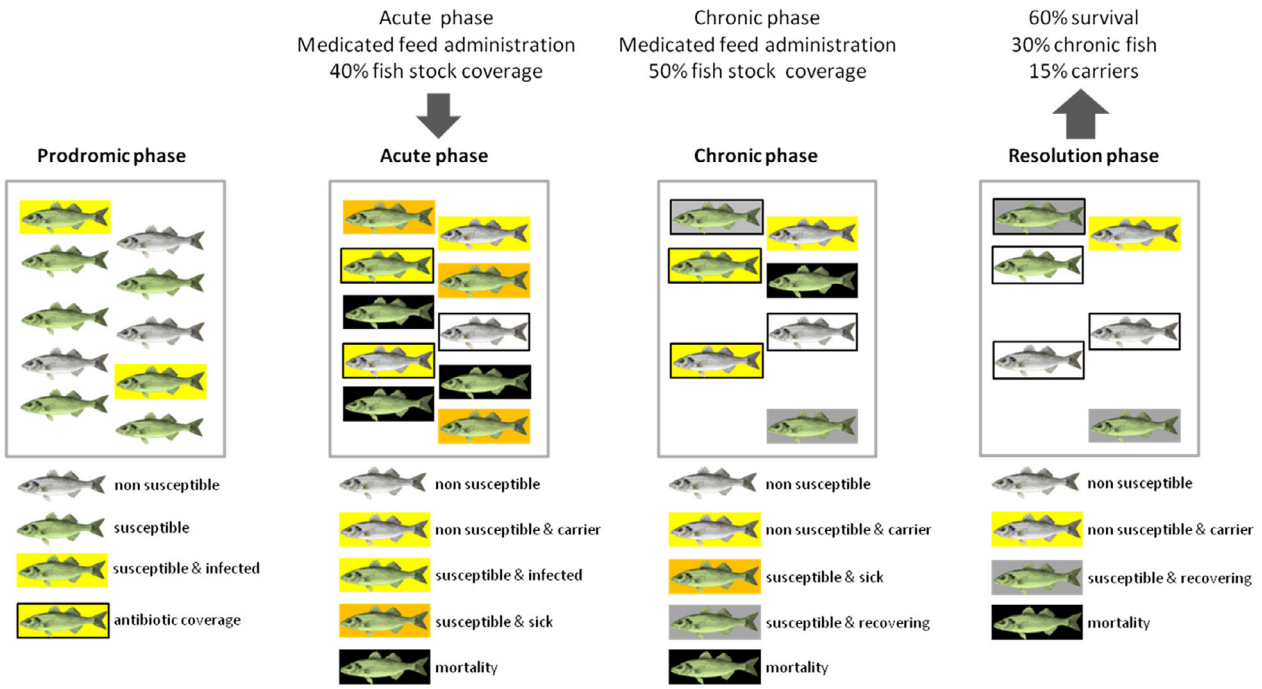


Figure 4 Late administration strategy: lower and more variable antibacterial coverage during the outbreak. This is the most frequent situation found in the field.

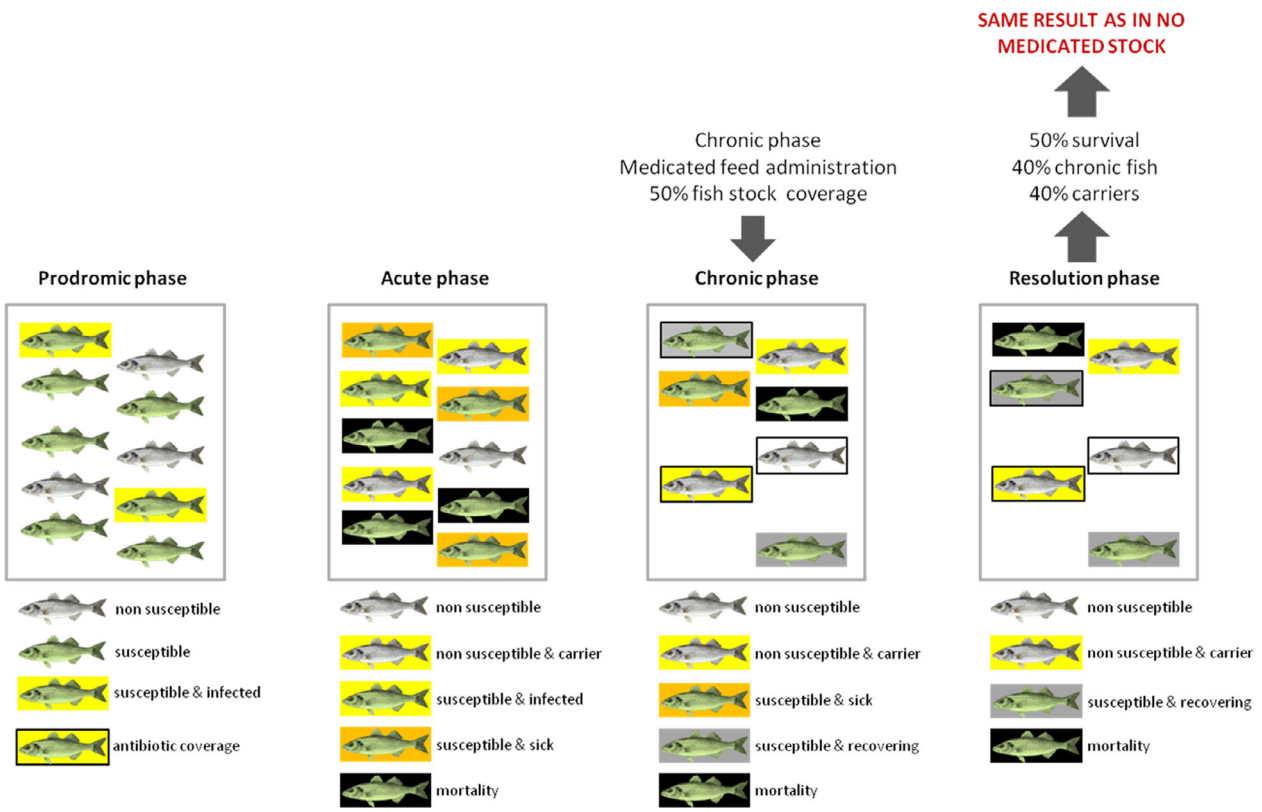


Figure 5 Very late administration: please notice that results in this case are the same to those obtained if fish do not receive any medication.

measures and alternative treatment methods where possible, Reduced in terms of quantity and frequency only if strictly necessary, and Refined in order to use the smallest amount of antibacterials for the highest efficacy in the treatments with the lowest impact on the environment.

The tools: antibacterials, main characteristics and pharmacokinetic properties

Antibacterials that are used or can be used in Mediterranean aquaculture and particularly for the production of gilthead seabream and European seabass is one of the most relevant part of this review. The legislation and antibacterial use pertaining to the European Mediterranean region, has been above and includes extensive updated information of the different antibacterials that can be used in gilthead seabream and European seabass under the current EU and national legal framework. The selection and use of these antibacterials in Mediterranean aquaculture require a complete update of the general and specific characteristics of each antibacterial. Knowledge of these characteristics is also very important for a complete understanding the pharmacokinetic (PK) and PD properties of these antibacterial compounds and of how these compounds should be used for the control of bacterial diseases in order to ensure the best levels of efficacy, safety and responsibility.

Tetracyclines

Tetracyclines, discovered as early as the 1940s, are a family of antibacterials that inhibit bacterial protein synthesis (mRNA translation) by binding to the bacterial 30S ribosomal subunit of microbial 70S ribosomes. They are broad-spectrum low-cost bacteriostatic agents (Table 4), produced by *Streptomyces* spp. fungi that exhibit a time-

Table 4 Properties of important antibacterial groups for animal farming/aquaculture in EU/Mediterranean countries

Antibacterial class	Type	Mechanisms of action	Mechanisms of resistance	PK/PD interactions	Goal of therapy	Predictive indices of PK/PD
Tetracyclines	Bacteriostatic	Inhibitors of protein synthesis	Efflux, ribosomal protection, drug modification	(Co-dependent) Concentration &Time-dependent killing	Maximize amount of drug	$AUC_{0\text{-}24}$/MIC
(Fluoro)Quinolones	Bactericidal	Inhibitors of DNA gyrase	Altered target, decreased uptake	Concentration-dependent killing	Maximize concentration	C_{max}/MIC
Sulphonamides &			*Diaminopyrimidines*	Bacteriostatic/in combination bactericidal	Inhibitors of folic acid synthesis	Altered drug penetration, altered target enzyme, plasmid transfer
Time-dependent killing	Maximize duration of exposure	$T_{C>}$MIC				
Penicillins	Bactericidal	Inhibitors of cell wall synthesis	Enzymatic destruction, altered target, decreased uptake	Time-dependent killing	Maximize duration of exposure	$T_{C>}$MIC
Phenicols	Bacteriostatic	Inhibitors of protein synthesis	Plasmid-mediated resistance, reduced membrane permeability, mutation of the ribosomal subunit	Time-dependent killing	Maximize duration of exposure	$T_{C>}$MIC
Lincosamides	Bacteriostatic	Inhibitors of protein synthesis	Plasmid-mediated resistance, ribosomal modification, efflux, and drug inactivation	Time-dependent killing	Maximize duration of exposure	$T_{C>}$MIC
Aminocyclitols	Bacteriostatic/ Bactericidal	Inhibitors of protein synthesis	Enzymatic modification, altered target, decreased uptake	Mostly concentration-dependent killing	Maximize concentration	C_{max}/MIC

$AUC_{0\text{-}24}$: area under the serum/plasma concentration curve at 24 h; MIC: minimum inhibitory concentration; C_{max}: peak serum/plasma concentration; $T_{C>}$MIC: percentage of the inter-dosing interval during which the serum/plasma concentration exceeds the *in vitro* MIC against the target bacterium.

dependent killing activity against a wide range of gram-positive and gram-negative bacteria, atypical organisms such as *Chlamydiae*, mycoplasmas and *Rickettsiae*, as well as protozoan parasites. Their beneficial antimicrobial properties and the absence of major adverse side effects has led to the extensive use of tetracyclines in the treatment of bacterial infections (Roberts 2003).

Oxytetracycline is perhaps the most common tetracycline used worldwide for the treatment of bacterial fish diseases. The PK of OTC has been thoroughly investigated in European seabass and gilthead seabream (Malvisi *et al*. 1996; Rigos *et al*. 2002a, 2003b, 2004a, 2006) (Table 5). The absorption of OTC in these two species is limited, with bioavailability (*F*) values as low as 9–22% (Rigos *et al*. 2003b, 2004a). Therefore, a significant fraction of the administered OTC remains unabsorbed in the gastrointestinal tract of euryhaline fish. This was also evidenced by the high amounts of unaltered OTC (40–73%) recovered in the faeces of euryhaline fish (Rigos *et al*. 1999). Chelate formations with divalent cations (Mg^{2+} and Ca^{2+}) in the feed and the intestinal environment of the fish, which apparently reduce solubility and, consequently, membrane permeability along the gastrointestinal tract, have been blamed for low OTC absorption (Rigos *et al*. 2004a). It should be taken into account that marine water contains high numbers of divalent cations that can increase the chelation effect of OTC in marine species. Some attempts to increase OTC oral bioavailability through self-emulsifying formulations for European seabass have been reported recently (Serdoz *et al*. 2010).

Although the *F* of OTC has been found to be low in both species, the maximum plasma concentration after a single oral administration of 75 mg kg^{-1} fish has been found to be around 2.5 μg mL^{-1} (Rigos *et al*. 2004a). The direct effect of water temperature on the elimination of OTC is considerably apparent in the circulatory compartment of euryhaline fish (Rigos *et al*. 2002a) and in edible tissues of gilthead seabream (Romero Gonzales *et al*. 2010), suggesting that more than one medicated meals per day should be administered at high water temperatures.

After 6 days of oral administration of 75 mg kg^{-1} fish for 14 days at 19–28°C in gilthead seabream, tissue levels can reach values of 7.7 and 14.7 μg/g in skin and liver, respectively (Malvisi *et al*. 1996). However, the same authors report lower and surprisingly decreasing concentrations of OTC in muscle. Slow removal of OTC from

Table 5 Selected pharmacokinetics of antibacterials in European seabass and gilthead seabream

Drug	Route	Dose (mg kg^{-1})	Duration (days)	Weight (g)	Temp (°C)	$t_{1/2\beta}$ (h)	*F*%	C_{max} (μg mL^{-1})	*WT* (h)	References
European seabass										
OTC	IV	40		110	13	69				Rigos *et al*. (2002a)
OTC	IV	40		110	22	10				Rigos *et al*. (2002a)
OTC	OR-S	50		120	22		22	2.6		Rigos *et al*. (2004a)
DOX	OR-M	100	5	122	22			0.7		Rigos *et al*. (2020)
OA	IV	10		100	15	87				Poher *et al*. (2003)
OA	IV	15		110	14	315				Rigos *et al*. (2002b)
OA	IV	15		110	22	55				Rigos *et al*. (2002b)
FLU	IV	10		120	18	11				Rigos *et al*. (2002d)
ENR	OR-G	5		200–300	15			1.4		Intorre *et al*. (2000)
THI	OR-S	15–30		250–300				5.6–9.4		Castells *et al*. (2000)
THI	OR-M	15–30	5	250–350	18–20			0.9–1.3	144–120	Intorre *et al*. (2002)
THI	OR-M	40	5	128–150	20–28				89–80	Malvisi *et al*. (2002)
DAN	OR-M	10	5	16–27	16.9–21.1				168–96	Vardali *et al*. (2017)
LIN	OR-M	100	5	93	23			13		Rigos *et al*. (2020)
SPE	OR-M	50	5	152	26			1.3		Rigos *et al*. (2020)
Gilthead seabream										
OTC	OR-M	75	14	50–70	19–28				480	Malvisi *et al*. (1996)
OTC	IV	40		100	20	53				Rigos *et al*. (2003b)
OTC	OR-S	75		100	20		9	2.5		Rigos *et al*. (2003b)
OTC	OR-M	30	10	150–200	14–19.5				288–48	Romero Gonzales *et al*. (2010)
OTC	OR-M	37.5–75	7	75	18					Rosa *et al*. (2018)
OA	IV	20		100	20	12				Rigos *et al*. (2002c)
OA	OR-S	30		100	20		14	1.0		Rigos *et al*. (2002c)

Table 5 (continued)

Drug	Route	Dose (mg kg^{-1})	Duration (days)	Weight (g)	Temp (°C)	$t_{1/2\beta}$ (h)	*F*%	C_{max} (μg mL^{-1})	*WT* (h)	References
OA	OR-M	30	10	120–170	19	13–19		0.9	<24	Rigos *et al*. (2003a)
OA	OR-M	30	10	150–200	14–19.5			24		Romero Gonzales *et al*. (2010)
OA	OR-M	6–12	7	75	18					Rosa *et al*. (2018)
FLU	OR-M	12	5	60–80	25–28					Malvisi *et al*. (1997)
FLU	IV	10		170	19	30				Rigos *et al*. (2003c)
FLU	OR-S	20		170			29	1.7		Rigos *et al*. (2003c)
FLU	OR-M	35	5	237–307	18–24	22.1–21.4			107–76	Tyrpenou *et al*. (2003)
FLU	OR-M	30	10	150–200	14–19.5				24	Romero Gonzales *et al*. (2010)
FLU	OR-M	6–12	7	75	18					Rosa *et al*. (2018)
SAR	OR-M	10	5	163–237	18–25	2.5–17.8			42	Tyrpenou *et al*. (2002)
ENR	OR-S	10		150	25–27			2.8		della Rocca *et al*. (2004a)
FLO	OR-M	10	10	150	27				96	Di Salvo *et al*. (2013)
THI	OR-M	40	5	110–140	20–28				88–86	Malvisi *et al*. (2002)
SDZ	OR-M	30	10	150–200	14–19. 5				48	Romero Gonzales *et al*. (2010)
SDZ	OR-M	25	5	230	24–26			2.9–3.2	118–103	Rigos *et al*. (2013)
SDZ	OR-M	110–220	7	75	18					Rosa *et al*. (2018)
TRI	OR-M	30	10	150–200	14–19.5				24	Romero Gonzales *et al*. (2010)
TRI	OR-M	22–44	7	75	18					Rosa *et al*. (2018)
SDZ + TRI	OR-M	25 + 5	5	230	24–26				144–120	Zonaras *et al*. (2016)
SMX + OMP	OR-M	50	5		26					Papapanagiotou *et al*. (2002)
AMO	OR-S	80		120–160	22		0.3	1		della Rocca *et al*. (2004b)
AMO	OR-M	80	10	50–180	22–26					della Rocca *et al*. (2004b)

OTC, oxytetracycline; DOX, doxycycline; OA, oxolinic acid; FLU, flumequine; ENR, enrofloxacin; THI, thiamphenicol; DAN, danofloxacin; LIN, lincomycin; SPE, spectinomycin SAR, sarafloxacin; FLO, florfenicol; SDZ, sulfadiazine; TRI, trimethoprim; SMX, sulfadimethoxine; OMP, ormetoprim; AMO, amoxicillin.

$t_{1/2\beta}$: elimination half time; *F*:bioavailability; C_{max}: maximum concentration after oral dosing (single or multiple); *WT*: withdrawal times

IV: intravascular; OR-S: oral single dose; OR-M: oral multiple dose; OR-G: gavage

edible tissues after oral treatment was associated with longer withdrawal times (*WT*) (20-day period) in the same OTC-treated fish. Long *WT* were also suggested (12 days) by Romero Gonzales *et al*. (2010), who administered 30 mg kg^{-1} fish for 10 days at 14°C, although the recommended *WT* were as low as 2 days at 19.5°C.

Due to the relatively slow elimination of OTC at least in medium/low water temperatures, a sequential (every other day) dosing schedule of OTC in these species might be a more prudent and cost-effective alternative if adequate tissue levels are maintained in the treated fish.

The use of chlortetracycline (CTC) in fish disease treatments is limited and rarely considered in fish farming since it is not authorized for aquaculture despite its antimicrobial potency; the spectrum of antimicrobial activity and its kinetic profile in other food-producing animal species is comparable to OTC (EMEA 1995a).

Doxycycline (DOX) is a widely used antibacterial in terrestrial animals and might be a promising alternative to OTC. It has been used empirically in fish farming in some South-East Asian countries (Phu *et al*. 2016; Adhikary *et al*. 2018) but scientific information on its use in aquaculture is scarce. In recent studies (Rigos *et al*. 2020), DOX was tested in European seabass plasma following 5-day oral administration of 100 mg kg^{-1} fish (Table 5). The highest plasma values measured were around 0.7 μg mL^{-1}.

Since tetracyclines are bacteriostatic drugs with co-dependent action, but mostly concentration-dependent killing, the $AUC_{0\text{-}24}$/MIC PK/PD index would be the most appropriate (Table 4). However, most available studies on Mediterranean farmed fish, with a few exceptions, do not include calculations of the $AUC_{0\text{-}24}$ of administered tetracyclines. For DOX in particular, the AUC/MIC_{90} was more than 20 during the first days of treatment, based on

Table 6 MICs values of several antibacterials recorded on bacterial pathogens from European seabass (ESB) and gilthead seabream (GSB) available in pertinent literature

Strains	Source	Country	No strains tested	Media/Temperature of incubation	MIC (mg L^{-1}) range	MIC_{50} (mg L^{-1})	MIC_{90} (mg L^{-1})	Break point (mg L^{-1})	References
Oxytetracycline									
P. damselae subsp. *piscicida*	GSB, ESB	Italy	24	BHI 20°C for 48 h	0.08–10	0.15	10		Mazzolini *et al*. (2000)
P. damselae subsp. *piscicida*	GSB	Spain	16	CSMHB 26°C ± 2 for 48 h	0.25–32			≤1–>32	Martínez-Manzanares *et al*. (2008)
P. damselae subsp. *piscicida*	ESB	Italy	9	NB + 3% NaCl	62.5–250				Laganà *et al*. (2011)
V. alginolyticus	ESB	Turkey	15	CAMHB + 1% NaCl 22 ± 2°C and 28 ± 2°C	4–≥16				Korun *et al*. (2013)
V. alginolyticus	GSB, ESB	Tunisia	17	MH broth + 1% NaCl 37°C for 24 h	0.48				Lajnef *et al*. (2012)
Vibrio spp.	GSB	Italy	150	CAMHB + 1% NaCl 35°C for 18–20 h	0.12–128	1	4	<4–>16	Scarano *et al*. (2014)
Aeromonas spp.	GSB	Italy	104	CAMHB + 1% NaCl 35°C for 20 h	0.12–≥128	0.5	16	≤1–≥8	Scarano *et al*. (2014)
Tetracycline									
P. damselae subsp. *piscicida*	GSB	Spain	16	CSMHB 26°C ± 2 for 48 h	0.25–32			≤8–>32	Martínez-Manzanares *et al*. (2008)
V. harveyi	GSB, ESB	Italy	30	CAMHB 22 ± 2°C for 24 h	0.0625–0.125	0.25	0.25		Pretto (2018)
V. alginolyticus	GSB, ESB	Tunisia	17	MH broth + 1% NaCl 37°C for 24 h	0.48–0.96				Lajnef *et al*. (2012)
Vibrio spp.	GSB	Italy	150	CAMHB + 1% NaCl 35°C for 18–20 h	0.06–64	0.5	2	<4–>16	Scarano *et al*. (2014)
Aeromonas spp.	GSB	Italy	104	CAMHB + 1% NaCl 35°C for 20 h	0.12–≥128	0.5	4	≤1–≥8	Scarano *et al*. (2018)
Doxycycline									
P. damselae subsp. *piscicida*	GSB, ESB	Italy, Tunisia	22	CAMHB 30°C for 48 h	0.125–0.5	0.125	0.25		Rigos *et al*. (2020)
V. anguillarum	ESB	Italy	11	CAMHB 30°C for 24–28 h	0.125–0.5	0.125	0.25		Rigos *et al*. (2020)
V. alginolyticus	GSB, ESB	Tunisia	17	MH broth + 1% NaCl 37°C for 24 h	0.24–1				Lajnef *et al*. (2012)
V. harveyi	GSB, ESB	Italy	10	CAMHB 30°C for 24–28 h	0.125–0.25	0.125	0.25		Rigos *et al*. (2020)
T. maritimum	GSB, ESB	Italy	7	FMM 25°C for 48 h	0.25–0.5	0.25	0.5		Rigos *et al*. (2020)
Flumequine									
P. damselae subsp. *piscicida*	GSB, ESB	Italy	24	BHI 20°C for 48 h	0.04–2.5	0.3	1.25		Mazzolini *et al*. (2000)
P. damselae subsp. *piscicida*	GSB			MH broth + 2% NaCl - CAMHB	0.3 (2% NaCl) or >38.25 (+cation)				Rigos *et al*. (2003a,b)

Table 6 (continued)

Strains	Source	Country	No strains tested	Media/Temperature of incubation	MIC (mg L^{-1}) range	MIC_{50} (mg L^{-1})	MIC_{90} (mg L^{-1})	Break point (mg L^{-1})	References
P. damselae subsp. *piscicida*	GSB	Spain	16	CSMHB 26°C ± 2 for 48 h	0.5–4				Martínez-Manzanares *et al.* (2008)
P. damselae subsp. *piscicida*	ESB	Italy	9	NB + 3% NaCl	0.97–62.5				Laganà *et al.* (2011)
V. anguillarum	GSB, ESB	Greece	1	MH broth + 2% NaCl - CAMHB	0.15 (2% NaCl) or 4.78 (+cation)				Rigos *et al.* (2003a,b)
V. alginolyticus	GSB, ESB	Greece	1	MH broth + 2% NaCl - CAMHB 22°C	1.2 (2% NaCl) or 38.25 (+cation)				Rigos *et al.* (2003a,b)
V. harveyi	GSB, ESB	Italy	30	CAMHB 22 ± 2°C for 24 h	<0.25–0.5	<0.25	0.5		Pretto (2018)
Vibrio spp.	GSB	Italy	150	CAMHB + 1% NaCl 35°C for 18–20 h	0.06–128	0.5	4	<2–>4	Scarano *et al*. (2014)
V. damsel	GSB, ESB	Greece	1	MH broth + 2% NaCl - CAMHB 22°C	0.019 (2% NaCl) or 0.15 (+cation)				Rigos *et al.* (2003a,b)
V. fluvialis	GSB, ESB	Greece	1	MH broth + 2% NaCl - CAMHB 22°C	0.15 (2% NaCl) or 4.78 (+cation)				Rigos *et al.* (2003a,b)
Aeromonas spp.	GSB	Italy	104	CAMHB + 1% NaCl 35°C for 20 h	0.06–≥128	0.12	16	≤2–≥4	Scarano *et al*. (2018)
Oxolinic acid									
P. damselae subsp. *piscicida*	GSB, ESB	Italy	24	BHI 20°C for 48 h	0.04–2.5	0.08	1.25		Achene *et al.* (2000)
P. damselae subsp. *piscicida*	GSB	Spain	16	CSMHB 26°C ± 2 for 48 h	0.25–16				Martínez-Manzanares *et al.* (2008)
P. damselae subsp. *piscicida*	ESB	Italy	10	NB + 3% NaCl	3.9–62.5				Laganà *et al.* (2011)
Vibrio spp.	GSB	Italy	150	CAMHB + 1% NaCl 35°C for 18–20 h	0.06–32	0.25	4	<4–>8	Scarano *et al*. (2014)
Aeromonas sp	GSB	Italy	104	CAMHB + 1% NaCl 35°C for 20 h	0.06–≥128	0.06	16	≤0.12–≥1	Scarano *et al*. (2018)
Enrofloxacin									
P. damselae subsp. *piscicida*	GSB, ESB	Italy	24	BHI 20°C for 48 h	0.04–0.15	0.04	0.15		Achene *et al.* (2000)
P. damselae subsp. *piscicida*	GSB	1996/2000 Spain	16	CSMHB 26°C ± 2 for 48 h	0.25–0.5				Martínez-Manzanares *et al.* (2008)
V. harveyi	GSB, ESB	Italy 2011–2018	30	CAMHB 22 ± 2°C for 24 h	0.03125–0.25	0.125	0.25		Pretto (2018)
Tenacibaculum maritimum	GSB, ESB	Spain-France	7	FMM 22°C for 24–48 and 44–48 h	0.5–32			4	Avendaño-Herrera *et al.* (2006)
Florfenicol									

Table 6 (continued)

Strains	Source	Country	No strains tested	Media/Temperature of incubation	MIC (mg L^{-1}) range	MIC_{50} (mg L^{-1})	MIC_{90} (mg L^{-1})	Break point (mg L^{-1})	References
P. damselae subsp. *piscicida*	GSB, ESB	Italy	24	BHI 20°C for 48 h	0.3–0.6	0.3	0.6		Mazzolini *et al*. (2000)
V. alginolyticus	GSB, ESB	Tunisia	17	MH broth + 1% NaCl 37°C for 24 h	0.48–0.96				Lajnef *et al*. (2012)
V. harveyi	GSB, ESB	Italy	30	CAMHB 22 ± 2°C for 24 h	<1–16	<1	<1		Pretto (2018)
Vibrio spp.	GSB	Italy	150	CAMHB + 1% NaCl 35°C for 18–20 h	0.06–32	0.5	4	<2–>8	Scarano *et al*. (2014)
Aeromonas spp.	GSB	Italy	104	CAMHB + 1% NaCl 35°C for 20 h	0.06–64	1	4	≤4–≥8	Scarano *et al*. (2018)
Thiamphenicol									
P. damselae subsp. *piscicida*	GSB, ESB	Italy	24	BHI 20°C for 48 h	2.5–100	5	100		Mazzolini *et al*. (2000)
Trimethoprim									
P. damselae subsp. *piscicida*	GSB, ESB	Italy	24	BHI 20°C for 48 h	0.15–>100	1.25	>100		Mazzolini *et al*. (2000)
V. alginolyticus	ESB	Turkey	15	CAMHB + 1% NaCl (22 ± 2 and 28 ± 2°C	≤8				Korun *et al*. (2013)
V. alginolyticus	GSB, ESB	Tunisia	17	MH broth + 1% NaCl 37°C for 24 h	0.48–16				Lajnef *et al*. (2012)
Vibrio spp.	GSB	Italy	150	CAMHB + 1% NaCl 35°C for 18–20 h	0.06–128	4	64	<8–>16	Scarano *et al*. (2014)
Aeromonas spp.	GSB	Italy	104	CAMHB + 1% NaCl 35°C for 20 h	0.12–≥128	32	64	≤8–≥16	Scarano *et al*. (2018)
Sulfadimethoxine									
Vibrio spp.	GSB	Italy	150	CAMHB + 1% NaCl 35°C for 18–20 h	0.12–>512	>512	>512	<256–>512	Scarano *et al*. (2014)
Aeromonas spp.	GSB	Italy	104	CAMHB + 1% NaCl 35°C for 20 h	0.12–≥256	≥256	≥256	≤38–≥76	Scarano *et al*. (2018)
Trimethoprim - Sulfadimethoxine									
P. damselae subsp. piscicida	GSB, ESB	Italy	24	BHI 20°C for 48 h	1.25–>100	5	>100		Mazzolini *et al*. (2000)
V. harveyi	GSB, ESB	Italy	30	CAMHB 22 ± 2°C for 24 h	<0.0625/1.1875–1/19	<0.0625/1.1875	0.125/2.375		Pretto (2018)
Amoxicillin									
P. damselae subsp. *piscicida*	ESB	Italy	9	NB + 3% NaCl	7.8–125				Laganà *et al*. (2011)
Vibrio spp.	GSB	Italy	150	CAMHB + 1% NaCl 35°C for 18–20 h	0.06–>128	>128	>128	<8–>32	Scarano *et al*. (2014)

Table 6 (continued)

Strains	Source	Country	No strains tested	Media/Temperature of incubation	MIC (mg L^{-1}) range	MIC_{50} (mg L^{-1})	MIC_{90} (mg L^{-1})	Break point (mg L^{-1})	References
Aeromonas sp	GSB	Italy	104	CAMHB + 1% NaCl 35°C for 20 h	0.06–≥128	16	≥128	≤8–≥32	Scarano *et al*. (2018)
Ampicillin									
P. damselae subsp. *piscicida*	GSB, ESB	Italy	24	BHI 20°C for 48 h	0.04–>100	0.04	100		Mazzolini *et al*. (2000)
V. alginolyticus	GSB, ESB	Tunisia	17	MH broth + 1% NaCl 37°C for 24 h	128–256				Lajnef *et al*. (2012)
V. harveyi	GSB, ESB	Italy	30	CAMHB 22 ± 2°C for 24 h	32–>32	>32	>32		Pretto (2018)
Vibrio spp.	GSB	Italy	150	CAMHB + 1% NaCl 35°C for 18–20 h	0.06–>128	>128	>128	<8–>32	Scarano *et al*. (2014)
Aeromonas spp.	GSB	Italy	104	CAMHB + 1% NaCl 35°C for 20 h	0.06–≥128	16	≥128	≤8–≥32	Scarano *et al*. (2018)
Streptomycin									
P. damselae subsp. *piscicida*	GSB, ESB	Italy	24	BHI 20°C for 48 h	1.25–>100	5	>100		Mazzolini *et al*. (2000)
Vibrio spp.	GSB	Italy	150	CAMHB + 1% NaCl 35°C for 18–20 h	0.25–>128	8	32	<6–>25	Scarano *et al*. (2014)
Aeromonas spp.	GSB	Italy	104	CAMHB + 1% NaCl 35°C for 20 h	1–≥128	16	64	≤6–≥25	Scarano *et al*. (2018)
Erythromycin									
V. alginolyticus	GSB, ESB	Tunisia	17	MH broth + 1% NaCl 37°C for 24 h	16–64				Lajnef *et al*. (2012)
Vibrio spp.	GSB	Italy	150	CAMHB + 1% NaCl 35°C for 18–20 h	0.06–>128	8	128	<0.5–>8	Scarano *et al*. (2014)
Aeromonas spp.	GSB	Italy	104	CAMHB + 1% NaCl 35°C for 20 h	0.06–≥128	16	64	≤0.5–≥8	Scarano *et al*. (2018)
Gentamicin									
V. harveyi	GSB, ESB	Italy	30	CAMHB 22 ± 2°C for 24 h	0.5–4	1	2		Pretto (2018)
Vibrio spp.	GSB	Italy	150	CAMHB + 1% NaCl 35°C for 18–20 h	0.06–>128	2	4	<4–>16	Scarano *et al*. (2014)
Aeromonas spp.	GSB	Italy	104	CAMHB + 1% NaCl 35°C for 20 h	0.12–32	2	8	≤4–≥16	Scarano *et al*. (2018)
Kanamycin									
V. alginolyticus	GSB, ESB	Tunisia	17	MH broth + 1% NaCl 37°C for 24 h	32–64				Lajnef *et al*. (2012)
Vibrio spp.	GSB	Italy	150	CAMHB + 1% NaCl 35°C for 18–20 h	0.25–>128	8	16	<16–>64	Scarano *et al*. (2014)
Aeromonas spp.	GSB	Italy	104	CAMHB + 1% NaCl 35°C for 20 h	0.5–≥128	8	32	≤16–≥64	Scarano *et al*. (2018)

Table 6 (continued)

Strains	Source	Country	No strains tested	Media/Temperature of incubation	MIC (mg L^{-1}) range	MIC_{50} (mg L^{-1})	MIC_{90} (mg L^{-1})	Break point (mg L^{-1})	References
Cefotaxime									
V. alginolyticus	GSB, ESB	Tunisia	17	MH broth + 1% NaCl 37°C for 24 h	0.48–0.96				Lajnef *et al.* (2012)
Cephalothin									
Vibrio spp.	GSB	Italy	150	CAMHB + 1% NaCl 35°C for 18–20 h	0.06–>128	16	>128	<8–>32	Scarano *et al.* (2014)
Aeromonas spp.	GSB	Italy	104	CAMHB + 1% NaCl 35°C for 20 h	0.06–≥128	>128	>128	≤8–≥32	Scarano *et al.* (2018)
Ceftiofur									
P. damselae subsp. *piscicida*	GSB, ESB	Italy	24	BHI 20°C for 48 h	0.04	0.04	0.04		Mazzolini *et al.* (2000)
Nalidixic acid									
V. alginolyticus	GSB, ESB	Tunisia	17	MH broth + 1% NaCl 37°C for 24 h	0.48–0.96				Lajnef *et al.* (2012)
Colistin									
V. harveyi	GSB, ESB	Italy	30	CAMHB 22 ± 2°C for 24 h	0.25–>8	>8	>8		Pretto (2018)
Apramycin									
V. harveyi	GSB, ESB	Italy	30	CAMHB 22 ± 2°C for 24 h	4.–8	4	8		Pretto (2018)
Aminosidine									
V. harveyi	GSB, ESB	Italy	30	CAMHB 22 ± 2°C for 24 h	2–8.	4	4		Pretto (2018)
Lincomycin									
P. damselae subsp. *piscicida*	GSB, ESB	Italy, Tunisia	22	CAMHB 30°C for 48 h	4–8	8	8		Rigos *et al.* (2020)
V. anguillarum	ESB	Italy	11	CAMHB 30°C for 24 h	8–32	32	32		Rigos *et al.* (2020)
V. harveyi	GSB, ESB	Italy	10	CAMHB 30°C for 24 h	32–128	64	128		Rigos *et al.* (2020)
T. maritimum	GSB, ESB	Italy	7	FMM 25°C for 48 h	0.12–0.5	0.12	0.5		Rigos *et al.* (2020)
Spectinomycin									
P. damselae subsp. *piscicida*	GSB, ESB	Italy, Tunisia	22	CAMHB 30°C for 48 h	8–32	16	32		Rigos *et al.* (2020)
V. anguillarum	ESB	Italy	11	CAMHB 30°C for 24 h	8–16	16	16		Rigos *et al.* (2020)
V. harveyi	GSB, ESB	Italy	10	CAMHB 30°C for 24 h	14–64	32	64		Rigos *et al.* (2020)
T. maritimum	GSB, ESB	Italy	7	FMM 25°C for 48 h	16–32	16	32		Rigos *et al.* (2020)

BHI (brain heart infusion), CSMHB (cation-supplemented Mueller-Hinton broth), NB (nutrient broth), CAMHB (cation-adjusted Mueller–Hinton broth), MH (Mueller–Hinton broth), FMM (*F. maritimus* medium)

relatively low MIC values of important bacterial pathogens (Table 6). These promising *in vitro* DOX values were observed in laboratory trials where the oral administration of compounds appeared to be very effective against heavy *V. harveyi* infections of European seabass juveniles (Rigos *et al.*, laboratory observations).

(Fluoro)quinolones

Quinolones, such as OA and fluoroquinolones (first-generation derivatives of quinolones) including FLU, sarafloxacin (SAR) and danofloxacin (DAN), are synthetic modern antibacterials that are effective against a broad spectrum of systemic infections by gram-negative bacteria. They act by interfering with bacterial DNA gyrase, preventing completion of the super-coiling of bacterial chromosomes, and are characterized by post-antibacterial action in a dose-dependent manner.

The kinetic profiles of some quinolones and fluoroquinolones have been widely investigated in European seabass and gilthead seabream (Table 5). Interestingly, OA absorption studies in European seabass have revealed higher digestion values (64–92%) (Rigos *et al.* 1999) compared with calculated *F* in gilthead seabream (14–15%; Rigos *et al.* 2002c), indicating species-specific absorption differences. This is a very important aspect for specific OA treatments, including dosage, in European seabass or gilthead seabream. Concerning the absorption of this antibacterial class, FLU has been shown to be more bioavailable (Rigos *et al.* 2003c) than OA in gilthead seabream, This might indicate that FLU is preferable to OA in diseased gilthead seabream, assuming there are no differences in bacterial sensitivity to these two drugs.

Accordingly, plasma OA levels following single or multiple oral dosing in gilthead seabream, were found to be <1 μg mL^{-1} (Rigos *et al.* 2002c, 2003a), while the respective values for FLU after single dosing in gilthead seabream were 1.7 μg mL^{-1} (Rigos *et al.* 2003c). This difference may be attributable to the greater *F* of FLU in gilthead seabream.

Regarding the removal of OA in gilthead seabream, it has been shown that other than muscle, tissues such as liver, bile and skin may act as reservoirs of the drug with rapid depletion below the MRL of 100 μg kg^{-1} (EMEA 2005), as fast as 24 h after completion of the treatment at 19°C (Rigos *et al.* 2003a). The rapid depletion of OA from the edible tissues of the same species was confirmed by Romero Gonzales *et al.* (2010) who administered 30 mg kg^{-1} fish for 10 days at 14–19.5°C. The fast clearance of OA is advantageous, allowing fish to enter the market more rapidly.

As in the case of OA, Malvisi *et al.* (1997) reported that the skin and vertebrae of gilthead seabream act as reservoirs for FLU for prolonged periods even after cessation of treatment (12 mg kg^{-1} fish for 5 days), although drug levels remain below the MRL of 600 μg kg^{-1} (EMEA 2002c). The same study revealed consumer safe levels in edible tissues (muscle plus skin) even 24 h post-treatment at 25–28°C. This finding is in agreement with Romero Gonzales *et al.* (2010) who administered 30 mg kg^{-1} fish for 10 days at 14–19.5°C. In contrast, higher FLU levels in the same tissues necessitated the calculation of *WT* of 106 and 76 h at 18–24°C, respectively (Tyrpenou *et al.* 2003).

Tissue distribution studies of SAR in gilthead seabream have revealed accumulation in liver and vertebrae, with the vertebrae acting as a reservoir for the drug since the levels persist after the treatment has ended (Tyrpenou *et al.* 2002). In edible tissues, *WT* were 42 h at 25°C for a MRL of 30 μg kg^{-1} (EMEA 1998). The rapid depletion of SAR from medicated fish tissues is a favourable characteristic, allowing shorter *WT* of treated fish. However, distribution of SAR in fish circulation, along with pharmacodynamic data, is required to obtain a complete picture of its potential efficacy against European seabass and gilthead seabream pathogens.

Oral treatment (gavage) with 5mg enrofloxacin (ENR) per kg/fish resulted in C_{max} of 1.4 μg mL^{-1} in European seabass plasma kept at 15°C (Intorre *et al.* 2000), while after IV injection or single oral administration of 2.5 and 10 mg kg^{-1} fish in the figure was 3.8 and 1.2 μg mL^{-1}, respectively, in gilthead seabream serum maintained at 25–27°C (della Rocca *et al.* 2004a).

DAN was investigated in European seabass after multiple (5 days) in-feed administration (10 mg kg^{-1}) at 16 and 27°C (Vardali *et al.* 2017). Withdrawal times in muscle plus skin were estimated to be 4 and 7 days for the high and low temperature, respectively (MRL = 100μg kg^{-1}; EMEA 2002a). As in the case of SAR, distribution of DAN in fish circulation and pharmacodynamic data are lacking for euryhaline fish species.

Rapid elimination of quinolones and fluoroquinolones (Table 5) from the tissues of gilthead seabream suggests that at least daily dosing (two meals per day in certain cases) is required, especially at higher water temperatures where depletion is faster, to maintain maximum tissue concentrations in euryhaline fish. Since quinolones are bactericidal drugs with concentration-dependent action ($AUC_{0\text{-}24}$/MIC, C_{max}/MIC), dosages should be maximised wherever possible (Table 4). Finally, it should also be taken into account that quinolones are recently considered by the World Health Organization as critically important high-priority antimicrobials (WHO 2017). Thus, their use in farm animals will decrease gradually and eventually banned.

Potentiated sulphonamides

Potentiated sulphonamides (SFM) (sulfadiazine: SDZ, sulfamethoxazole: SMX and sulfadimethoxine: SDM) are generally administered in combination with

diaminopyrimidines (DAP), such as trimethoprim (TRM) and ormetoprim (OMP), in a concentration ratio of 5:1 in order to increase the SFM antibacterial potency due to DAP inhibition of tetrahydrofolic acid formation. Potentiated sulfadiazine antibacterials have a broad spectrum of bactericidal activity against bacterial pathogens, and their combined efficacy is greater than the sum of the potencies of any two separate drugs. They interfere with the nucleic acid metabolism of bacteria, by acting as competitive inhibitors of folic acid metabolism. Sulfadiazine plus TRM is the most commonly used combination of potentiated sulphonamides in veterinary medicine, and they are widely used in fish medicine. The recommended dosage of potentiated sulfadiazine in fish treatments is 25 and 5 mg kg^{-1} fish (for 5–10 days) for SDZ and TRM, respectively (EMEA 1995b, 1997b).

There are several PK studies on sulphonamides/potentiated sulphonamides in gilthead seabream but none in European seabass (Table 5). The distribution of SDZ (25 mg kg^{-1} fish for 5 days at 24–26°C) in gilthead seabream circulation was promising, reaching values as high as 2.9–3.2 μg mL^{-1} plasma in fish receiving fish or plant-oil based diets (Rigos *et al.* 2013). The *WT* to reach consumer safety levels (MRL of 100 μg kg^{-1}: (EMEA 1995b) were 103 and 118 h for the two treated groups, respectively. Interestingly, in the same study, N4-acetylation was found to be the major metabolic pathway of SDZ in gilthead seabream fillet. Depletion of both SDZ (30 mg kg^{-1} fish for 10 days) and TRM (30 mg kg^{-1} fish for 10 days) was reported to be rapid in gilthead seabream maintained at either 14 or 19.5°C, with levels falling below MRLs, 2 and 1 day post-administration for the two antibacterials, respectively (Romero Gonzales *et al.* 2010). Similarly, fast removal of SDM delivered in combination 5:1 with OMP at 50 mg kg^{-1} fish for 5 days was apparent in gilthead seabream kept at 26°C. The drug levels dropped below MRLs (50 and 100 μg kg^{-1} MRLs for OMP and SDM, respectively, (EMEA 1995b, 1997b) 24 h following treatment (Papapanagiotou *et al.* 2002). Longer *WT* (5–6 days) for SDZ and TRM have been recommended for this fish species by Zonaras *et al.* (2016) at 24–26°C.

Potentiated SFM drugs possess bactericidal effects with a time-dependent killing profile ($T_{C>}$MIC) (Table 4). In this case, the dosing schedule must reach drug concentrations at the infected sites exceeding the MIC for the longest possible time. Consequently, the dosage regime should aim at dividing the daily doses into two or more administrations.

Penicillin derivatives

Penicillin derivatives (β-lactams), including amoxicillin (AMO) and ampicillin (AMP), are broad-spectrum antibacterial agents widely used in human and many domestic and livestock animals. β-lactams exhibit bactericidal-time-dependent action by inhibiting bacterial cell wall synthesis (Table 4). The usual dosage of β-lactams in fish treatments is 40–80 mg kg^{-1} fish for 5–10 days (della Rocca *et al.* 2004b).

Penicillin derivatives have not been widely employed in euryhaline fish farming, probably due to the fact that they have not been authorized for use in aquaculture in most Mediterranean countries and that there is a lack of relevant PK fish studies. The kinetic profile and efficacy of AMP have not been investigated in euryhaline fish species. However, published pharmacodynamics for AMO indicate that this drug is a promising antibacterial against important bacterial pathogens of euryhaline fish (Mazzolini *et al.* 1997). Amoxicillin displayed negligible bioavailability (0.33%) in gilthead seabream (della Rocca *et al.* 2004a; Table 5), thus questioning its use in this species, at least by oral administration. However, kinetic studies in European seabass may demonstrate improved AMO absorption, but this remains to be scientifically proved.

As for SFM drugs, penicillin drugs have a bactericidal effect with a time-dependent killing profile ($T_{C>}$MIC; Table 4). The same suggestion as for potentiated sulphonamides, must be given herein for the dosage regimen in order to divide the daily dose during the daytime.

Phenicols

Chloramphenicol (CAP) derivatives, including florfenicol (FLO) and thiamphenicol (THI), are primary bacteriostatic broad-spectrum compounds that inhibit bacterial protein synthesis by binding to the 50s subunit of the bacterial ribosome. Both antibacterial agents have been used in veterinary medicine without serious adverse effects, for example aplastic anaemia that has been observed with the use of CAP, leading to a ban on its use in food-producing animals (EC 1430/94). The recommended dosage of FLO and THI against bacterial fish diseases is 10–15 and 15–40 mg kg^{-1} fish for 10 and 5 days, respectively (EMEA 2002d, 2006).

There are no publications on the kinetic profile during treatment and the efficacy of FLO in euryhaline fish species. However, preliminary trials on orally administered FLO in European seabass revealed plasma concentrations of around 1.6 μg mL^{-1} following dosing of 10 mg kg^{-1} fish for 7 days (Kogiannou *et al.* 2021). In the same work, the *WT* in edible tissues was less than 24 h in fish kept at 20°C. Similarly, in gilthead seabream, FLO levels in muscle plus skin dropped below the MRL (1000 μg kg^{-1}; EMEA 2002d) on day 2 post-treatment after a dosing of 10 mg kg^{-1} fish for 10 days at 27°C (Di Salvo *et al.* 2013).

Nevertheless, there are several studies on the kinetics of THI in European seabass (Castells *et al.* 2000; Intorre *et al.* 2002; Malvisi *et al.* 2002) and gilthead seabream (Malvisi *et al.* 2002; Table 5). Following oral administration of THI (gavage), peak plasma THI concentration was as high as 5.6

and 9.4 μg mL^{-1} in European seabass, following 15 and 30 mg kg^{-1} dosing, respectively, indicating dose-dependent absorption (Castells *et al.* 2000). However, maximal plasma THI levels following 5-day treatment administered in-feed, were found to be considerably lower for both dosing levels at 0.8 and 1.3 μg mL^{-1}, respectively (Intorre *et al.* 2002).These differences may be due to the different routes of administration employed, and the influence of feed components on THI absorption. The lower drug levels attained in treated fish in the latter study are more representative of 'at site' treatments where drugs are delivered via the feed, indicating that the absorption of THI is inhibited in the gut environment.

Regarding the distribution of THI in gilthead seabream, it was found that it is well-distributed in the tissue compartments following 5-day dosing at 40 mg kg^{-1} at 20–28°C (Malvisi *et al.* 2002). Intorre *et al.* (2002) suggested *WT* of 120 and 144 h for a 5-day dosing of 15 and 30 mg kg^{-1}, respectively, at 18–20°C, considering an MRL of 50 ng/g (EMEA 2006). Similar *WT* (80 and 89 h) were proposed for THI by the trial of Malvisi *et al.* (2002) in European seabass and gilthead seabream, respectively (Table 5). Phenicols are bacteriostatic compounds with time-dependent action as in the case of SFM and penicillins (Table 4), thus, the same conclusions must be drawn as regards to the dosage regimens.

Lincosamides

Lincomicin (LCM) is a natural antibacterial drug obtained from *Streptomyces lincolnensis*. It interferes in protein synthesis by binding to the 50s ribosomal subunit at the same site where phenicols and macrolide drugs bind. It is active against gram-positive bacteria and some gram-negative anaerobes. No data about the PK of this drug in fish was found in the accessible literature.

The absorption of LCM in the circulation of European seabass has been studied recently (Rigos *et al.* 2020). Specifically, LCM displayed a promising distribution profile in fish plasma following 5 days oral administration of 100 mg kg^{-1} fish (Table 5). The highest concentrations were as high as 13 μg mL^{-1}. The $T_{C>}$MIC has been suggested as the most appropriate for lincosamides (Table 4).

Aminocyclitols

Spectinomycin (SPE) is an aminocyclitol closely related to the aminoglycoside antibacterial group. It binds to the 30s ribosomal subunit of the bacteria inhibiting protein synthesis. It is a broad-spectrum drug acting against gram-positive and some gram-negative aerobic bacteria.

Absorption has also been measured in European seabass plasma (Rigos *et al.* 2020). In that trial, SPE displayed an adequate distribution profile in 5 days following oral administration of 50 mg kg^{-1} fish (Table 5). The highest concentrations reached 1.3 μg mL^{-1}. The C_{max}/MIC ratio has been suggested for aminocyclitols (Table 4).

Pharmacodynamics (PD) of antibacterials used against bacterial pathogens of european seabass and gilthead seabream

Minimum inhibitory concentrations (MIC)

The MIC values against the target bacterium have, almost universally, been treated as the key PD parameter with respect to dose optimization (Lees *et al.* 2006). Generally, in medicine, there have been attempts to design dosage regimes that would allow treatment of species-specific bacterial infections that manifest less than full susceptibility. In these attempts, the MIC_{50} or MIC_{90} has been used. If the design criteria for any therapy should be capable of achieving clinical success when applied to infections by sensitive bacteria, the MIC data required would be those for fully sensitive strains. Unfortunately, with respect to the bacteria encountered in aquaculture there are, at present, no validated clinical breakpoints allowing empirical identification of sensitive strains, with the exception of *A. salmonicida* in salmonids (CLSI M-42M-49 S1, 2010). In this situation, the MIC of fully susceptible strains would be the most valuable data set. Fully susceptible strains can be identified by using ECOFF values determined from the distribution of susceptibility measurements for a number of strains (Kahlmeter *et al.*, 2003; Miller & Reimschuessel 2006).

When determined in this way, these fully susceptible strains are referred to as wild type. However, the available information on MIC values and ECOFF values for the antibacterials used and the relevant bacterial pathogens in gilthead seabream and European seabass or for Mediterranean cultured marine finfish species in general, is scarce. Most of the available data is from specific research studies and these studies frequently focus on specific antibacterials or bacteria. In contrast, background data is perhaps available, namely, data owned by private companies, microbiology laboratories, fish health consultants and specialists, as most of them perform antibiogram tests (Kirby-Bauer-disc diffusion method), as a complementary technique in their routine microbiological checks. Unfortunately, the Kirby-Bauer-disc diffusion method has not been fully standardized for all fish bacteria.

Another limitations as regards the use of data obtained by the Kirby-Bauer method is that the results are usually qualified as 'resistant', 'sensitive' or 'intermediate', that is according to a qualitative classification (without recording the size of the inhibition zone in mm), based on the recommendations of the company supplying commercial discs for the diffusion tests. These recommendations establish different 'breakpoints', according to the inhibition diameter of each antibacterial and concentration. However, it

should be noted that most of this information is obtained from the extrapolation of breakpoints in human and, sometimes, veterinary medicine (with regard to terrestrial animals). In addition, this is based on data obtained from the European Committee on Antimicrobial Susceptibility Testing, the BSAC (1991), or similar platforms and not from specific databases based on microbiological, pharmacological and clinical data relating to fish and fish pathogens. This is a very relevant handicap for antibacterial fish disease treatment as these 'commercial breakpoints' does not take into account the differences between human, terrestrial animal and fish bacterial pathogens, and also the differences between PK and the therapeutic efficacy of the treatments. In some cases, clinical fish disease practitioners have to rely on their own experience in for more realistic interpretation of these values.

Thus, the practical approach suggested by Bonev *et al.* (2008) as regards the use of regression analysis of the inhibition zone sizes plotted against the natural logarithm of antibacterial concentration to provide an estimate of the MIC is no longer considered applicable. In light of these issues, the definition of MIC values obtained by standard methods is an essential tool to define breakpoint values, which are necessary in diagnostics for correct and responsible antibacterial use in aquaculture. It is also important to bear in mind that the definition of MIC values in routine diagnostics also allows monitoring of antimicrobial resistance trends in aquaculture (in our case, Mediterranean Aquaculture). Increasing antibacterial resistance is one of the most relevant problems set out in the WHO framework (http://www.euro.who.int/en/health-topics/disease-prevention/antimicrobial-resistance/about-amr/one-health), and many scientific works have addressed the emergence of antibacterial resistances in aquaculture (Miranda *et al.* 2018), including Mediterranean aquaculture (Chelossi *et al.* 2003). It should be noted that limited MIC information is currently reported in the literature about the impact of antibacterials on the different bacterial pathogens of European seabass and gilthead seabream (Table 6).

In general, most of the information does not include a species identification or the source of infection. Research on the MIC values of *P. damselae* subsp. *piscicida* (Mazzolini *et al.* 2000; Rigos *et al.* 2003, 2020; Martínez-Manzanares *et al.* 2008; Laganà *et al.* 2011), *V. anguillarum* (Rigos *et al.* 2003, 2020), *V. harveyi* (Pretto 2018; Rigos *et al.* 2020) and *T. maritimum* (Avendaño-Herrera *et al.* 2006; Rigos *et al.* 2020) in European seabass and gilthead seabream in the Mediterranean area and the number of strains tested is often limited.

With respect to the media used and the incubation time, the data are not homogeneous. The MIC value testing for *P. damselae* subsp. *piscicida* was performed in Brain Heart Infusion Broth (BHI; Mazzolini *et al.* 2000), Nutrient Broth (NB) supplemented with 3% NaCl (Laganà *et al.* 2011), Mueller-Hinton Broth + 2% NaCl or Mueller-Hinton Cation Adjusted (CAMHB or CSMHB) Broth (Rigos *et al.* 2003, 2020; Martínez-Manzanares *et al.* 2008). Concerning *V. anguillarum* and *V. harveyi*, CAMHB and MH + 2% NaCl were used (Rigos *et al.* 2003, 2020; Pretto 2018), while for other *Vibrio* species MH broth + 1% or 2% NaCl was used (Rigos *et al.* 2003; Lajnef *et al.* 2012), CAMHB + 1% NaCl (Korun *et al.* 2013; Scarano *et al.* 2014). *Flexibacter maritimum* media (FMM) was used for *T. maritimum* (Avendaño-Herrera *et al.* 2006; Rigos *et al.* 2020). In addition, the breakpoints (both clinical and epidemiological) relating to the different molecules tested are not always reported.

What is apparent is the huge variation of the MIC values (Table 6) of a single antibacterial against several strains of the same bacterial pathogen. This is clearly the case of OTC MICs with *P. damselae subsp. piscicida* and several *Vibrio* spp. with few exceptions. On the other hand, DOX has exhibited low (small range) values in Lajnef *et al.* (2012) and Rigos *et al.* (2020), when tested against the above list of pathogens, including *T. maritimum*. Similar findings are evident in quinolone drugs such as OA, FLU, ENR and FLO, sulphonamides, potentiated sulphonamides and other antibacterials reviewed in Table 6. In Rigos *et al.* (2020), the MICs of LIN and SPE against *V. anguillarum*, *V. harveyi* and *P. damselae* subsp. *piscicida* were relatively high as opposed to the one observed for *T. maritimum* in SPE. For all these reasons, the data available in scientific literature are insufficient and incomparable. Therefore, actions aimed at increasing the number of bacteria strains isolated from European seabass and gilthead seabream during recent outbreaks or stored in bacterial collections appear necessary. These need to be subjected to MIC studies using several antibacterial molecules, whether authorized or candidates for future application in aquaculture, and the procedures should be standardized, as suggested by Smith (2019).

Optimization of antibacterial treatments and application of PK/PD to the rational design of treatment regimen in fish

The primary aim of antibacterial therapy in aquaculture is to limit the economic losses that might result from the impact of bacterial infections on the health and survival of farmed animals. It follows that the goal of dose regime design in aquaculture must be not only to reduce losses but also, and importantly, to do so in a cost-effective manner respecting environmental and social responsibility. There are also other issues to be considered as to the decision on the treatment, such as the severity of the disease and the condition of the sick fish and their ability to withstand the treatment.

In contrast to human medicine and clinical veterinary medicine (e.g. dogs, cats, horses), most treatments in aquaculture are '*sensu stricto*' metaphylactic in that they are administered to particularly large populations, which include both infected and uninfected individuals. The concept of metaphylaxis is not always well understood as, in some cases, it is mistakenly defined as mass treatment of a group of animals in advance of an expected disease outbreak. Based on a recent Regulation of the European Parliament and of the Council (2019/4) regarding the use of veterinary medicinal products, metaphylaxis means 'the administration of a medicinal product to a group of animals after a diagnosis of clinical disease in part of the group has been established, with the aim of treating the clinically sick animals and controlling the spread of the disease to animals in close contact and at risk and which may already be subclinically infected'. Metaphylactic treatments should always be understood as a control treatment and should be clearly differentiated from therapeutic treatments (curative) and prophylactic treatments (preventive): i) metaphylactic treatment (control) is actually delivered to a group of animals that is developing a disease, while ii) prophylactic treatment (preventive) is delivered to a group of animals that are still healthy but with a real risk to develop a disease, and lastly iii) therapeutic treatment (curative) is delivered individually to sick animals. This differentiation is particularly important as metaphylactic treatments present many unique and to a large extent, unresolved problems for the design of therapy regime (Smith 2008).

The use of medicated feed in finfish aquaculture is perhaps the most paradigmatic and clear example of the application of metaphylaxis in animal medicine. It is important to stress the fact that the main target in metaphylactic treatments in aquaculture is not the affected or sick fish in a specific stock. The main target of these treatments is the fish incubating the disease when an infectious disease outbreak has been triggered in a farm.

Any attempt to place the dose regime used in oral antimicrobial therapy on sound, empirical and rational grounds requires a combination of PK and PD data on the interaction between the antimicrobial agent and the target bacterium (Craig 2002; Drusano 2004). The simplest approach to combining PK and PD data would be to suggest that a successful clinical outcome would require that the concentration in all the treated animals should exceed the concentration needed to inhibit the bacterium. In experimental terms, this could be translated into a requirement for plasma concentrations to reach the MIC values during a long period or exceed the MIC values. It has been claimed that it is the nonprotein-bound drug concentration at the site of bacterial infection that determines the success of therapy (Shojaee AliAbadi & Lees 2000; Drusano 2004). Thus, although the amount of bound drug is occasionally considerable, the free amount appears to be the most relevant. This approach is valid in a therapeutic treatment scenario but presents some concerns in a metaphylactic treatment. As mentioned before, the target of the treatment in metaphylactic treatments is not the sick fish but the fish in the affected stock that most probably will be infected and sicken in the next hours and days. Thus, there is still no infection or asymptomatic animals, while the paradigm and scenario as regards the efficiency (site of action) of the antimicrobial substances can differ as in the case of the classic approach to therapeutic treatments. In metaphylactic treatments, the plasmatic levels of antimicrobial substances may also play a relevant role, but other protection mechanisms should also be considered.

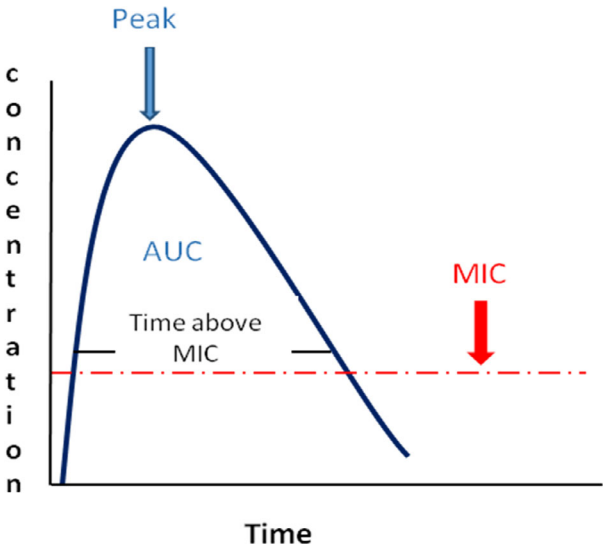


Figure 6 Pharmacokinetic/Pharmacodynamic parameters affecting antibacterial potency.

MIC and PK/PD approaches

In the past, the efficacy of antibacterial treatments was evaluated solely by integrating MIC values with maximum drug concentrations (C_{max}:MIC) in plasma (Figure 6) (Blaser *et al.* 1987; Stamm 1989). The idea was that *in vitro* MIC data could be used for *in vivo* application to predict the treatment efficacy of antibacterials (Bruun *et al.* 2003). However, MIC values reflect a quantitative measure of bacterial sensitivity to drugs and are determined *in vitro*, which does not necessarily represent the biological activity of the drug in the target animal *in vivo*, so their validity is questioned (Smith *et al.* 1994; Branson 2001). These are based on a theoretical assessment of a drug's efficacy against a bacterial pathogen based on the requirement that a drug's C_{max} plasma (maximum plasma concentration) following

administration in the target species, exceeds a factor of 4:1 (C_{max}:MIC) (BSAC 1991) or even 8:1 (Blaser *et al.* 1987), to ensure effective antibacterial action. However, there is concern regarding the generalized over-simplicity of these proposals (Smith *et al.* 1994) and later aquaculture works have challenged these guidelines (Coyne *et al.* 2004a,b) and have proposed other solutions in an attempt to particularize drug treatments, generally in medicine (Shojaee AliAbadi & Lees 2000; Craig 2007). At a later date, very significant and empirically validated advances in the use of PK/PD data for setting optimal dose regimens in human and veterinary medicine were implemented (Toutain *et al.* 2007; Schmidt *et al.* 2008).

Shojaee AliAbadi and Lees (2000) initially proposed that an optimum dosage schedule should achieve drug concentrations at infected sites in excess of MIC in the case of bacteriostatic and some bactericidal drugs (time-dependent effect) and drugs whose action depends on high AUC or C_{max}/MIC ratios (concentration-dependent effect). On the other hand, Smith (2008) has stressed that the PK/PD approaches developed for therapeutic administration to individual large animals and humans, in particular, are difficult to apply in aquaculture. He noted that the theoretical, practical and logistical problems raised by metaphylactic administration of antimicrobial agents to large populations of relatively small animals have received much less attention. He concluded that until these problems have been addressed in a satisfactory way, it would not be possible to apply the PK/PD approaches that have been used in human medicine for optimization of dose regimes in aquaculture. However, the published literature on the PK and PD of antibacterials in aquaculture could be examined in light of the data requirements of the PK/PD approaches currently being developed for human therapies. A key component of these approaches has been the identification of the combination of PK and PD data (the PK/PD index) that is best correlated with bacteriological and clinical outcomes.

In this regard, three PK/PD indices (Figure 6) have been found to be of greatest value (Craig 2007). The first one is the percentage of the inter-dosing interval during which the serum concentration exceeds the *in vitro* MIC against the target bacterium ($T_{C>}$MIC). The second one is the ratio of peak serum concentration (C_{max}) to MIC (C_{max}/MIC). The third one is the ratio of the area under the serum concentration curve at 24 h ($AUC_{0\text{-}24}$) to the MIC ($AUC_{0\text{-}24}$/MIC). If there is a lack of data from experimental infections, the general PD properties of an agent or class of agents can be used as a guide for the most appropriate PK/PD index. For agents whose effect is primarily time-dependent, the optimal PK/PD index is normally $T_{C>MIC}$. For agents whose effects are concentration-dependent, C_{max}/MIC appears to be the most appropriate. However, for those agents that show significant post-antibacterial effects (PAE; Craig & Gudmundsson 1996) or subinhibitory effects (Odenholt 2001), the $AUC_{0\text{-}24}$/MIC ratios may be more useful.

Potency

From a theoretical point of view, PK/PD models should be taken into account in the design of the therapeutic strategy. However, the implementation of these approaches, which are valid for individual therapeutic treatments in humans and large animals, to fish raises considerable scepticism (Smith 2008). As indicated above, aquaculture treatments should be based primarily on the metaphylaxis concept, thus aiming at the protection of fish at real risk and not sick fish. This is due to the decreased appetite of sick fish. In this case, however, the main example of MIC and antibacterial plasma levels is no longer valid in a metaphylactic scenario or at least is not valid for the sick fish in a stock. The real treatment target should be healthy fish, still eating but in imminent jeopardy and at risk of death within a few days after the start of an infectious outbreak. In terms of total biomass, these fish represent the highest population at risk and the population that can be saved or protected by applying an appropriate therapeutic strategy. In these early stages of many bacterial diseases in fish (infections by *Vibrio* or *Tenacibaculum* spp.), the presence and activity of the pathogens in the digestive system, skin or gills before disease becomes septicaemic can be significant. For this reason, sufficient levels of the selected antibacterials should reach these structures as fast as possible. In this scenario, the local effect of antibacterials in the digestive system and in intestinal microbiota should be taken into account, as it is an indirect way of controlling bacterial pathogens in carrier fish or in early prodromic stages, before the infection spreads to the organism. In any case, the potential development of antibacterial resistance in this specific site during antibacterial oral delivery should not be disregarded as described in other farmed animals (Simoneit *et al.* 2015).

The delivery method

Use of medicated feeds in fish farms: best practices

Antibacterials in aquaculture can be delivered using five main routes of administration: in-water, in-feed, injection, topical application and gavage (Sekkin & Kum 2011). The last three methods have a clear clinical approach. They are widely used in ornamental fish for individual fish cases, but only in very specific and occasional cases in aquaculture, such as brood stock management or research. Antibacterial in-water administration is used in ornamental fish treatments or in research but use is very limited in aquaculture for different reasons (e.g. hatcheries). Antibacterial bath treatments were described in the past as a potential

antibacterial delivery method for aquaculture (O'Grady *et al.* 1988), but this route of administration is no longer recommended since it involves large volumes of water, changes in antibacterial chemical availability associated with water quality and, consequently, large quantities of antibacterials and serious environmental issues.

Oral administration of antibacterials via the feed is the most frequently used route of administration in Mediterranean aquaculture (Rigos & Troisi 2005). Oral treatments with antibacterial-medicated feed are very similar to the treatments used in other technically-advanced finfish product facilities, such as salmon farming. The general principle of medicated feed consists in the use of a specific feed carrier as a basis and mixing with the antibacterial (Daniel 2009). Medicated fish feed producers usually have their own special standard feed formulations for medicated feeds and for each species as this food formulation needs to fulfil the nutritional requirements of the fish species at the same time. Moreover, they have appropriate physical and chemical properties for an optimal combination with the antibacterial premix.

Modern drug delivery approaches are related to pre-programmed and time-controlled smart nanotech systems (Aklakur *et al.* 2015), where nano delivery seems to be a potential route for nutraceuticals and drug administration. Microencapsulation and microparticles such as marine polysaccharides could be effective vehicles for drugs, vaccines and other compounds in aquaculture (Borgogna *et al.* 2011). Approaches involving microencapsulation provide a protective environment for the delivered compounds against the adverse conditions of the digestive system, resulting in enhanced bioavailability in the target organisms.

Premix and dose selection

In general, the recommended dose of an antimicrobial for an oral treatment is given in mg of active ingredient per kg of fish body weight (BW) daily (mg kg^{-1} BW/d). This is specific to the antimicrobial agent and is determined by the manufacturer of the premix. The recommended dose is based on studies carried out by the manufacturer in view of obtaining authorization from EMA or national authorities, according to specific guidelines and procedures. However, these studies are performed under specific experimental conditions (temperature, biomass per tank, species and diseases, etc.) and using limited information from field trials. Moreover, in many cases, these recommended doses are based on studies in very different fish species (salmonids), simply referred to as 'for fish' or in the worst cases, when cascade prescription is required, in terrestrial vertebrates. Thus, we suggest that the 'recommended' dose is treated as a general reference and that this dosage is adapted according to the different scenarios and available knowledge. Secondly, the desired dosage of the premix in feed is usually calculated in grams of premix per tonne of feed. Three important factors are involved in this calculation:

(1) Concentration of the active substance of the antimicrobial in the premix. In general, the concentration of commercial premixes is less than 100% and this should be taking into account when calculating the dosage in the feed. The excipients used in the premixes (quantitative and qualitatively) are also important. Under certain conditions, lower concentrations of active antimicrobial substances may require a large amount of premix in the medicated feed and this can lead to severe production problems.

(2) Daily feed intake depends on fish species, size and temperature. It is important to have real data about how the fish eat, as stated above. For example, trout fingerlings can eat up to 2% of the specific feeding ratio (SFR) under standard conditions; in comparison, the SFR of a 1 kg gilthead seabream in winter is very low, around 0.2%.

(3) Biomass to treat. The number and average weight of the fish requires monitoring on a regular basis, because the daily antimicrobial dose depends on the quantity of fish (kg) being treated (see example below). The biomass of a specific fish stock (cage, pond, tank) can be determined using different assessment methods and the evolution and growth of this biomass can be estimated using different mathematical models. In addition, disease-affected stocks frequently present deviations from the norm due to mortalities or growth slowdown. If mortalities and growth reduction are not properly evaluated, then mistakes in biomass assessment can lead to inefficient treatments or waste and environmental impact due to uneaten medicated feed. Depending on daily feed intake (SFR), the quantity of premix is mixed into a larger or smaller quantity of feed. Feed suppliers should be able to prepare medicated feeds with different doses of the same premix in order to provide the exact quantity of antimicrobial regardless of SFR variations.

Manufacturing of medicated feeds

Medicated fish feeds present are more complex compared with medicated feeds for terrestrial animals (pigs, poultry) due to specific characteristics and higher technical specifications of the feed pellets used in aquaculture. The fact that these medicated feeds need to keep and protect the pharmacological substances in the aqueous interface between its delivery and the ingestion by the fish, adds to the difficulties in the production of medicated fish feed (Daniel

2009).The preparation of medicated diets is based on the following procedures (Ranjan *et al.* 2017):

(1) *Pelleted or extruded*: In the past, medicated feed was produced on the same line as normal feed by adding the premix at the beginning of the process to the rest of the raw materials. This method is now outdated because it caused important problems of contamination and carry-over, and it was also impossible to predict the final concentration of the antimicrobial agent in the feed due to the great loss resulting from high temperatures and the pressure used during the manufacturing process. This is the reason why medicated feeds are produced using the following two methods (b, c):
(2) *Top coating or surface coating*: The medicine premix is mixed with the base feed in an industrial or pharmaceutical mixer/blender with the help of a binding agent, generally fish or vegetable oil;
(3) *Vacuum-coating*: The medicine premix is mixed with the oil, and the mixture is coated onto the pellet, with the help of a vacuum process.

In both cases (b and c), a surface layer of medicine coats the feed. In the vacuum-coating system, depending on the solubility and particle size of the medicine, part of the mixture (medicine plus oil) can penetrate the pellet. The medicated feed is produced on a dedicated line of the feed factory. It has been demonstrated that oil-coated medicated fish diets suffer leaching to a greater degree compared with pelleted or extruded pellets, and may also create higher palatability problems for farmed fish (Xu & Rogers 1994; Rigos *et al.* 1999). Both these factors may result in a reduction in amount of drug available to the treated population. Premixes should be 100% pure (or at least have a high concentration of active substances) and made of fine-sized particles in order to use low doses and avoid problems relating to homogeneity and palatability. However, in general, they are not 100% pure and they are coarse (rough particles), a very typical situation when we have to use by exceptional prescription a non-fish premix. In such situations, serious problems of physical quality, homogeneity and palatability may arise.

Palatability of the medicated feed can be also an issue (Ranjan *et al.* 2017), causing a dramatic reduction in feed intake due to bad taste of the premix that is sometimes combined with decreased appetite due to infection. Moreover, if the fish do not eat the medicated feed quickly or reject it, the loss of antimicrobial agent into the water via leaching may be important (Rigos *et al.* 1999). Reducing the availability of the agent in the gut and dispersing it in the environment can be minimized by starting the treatment as early as possible, thanks to efficient and prompt diagnosis. Moreover, it is possible to increase the palatability of the medicated feed by the addition of attractants such as fish oil or other feed components during the manufacturing process (Partridge *et al.* 2014).

The therapeutic regime/therapeutic strategy

Medicated feed administration

Fish can be fasted for 12 to 24 h to increase their appetite without inducing any welfare issues; this could be coordinated with the time of arrival of the medicated feed at the farm. In order to maximize the quality of the medicated feed delivered to the sick fish, manual administration of the feed is preferable rather than the use of automatic feeding systems. By distributing the feed by hand, the feeders have to follow procedures and monitor closely whether the sick fish are actually eating the medicated feed. This is even more important for fry/juveniles as the number of fish per rearing unit is usually very high and feeding behaviour and efficient distribution to all the stock is key to the efficacy of the treatment. When manual delivery is not possible due to operational reasons, efficient and supervised automatic fish feed delivery systems are used in the farms. In this situation, monitoring of the medicine content in the feed is recommended, as automatic systems (silo transportation, pipelines, etc.) can increase loss of medicine from the feed due to the traumatic/mechanical process. Careful supervision of the medicated fish distribution is the best option to ensure fast and homogeneous distribution of the medicated pellets in the cage, pond or tank under treatment, but it is important to do this carefully in cages in order to minimize the loss of medicated pellets outside the cages. In any case, detailed feedback and reports on the feeding behaviour after each treatment (products, dose, conditions, feed rejection, feed delivered vs feed not delivered to the stock) is very important and should be recorded and given due consideration in future treatments.

Therapeutic procedures: medicated feed delivery guidelines

The number of daily feedings should be adapted not only to the species, size and culture system, but also to the daily logistics of a farm and environmental conditions. It is also critical to take into account the PK of each medicine. For example, with FLO at least two (2) meals /day are recommended at high water temperatures due to the rapid absorption and depletion in European seabass. On the other hand, a sequential (every other day) dosing schedule is suggested for OTC due to slow removal in the same fish species (Rigos *et al.* 2002a).

Concentration of all the recommended daily doses of the medicine in a single meal intake is advisable under certain conditions: high temperatures and high feed intake when

specific PK data is implemented. On the other hand, distribution of all recommended daily doses in several small meals throughout the day may result in more even drug distribution among the infected stock, perhaps due to decreased effects of hierarchies on feed behaviour in the cage.

Pharmacokinetics at different temperatures

Temperature is an important factor that may significantly affect PK parameters. Specifically, studies have revealed significant differences in OA and OTC PK with increasing temperature in European seabass (Rigos *et al.* 2002a,b). Although there is little direct evidence, this temperature effect may be related to increased gastric emptying rate. Generally, in the above studies, a faster kinetic profile (absorption, elimination) and lower tissue concentrations of the drugs have been observed with increasing temperature.

Duration of treatment

The duration of treatment is recommended by the premix manufacturer and can be prescribed by a veterinarian according to current knowledge and practical experience with the antibacterial and the diseases. Typically, antibacterial treatments last around 5 to 12 days, but duration of the antibacterial treatments is still under debate in human and also animal medicine (Rigos & Troisi 2005). Sometimes, in chronic disorders, such as furunculosis in turbot (*Scophthalmus maximus*, Linnaeus 1758) (Bjornsdottir *et al.* 2005) or bacterial kidney disease in rainbow trout (*Oncorhynchus mykiss*, Walbaum, 1792) (Elliott *et al.* 1989), it can be longer. From field experience, if after 15 days of treatment, there is no improvement, it is time to stop the treatment and analyse the fish again; the diagnosis at the beginning of the process might have been wrong. New studies in human medicine, based on clinical results, demonstrate that for certain diseases, shorter courses of treatment are as effective as longer ones (7–14 days; Spellberg 2016).

Suitability of the treatments and treatment efficacy evaluation

Other issues regarding medication practices in Mediterranean aquaculture, which are frequently underrated, include the evaluation of the suitability of the treatments and the efficacy of the treatments. In the case of antibacterial treatments, both concepts (suitability and efficacy) should be reviewed given that they are associated with the improvement of therapeutic adjustment and the global sustainability of treatments.

The nature of the disease can also be underestimated. For example, infections with slow progression or asymptomatic infections that may persist for long periods and reappear under optimum conditions for the pathogen require special attention. This could be the case, for instance, of *P. damselae* subsp. *piscicida*. It is known that this pathogen can persist in asymptomatic fish (Osorio *et al.* 1999).This could be related to the ability of this bacterium to survive and multiply in macrophages (Elkamel *et al.* 2003). As chronic forms in pasteurellosis/photobacteriosis are very common and recurrence of the infection is frequent, particular long-term therapeutic strategies and adequate antibacterial dosage should be taken into account in these cases. Similar approaches should be adopted in the case of other common or occasional persistent bacterial infections in Mediterranean aquaculture, such as nocardiosis, epitheliocystis or mycobacteriosis.

Furthermore, it is important to emphasize the need to gain a better understanding of the downstream effects of antibiotics on fish microbiota, which play a vital role in supporting host health and nutrition (e.g. loss of important beneficial microbes following treatment may lead to increased disease susceptibility). As a result, care should be taken, if possible, in choosing a antimicrobial, as different antibacterials have different effects on fish microbiota (Rosado *et al.* 2019; Kokou *et al.* 2020) and prolonged administration may cause adverse effects on target fish.

Efficacy control

The concept of therapeutic treatment as discussed is the most widely known in animal husbandry and is based in the use of medicines in animals that are still sick. Prophylactic treatments aim at prevention but always in the absence of infection. The extreme case involves continuous routine control using subtherapeutic doses, a totally undesirable practice used in the past for several terrestrial farming activities and closely related to the concept of antibacterials as growth promoters.

Metaphylaxis is a much more controversial word that is used to describe two apparently similar, but significantly different concepts. Some authors (Bousquet-Melou *et al.* 2010) define metaphylaxis in association with a risk factor, that is the use of medicines/antibiotics when one or several risk factors are present in a specific population. Other organizations (EMA & EFSA 2017), however, describe metaphylaxis in association with a population at real risk, that is the use of medicines/antibacterials when a certain population stock is experiencing or has already been diagnosed with a certain (usually early) level of bacterial disease and a high probability of disease outbreak. In this case, the medicines do not target the sick animals (fish, in our case) but rather the still-not-infected ones or the close-to-be-sick fish that are in the same cage, tank or pond as the sick fish or in cages, tanks or in the vicinity of the affected stock (depending on the isolation between rearing units).

The differences between the two definitions are important because in the affected-stock metaphylaxis concept, the disease is yet present in the stock, but in the risk metaphylaxis concept, the disease is not yet present in the stock. In other words, metaphylaxis in an affected stock should be considered as a curative method, but risk metaphylaxis should be understood as being much closer to prevention. In Mediterranean aquaculture, antibacterial treatments are always applied according to the concept of metaphylaxis associated with populations at real risk, given that treatments are always applied after the identification of a bacterial disease in the population at risk. However, in aquaculture treatments rely on delivery of the antibacterials through the medicated feeds and also occasionally on the fact that the feed ingestion is fully suppressed in sick fish. These two facts mean that the supplied antibacterial only reaches the part of the stock that is not affected. This point is important for understanding how medicated feeds control a bacterial outbreak in a fish population and has implications for the efficient and sustainable use of antibacterial-medicated feeds. A summary of some implications is given below:

- In antibacterial treatments using medicated fish feeds, the amount of feed and antibacterial to be used should be calculated using the estimated 'healthy' biomass in the affected population, and not the total estimated or recorded population in this population (Smith 2008). This can be estimated by a precise calculation of the current food intake of this population and compared with the theoretical estimated feed consumption of the fish stock under normal conditions. Hand feeding and close evaluation of feeding behaviour is highly recommended in this situation
- As medicated feed will be delivered to healthy fish, antibacterial plasma values constitute a reference (Chen *et al.* 2017), but are not as relevant as the classical therapeutical approach to sick fish, where bacteria can be found in blood. In this sense, there are some observations in terrestrial animals indicating that total doses were much lower for early treatments and the bacteria load is lower, indicating the relevance of the so-called 'inoculum effect' (Kesteman *et al.* 2009). As medicated feeds are processed in the digestive system before the antibacterial is absorbed in the intestine and, in some cases, systemic bacterial infections such as vibriosis can originate in the intestine, the effect of antibacterial concentrations in the intestinal microenvironment should not be disregarded.

A real evaluation of the efficacy of the treatments is another handicap in Mediterranean aquaculture. Very frequently, the only way to evaluate the apparent efficacy of a treatment is to estimate the decreases in mortalities or signs of the diseases, including appetite. In very few cases, it is possible to assess with a high level of confidence whether the recovery of the stock is due to the effects of the antimicrobial substance or is simply part of natural recovery after an infectious disease outbreak. In very few cases in field conditions, it is possible to have a comparable non-treated control group of fish or different groups affected to the same extent at the same time. This is a very important difference from the antibacterial efficacy studies that are based on laboratory-scale trials under controlled conditions, where results are robust and easily comparable between treated and control groups. This lack of predictive indicators reinforces the relevance of extensive (in number of batches examined in different condition) field efficiency monitoring. Another very frequent handicap of Mediterranean finfish aquaculture are the problems associated with the very complex logistics of administration of medicated feeds, including diagnosis, prescription, production, transport to the farms and distribution to the affected stocks.

Environmental impact

Amongst the different potential impacts of aquaculture activities, the use of antimicrobial drugs has been considered one of the most relevant issues due to the implications for human health (Alderman & Hastings 1998) and the environment. Concern about the implications for human health is related mainly to the misuse of antimicrobial medicines in intensive terrestrial animal husbandry and aquaculture, and the emergence of antibacterial resistance. This is a global problem that is not related solely to aquaculture. Effects on the aquatic environment are unquestionable, but their importance varies greatly between areas and activities. Moreover, in many cases, they are combined with significant impacts on the antibacterials from human origin and terrestrial animal farming. It is well known that advanced aquaculture systems like salmon aquaculture in Norway have successfully minimized some of these impacts (Taranger *et al.* 2014), but the situation in other areas with lower technological development and less advanced regulation and supervision policies remains alerting.

In view of the above, EU policies have set high standards that are implemented through the Marine Strategy Framework Directive (MSFD) and the Water Framework Directive (WFD). Planning and development of new aquaculture sites fall under the Environmental Impact Assessment Directive (EIA) and Strategic Environmental Assessment Directive (SEA), amongst others.

The potential environmental impacts of antibacterials in Mediterranean aquaculture have already been identified and commented by Rigos and Troisi (2005). In this work, the release mechanisms of antibacterial substances (e.g. OTC) were clearly explained. Antibacterial substances (and

their metabolites) released in the environment are mainly found in sediments, redistributed in the water column and/or can be transferred to other organisms, causing three different types of potential problems, as described by Rigos and Troisi (2005). This process may cause:

- Generation of antibacterial resistance and escape group or multidrug-resistant bacteria
- Accumulation of antibacterials in other organisms and the environment
- Toxic effects in several organisms.

The presence of all three types of environmental problems has also been described with reference to Mediterranean aquaculture in scientific and technical papers or specific studies (Chelossi *et al.* 2003; Di Cesare *et al.* 2013). However, as noted previously, the presence of antibacterials in the Mediterranean marine aquatic environment should be seen as a pool of different sources, not only due to aquaculture activities. In any case, the Mediterranean aquaculture industry and the European and national regulatory bodies are fully aware that the only possible way forward is to design strategies aimed at the reduction and minimization of the use of antibacterials (IUCN 2007). Such strategies shall also include rationalization of treatments, one of the main objectives of this document.

Conclusions & recommendations for best practices

Fast and accurate diagnosis of the problem associated with bacterial disease outbreaks is undoubtedly essential for successful treatment. Prompt diagnosis is also the first step for confronting the bacterial pathogen given that sick fish will inevitably display decreased appetite, while response time is crucial for the selection of the most convenient substance and dosage, coupled with preparation and delivery of the medicated feed to the farm. The handling stress and the cost associated with a particular treatment must be balanced against the expected benefits before deciding on the treatment to be administered. The fact that that in some cases of disease the cost of treatment might exceed the benefits should not be ignored.

Clearly, empiric administration of medicines should be avoided. The choice of medicine should be based on sensitivity tests (disc diffusion, MIC), and if treatment is urgent, it should be based initially on historical data kept by the farm and/or treatment response in neighbouring farms, and corrected if necessary, according to the *in vitro* results. The MRL of the target or of other fish species should be taken into account in the design of the treatment schedule while considering an adequate withdrawal period if the fish are to be consumed soon.

With regard to published PK, tetracyclines, mainly represented by OTC, have received wide attention as reflected by the large number of publications regarding Mediterranean farmed fish. Its slow elimination suggests that a sequential dosing schedule is needed, namely a more prudent and economic treatment strategy, at least in medium/low water temperatures. Interestingly, DOX appears as a promising alternative considering its circulatory levels in European seabass, the low MIC values and the encouraging clinical outcome mainly based on small laboratory trials. Although some quinolones/fluoroquinolones showed promising PK profiles (mainly FLU over OA) according to the significant amount of published data, this group is considered of highest priority by current legislation. Thus, their use in farm animals will gradually decrease and eventually be banned. Information on the absorption of ENR, SAR and DAN in the circulation of Mediterranean fish species is missing. Concerning sulphonamides/potentiated sulphonamides, while a large amount of data on PK-depletion is available, absorption data are limited for gilthead seabream; surprisingly, such information is lacking for European seabass. Penicillin derivatives have little use in euryhaline fish farming, probably due to the lack of registration for use in aquaculture. PK studies of AMP in euryhaline fish species are limited. Amoxicillin displayed negligible bioavailability in gilthead seabream, although its MIC values are rather promising. Further PK studies in European seabass may demonstrate improved AMO absorption, where it may be used more appropriately for antibacterial therapy. With respect to phenicols, preliminary trials with FLO in European seabass revealed promising findings regarding absorption, removal and clinical outcome. More research is required to obtain a complete picture of FLO PK in both European seabass and gilthead seabream. On the other hand, there are plenty PK studies on THI in both species. Overall, clinical studies are generally lacking in the pertinent literature and proper PK parameters for integration in modern predictive indices are missing.

Concerning PD, the data available in scientific literature are not sufficient and there are hardly any comparisons. Therefore, it is paramount for the next actions to focus on increasing the number of bacteria strains isolated from European seabass and gilthead seabream during recent outbreaks. These new strains, along with stored bacterial collections, should be subjected to MIC studies under standardized procedures using several antibacterial molecules already authorized or potential promising candidates for their future application in aquaculture. For the main fish pathogens of European seabass and gilthead seabream at least, determination of ECOFF breakpoints using valid protocols will be of primary importance.

Consideration of species-dependent differences is recommended when applying/adopting dosing schedules after selection of the appropriate antibacterial. Special attention should also be given to other important factors such as fish

size, fish density, growing environment and especially water temperature, which appears to totally alter the PK of antibacterials and thus necessitates substantial changes in the treatment regimes. Disease evolution and consideration of the multi-phase model are of tremendous importance for designing dosing schedules and avoiding considerable financial loss and environmental side effects. Adjustments to the initial treatment plan is recommended based on the concept of metaphylactic treatment and the anticipated anorexia of the sick population.

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