

Acronym: PerformFISH

***Title: Consumer-driven production: Integrating Innovative Approaches
for Competitive and Sustainable Performance across the
Mediterranean Aquaculture Value Chain***

Grant Agreement: 727610

Deliverable 3.3

Best therapeutics practices for Mediterranean farmed fish

Lead parties for Deliverable: HCMR

Due date of deliverable: 30 April 2019

Actual submission date: 15 July 2019

Submission of Revised Version: 15 September 2020

Dissemination level: PU (Public)

Version: V2- Revised

All rights reserved

This document may not be copied, reproduced or modified in whole or in part for any purpose without the written permission from the PerformFISH Consortium. In addition to such written permission to copy, reproduce or modify this document in whole or part, an acknowledgement of the authors of the document and all applicable portions of the copyright must be clearly referenced.



This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 727610. This output reflects the views only of the author(s), and the European Union cannot be held responsible for any use which may be made of the information contained therein.

Table of Contents

CHAPTER ONE: use of antibacterial agents	6
1. Introduction.....	6
1.1. Overview of the main diseases and conventional health management of European seabass and gilthead seabream in Mediterranean aquaculture	6
2. ANTIBIOTICS AND ANTIBIOTIC MANAGEMENT FOR FISH DISEASE CONTROL: KEYPOINTS AND LEGISLATION ASPECTS.....	10
2.1. General considerations.....	10
2.2. Antibiotic therapy in Mediterranean finfish farming	10
3. Antibiotic selection, prescription and delivery: main criteria, technical aspects and constraints	20
3.1. The affected fish stock.....	20
3.2. The disease and the causative agent: the pathogen or pathogens and the specific characteristics.....	30
3.3. The tools: antibiotics, main characteristics and pharmacokinetic properties	30
3.4. The delivery method.....	60
3.5. The therapeutic regime / therapeutic strategy	64
3.6. Environmental impact	70
4. Conclusions & recommendations for best practises.....	73
CHAPTER TWO: use of antiparasitics and parasite control strategies	75
1. Introduction.....	75
2. Overview of the main parasitic diseases	75
3. Parasitic diseases and health management and control of the European sea bass and gilthead sea bream in Mediterranean aquaculture	78
4. Antiparasitic substances	79
5. PerformFISH: New developments: Mebendazol & Toltrazuril	85
6. Treatments: delivery method.....	85
6.1. Oral treatments / in-feed administration.....	85
6.2. Bath treatments / In-water medication	88
7. Disease remediation through nutritional intervention	97
8. Parasitic disease management through environmental control.....	99
9. Specific management and integrated control strategies	102
10. Potential quantitative environmental pollution of different antiparasitic approaches	105
11. Conclusions & recommendations.....	107
List of Tables	107
List of Figures.....	108

ANNEX I Pharmacokinetics of Oral Lincomycin in European sea bass (<i>Dicentrarchus labrax</i>)	110
1. Introduction	110
2. Materials and methods	110
2.1. Fish	110
2.2. Medicated feed & distribution	110
2.3. Blood sampling and sample preparation	111
2.4. Analytical method	111
3. Results	114
3.1. Validation results	114
3.2. PK Study results	114
4. Discussion	115
ANNEX II Pharmacokinetics of Oral Doxycycline in European sea bass (<i>Dicentrarchus labrax</i>)	116
1. Introduction	116
2. Materials and methods	116
2.1. Fish	116
2.2. Medicated feed & distribution	116
2.3. Blood sampling and sample preparation	117
2.4. Analytical method	117
3. Results	119
3.1. Validation results	119
3.2. PK Study results	120
4. Discussion	121
ANNEX III Pharmacokinetics of Oral Spectinomycinin European sea bass (<i>Dicentrarchus labrax</i>)	122
1. Introduction	122
2. Materials and methods	122
2.1. Fish	122
2.2. Medicated feed & distribution	122
2.3. Blood sampling and sample preparation	123
2.4. Analytical method	123
3. Results	126
3.1. Validation results	126
3.2. PK Study results	126
4. Discussion	127

ANNEX IV Pharmacokinetics of Oral Mebendazole in gilthead sea bream (*Sparus aurata*) ..129

1. Introduction.....129
2. Materials and methods129
 - 2.1. Fish.....129
 - 2.2. Medicated feed & distribution129
 - 2.3. Blood sampling and sample preparation.....130
 - 2.4. Analytical method.....130
3. Results & Discussion133
 - 3.1. Validation results133
 - 3.2. PK Study results134

ANNEX V Oral Tortrazuril in gilthead sea bream (*Sparus aurata*)135

1. Introduction.....135
2. Materials and methods135
 - 2.1. Fish.....135
 - 2.2. Medicated feed & distribution135
 - 2.3. Blood sampling and sample preparation.....136
 - 2.4. Analytical method.....136
3. Results & Discussion139
 - 3.1. Validation results139
 - 3.2. PK Study results139

REFERENCES141

Contributors



(from left to right)

George Rigos is Research Director (Fish Pathology/Pharmacology) in the Hellenic Centre for Marine Research

Carles Cristòfol is Associate professor and Manager of the ‘Servei d’anàlisi de Fàrmacs’ of the Veterinary School (Universitat Autònoma de Barcelona)

Carlos Zarza is fish health expert of the Fish Health Department, Skretting Aquaculture Research Centre

Francesc Padrós is Associate professor and technical manager of the ‘Servei de Diagnòstic Patològic en Peixos’ of the Veterinary School (Universitat Autònoma de Barcelona)

Daniella Florio is veterinarian, working at the Fish Pathology Unit of the Department of Veterinary Medical Sciences, Bologna University (UNIBO)

Acknowledgements:

We would like to express our deepest appreciation to all the colleagues from PerformFISH WP3 and a special gratitude to our colleagues at the University of Bologna, particularly Renato Giulio Zanoni and Letizia Fioravanti for their work on MICs, our colleague Dr Carlo Godoy from the Universitat Autònoma de Barcelona for his comments about breakpoints and Dr Ariadna Sitjà for her final editing of the document and her coordination efforts of ParaFish Control.

CHAPTER ONE: use of antibacterial agents

1. Introduction

Current farming of European sea bass and gilthead sea bream, as in other finfish farming activities, involves facilities where large numbers of fish are kept together for a relatively long period of time. In hatcheries and land-based pre-growth systems using open or close-cycle water supply, millions of larvae and juveniles are packed for few months before being transferred to the open cages. There, a high number of grown animals (occasionally 100.000-500.000 per cage) are struggling for survival against several stressors for 12-15 months. Therefore, disease outbreaks are inevitable and they can become a serious risk if farm management is inadequate. Infectious diseases in caged farming may become a substantial problem not only via substantial extra financial costs due to mortalities, decrease of the performance of the fish and therapeutic costs but also due to depreciation of product value and welfare issues. This particular issue was clearly identified in PerformFISH and this is the reason why antibacterial and antiparasitic medicines have been particularly addressed in Task 3.4: Medicines, Biocides and Bioactive Substances of the WP3 (Boosting Fish Health at allLifecycleStages) and particularly in the subtasks Task 3.4.1: Medicines: Current Use and Task 3.4.2: Developing Future Treatments. Among the potential disease agents, bacterial pathogens are the most frequently diagnosed.

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

In the Mediterranean aquaculture, the dominant bacterial pathogens in European seabass and gilthead sea bream production since the 80's have been traditionally reported to be *Vibrio anguillarum* and *Photobacterium damsela* subsp. *piscicida* and later *Tenacibaculum maritimum*, *V. harveyi* and *V. alginolyticus* (Table 1). More recently, the primary pathogenic potential of *Aeromonas veronii* bv. *sobria* and *A. salmonicida* was emerged. Commercial vaccines have been readily available the last two decades to confront *V. anguillarum* and *P. damsela* subsp. *piscicida* (Le Breton, 1999), while vaccination for *T. maritimum* and *A. veronii* bv. *Sobria* (autogenous products) was based on more recent developments. Other bacteria implicated in infections include several *Vibrio* species such as *V. harveyi*, *V. ordalii*, *V. parahemolyticus*, *V. splendidus*, *V. vulnificus*, *Pseudomonas* spp. and *A. hydrophila* (Table 1). Disease outbreaks in European sea bass and gilthead sea bream have also been triggered by other emerging species such as *Mycobacterium marinum*, *Streptococcus iniae* and *Staphylococcus epidermidis*. Interestingly, some of the above bacteria have been occasionally considered as primary pathogens in specific cases. Important bacterial diseases of European sea bass and gilthead sea bream are also summarised in Colorni & Padros (2011), Sitjà-Bobadilla, Zarza et al. (2014), N. Vendramin, Zrncic et al. (2016) and also in the reports of the annual meetings of the European Union Reference Laboratory for Fish and Crustacean Diseases (EURL).

Appropriate health management practices and high standards of hygiene are the most effective preventive control methods to reduce the risks of an outbreak of an infectious bacterial agents and minimize their impact. These practices and standards are particularly relevant at hatchery and nursery level but more difficult to implement in on-growing farms using open sea cages or in large ponds or lagoons. In these conditions vaccination has been demonstrated to be very effective preventive strategy particularly for bacterial diseases. However, commercial vaccines are still available for a limited range of pathogens and occasionally become ineffective due to evolution of the bacteria antigenicity and its use and efficacy is very limited in larvae, post-larvae and small size juveniles. Autovaccines (CMDv of the European Medicines Agency) can surpass these handicaps and had been the only way to control and reduce the spread of some diseases given the limitations on the availability of commercial vaccines, but should be always considered as emergency and/or temporal strategies as they do not have the same guarantee as commercial vaccines. In some cases, and despite the preventive measures adopted, bacterial disease outbreaks are in some cases inevitable and require antibacterial treatments. Antibacterial treatments based on drug delivery are also a therapeutic tool in worldwide finfish aquaculture, also in Mediterranean aquaculture. Due to its relevance at environmental and human health level is developed under a strict regulatory framework (Regulation (EU) 2019/6). Antibiotic treatment efficacy and also safety for the fish, humans and the environment are two of the main axis to assess the correct, conscious and sustainable use of antibiotics.

With all that, knowledge on the efficacy of these therapeutic practices in gilthead seabream and European seabass is not so well developed as in other farmed fish species such as salmonids or terrestrial vertebrates and still requires substantial improvement based in scientific data. This is one of the main aims of the activities in Task 3.4 of Performfish WP3. These actions require a wide and critical review on the available technical and scientific information along with the production of new therapeutic knowledge on promising new candidate antibacterials. These actions has also been taken into account in the FishMedPlus initiative: (<https://www.fve.org/publications/fishmedplus/>)

It is anticipated that this review will a) direct future research in support of good practice in the industry and b) provide a scientific foundation to aid the design of future therapeutic schedules in Mediterranean marine fish farming.

Table 1. Important bacterial diseases of European sea bass and gilthead sea bream

Bacterial pathogens	Fish species	References
Primary		
<i>Vibrio anguillarum</i>	<i>D. labrax</i> , <i>S. aurata</i>	(Balebona, Zorrilla, Moriñigo, Borrego, 1998; Korun, Timur, 2008; Öztürk, Altinok, 2014)
<i>Photobacterium damsela</i> subsp. <i>piscicida</i>	<i>S. aurata</i> , <i>D. labrax</i>	(Balebona, Zorrilla, Moriñigo, Borrego, 1998; Candan, Kucker, Karatas, 1996; Essam, Abdellrazeq, Tayel, Torky, Fadel, 2016; Toranzo, Barreiro, Casal, Figueras, Magarinos, Barja, 1991)
<i>Tenacibaculum maritimum</i>	<i>D. labrax</i> , <i>S. aurata</i>	(Avenidaño-Herrera, Toranzo, Beatriz, 2006; Balebona, Zorrilla, Moriñigo, Borrego, 1998; Bernardet, Kerouault, Michel, 1994; Bernardet, 1998; Kolygas, Gourzioti, Vatsos, Athanassopoulou, 2012; Pepin, Emery, 1993; Yardimci, Timur, 2015)
<i>Aeromonas veronii</i> bv. <i>sobria</i>	<i>D. labrax</i>	(Smyrli, Prapas, Rigos, Kokkari, Pavlidis, Katharios, 2017; Uzun, Ogut, 2015)
<i>V. alginolyticus</i>	<i>D. labrax</i> , <i>S. aurata</i>	(Abdel-Aziz, Eissa, Hanna, Okada, 2013; Balebona, Zorrilla, Moriñigo, Borrego, 1998; Öztürk, Altinok, 2014; Zorrilla, Moriñigo, Castro, Balebona, Borrego, 2003)
<i>V. harveyi</i>	<i>D. labrax</i> , <i>S. aurata</i>	(Balebona, Zorrilla, Moriñigo, Borrego, 1998; Haldar, Maharajan, Chatterjee, Hunter, Chowdhury, Hinenoya, Asakura, Yamasaki, 2010; Korun, Timur, 2008; Pujalte, Sitjà-Bobadilla, Macián, Belloch, Álvarez-Pellitero, Pérez-Sánchez, Uruburu, Garay, 2003)
Secondary		
<i>V. parahemolyticus</i>	<i>D. labrax</i> , <i>S. aurata</i>	(Abdel-Aziz, Eissa, Hanna, Okada, 2013)
<i>P. damsela</i> subsp. <i>damsela</i>	<i>D. labrax</i> , <i>S. aurata</i>	(Öztürk, Altinok, 2014; Terceti, Ogut, Osorio, 2016)
<i>V. ordalii</i>	<i>D. labrax</i> , <i>S. aurata</i>	(Korun, Timur, 2008; Öztürk, Altinok, 2014)
<i>V. splendidus</i>	<i>S. aurata</i> ,	(Balebona, Zorrilla, Moriñigo, Borrego, 1998)
<i>V. vulnificus</i>	<i>D. labrax</i> , <i>S. aurata</i>	(Öztürk, Altinok, 2014; Uzun, Ogut, 2015)
<i>Aeromonashydrophila</i>	<i>D. labrax</i>	(Doukas, Athanassopoulou, Karagouni, Dotsika, 2008; Öztürk, Altinok, 2014)

<i>Streptococcus iniae</i>	<i>D. labrax, S. aurata</i>	(Aamri, Caballero, Real, Acosta, Déniz, Román, Padilla, 2014; Zlotkin, Hershko, Eldar, 1998)
<i>Pseudomonas spp.</i>	<i>S. aurata</i>	(Öztürk, Altinok, 2014; Zorrilla, Moriñigo, Castro, Balebona, Borrego, 2003)
<i>Staphylococcus epidermidis</i>	<i>D. labrax, S. aurata</i>	(Öztürk, Altinok, 2014)
<i>Mycobacterium marinum</i>	<i>D. labrax, S. aurata</i>	(Avsever, Çavuşoğlu, Eskiizmirli, Türe, Korun, Çamkerten, 2016; Colorni, 1992; Ucko, Colorni, 2005; Ucko, Colorni, Kvitt, Diamant, Zlotkin, Knibb, 2002)

2. ANTIBIOTICS AND ANTIBIOTIC MANAGEMENT FOR FISH DISEASE CONTROL: KEYPOINTS AND LEGISLATION ASPECTS

2.1. General considerations

Finfish aquaculture usually manages substantial larger numbers of individuals (hundreds of thousands or even millions) compared with other terrestrial animal productions such as poultry or pigs. When husbandry and prophylactic measures are not sufficient or adequate to prevent the entrance of pathogens and the development of diseases in the facilities, disease outbreaks tend to trigger in a particularly aggressive way due to the particularly high number of individuals and other relevant epidemiological factors.

When inevitable, disease outbreaks caused by bacteria in aquaculture are normally confronted with mass therapy strategies, ideally and obligatorily administered through incorporation of antibacterials into the feed (see section 3.4). This delivery system as therapeutic strategy should be then seen, for different reasons that will be discussed later on (see section 3.3.8.2), as a mainly metaphylactic treatment on the affected fish stock rather than a true curative treatment for the sick fish.

As in terrestrial animal veterinary medicine, the best therapeutic approach in aquaculture is to select the most suitable and available treatment when the responsible pathogen or pathogens have been appropriately identified and its or their antibacterial sensitivities have been clearly determined. Unfortunately, antibacterial drugs are not always used and delivered in the most appropriate, rational and efficient manner. However, this is clearly not due to negligent conduct of fish health veterinarians and fish health care staff but for many other external factors, including regulatory constraints, out of their control. The urgency of the farmer's for an immediate response to an outbreak and the special characteristics of the particularly complex logistics associated to the production, transportation and delivery of medicated fish feed, often results in ill-informed decision-making based on a rushed diagnosis that is followed by possible selection and use of not the most appropriate drugs and available pharmaceutical products, often coupled with choice of inefficient delivery systems. This will inevitably lead to the increase of the risk of suboptimal or unsuccessful therapy and this failure also may impact on the environment. Several international organizations including the Food & Agriculture Organization (FAO), the World Health Organization (WHO) and the World Organisation for Animal Health (OIE) and also European (EFSA, EMA) and national institutions and organizations have all raised the issues associated with inefficient and irresponsible use of antibacterial drugs in all production sectors including aquaculture, with particular concern for potential risks to public health.

2.2. Antibiotic therapy in Mediterranean finfish farming

The aquaculture industry in the Mediterranean European countries, as any other aquaculture farming activities in the European Union (EU), is currently subjected to EU and also national regulatory legislations. Discussions on the control of trade, development and legal use of veterinary medicines among countries members of the

EU has been ongoing for almost 30 years. The establishment of the open market within the EU in the 90's further increased the importance of regulating the use of medicines throughout the EU. Consequently, legislation of EU countries has had to bear a common regulatory environment across all member countries. Directive 81/851/EEC, relating to veterinary medicinal products, was the initial stage of this process in 1983. The availability and authorization of drugs for European veterinary medicine, including aquaculture, was regulated by the EU in 1990 via Council Regulation 2377/90 which was superseded by Council Regulation 2309/93 in 1993. Several EU Directives are applicable to the use of drugs for farming purposes. These directives were replaced by Regulation (EU) 2019/6 on veterinary medicinal products and Regulation (EU) 2019/4 on medicated feed. These new regulations will apply from 28 January 2022. As part of their implementation, the two Regulations require the European Commission to adopt delegated and implementing acts. Regulation (EC) No 470/2009 sets out the Union rules for the establishment of maximum residue limits (MRLs) in foodstuffs of animal origin of pharmacologically active substances included in veterinary medicinal products intended for food-producing animals or in biocidal products used in animal husbandry. Commission Regulation (EU) No 37/2010 sets out the classification of the pharmacologically active substances regarding maximum residue limits in foodstuffs of animal origin.

Veterinary medicine is currently regulated by the Committee for Medicinal Products for Veterinary Use (CVMP) of the European Medicines Agency (EMA, previously EMEA; <http://www.ema.europa.eu>) which is responsible for the scientific evaluation, supervision and safety monitoring of medicines in the EU. The CVMP plays a vital role in the authorisation of veterinary medicines in the European Union having several important tasks including:

- Initial assessment of EU-wide marketing authorisation applications
- Post-authorisation and maintenance activities, including the assessment of any modifications or extensions to an existing marketing authorisation
- Safety monitoring of veterinary medicines on the market and when necessary, recommending to the European Commission changes to a medicine's marketing authorisation, or its suspension/withdrawal from the market.
- Evaluation of veterinary medicines authorised at national level referred to EMA for a harmonised position across the EU
- Recommendation of safe limits for residues of veterinary medicines used in food-producing animals and biocidal products used in animal husbandry, for the establishment of maximum residue limits by the European Commission
- Scientific advice to companies researching and developing new veterinary medicines
- Preparation of scientific guidelines and regulatory guidance to help pharmaceutical companies prepare marketing authorisation applications for veterinary medicines

- Cooperation with international partners on the harmonisation of regulatory requirements

From this CVMP regulatory background, several antibacterial substances regulated by EU legislation are currently used in Mediterranean fin fish farming (**Table 2**). It should be highlighted that this regulation focuses mainly on approved active substances and medicaments and not so much in detail in the prescription and delivery processes. These processes are usually developed under different national regulations and follow common directives with terrestrial veterinary medicines.

In many instances of disease outbreak in farmed fish and particularly in Mediterranean aquaculture, there are still limited guidelines for selecting the appropriate antibacterial, suitable dose and treatment schedule. In these cases, fragmented existing scientific knowledge, field experience and research for other similar drugs and/or other closely-related fish species are used to select a treatment. Although this approach is far from ideal, it is an attempt to overcome the lack of data, and is unfortunately usually done without any formal guidance leading sometimes to unreliable extrapolation.

The availability of suitable pharmaceutical products for finfish aquaculture is low and for the specific case of gilthead seabream and European seabass is extremely reduced (**Table 2, Table 3**). The number of medicines that can be used 'on label' is extremely low. Only very few pharmaceutical antibiotic-based products has been licensed at different national level for specific use against gilthead seabream or European seabass diseases. The differences between countries regarding licensed products are also an add-on problem at Mediterranean level. If no licensed medicine for fish are available in a country but available in other EU countries, then it is also possible to use another legal mechanism known as importation and use of veterinary medicines under exceptional circumstances. In these cases, it is possible to apply to the responsible national authorities for a particular import authorization. Unfortunately, given the lack of licensed medicines for gilthead sea bream and European sea bass, this mechanism becomes common instead of exceptional and requires additional bureaucratic efforts.

The scarcity of specific 'on label' medicines, also problematic for other animal species so-called 'minor species' (European sea bass and gilthead sea bream are also 'minor species'), has also been alleviated by the 'cascade principle' mechanism. The European Union Directive 90/676/EEC replaced by Reg 37/2010, provides a 'prescribing cascade' system to support the use of drugs authorised for other farmed animals, when no suitable registered compound has been recommended to treat diseases in fish. In such cases, a standard withdrawal period is imposed, corresponding to 500-degree days in fish. This is to ensure consumer safety 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. Under this skepticism, a Council Regulation (2377/90) was established laying down a Community procedure for the establishment of MRL of veterinary medicinal products in food stuffs of animal origin. This generalised use of 'cascade prescription' leads to the frequent

use of licensed medicines (usually antibiotic premixes) designed for use in other species and not specifically in fish. In these cases, problems associated to the concentration of the active substance within the medicine or the type and amount used are transferred to the medicated fish feed manufacturing processes, with frequent problems about binding capability and stability in the feed pellets or failure to achieve the targeted concentration of the antibiotic, mainly when feeding rates are low (wintering) (see section 3.4). Finally, it is also necessary to highlight the complexity of the scenario, with different diseases and also the different scenarios related to different environmental conditions and fish stock characteristics. These diverse scenarios very frequently require modifications in the dosage from the original technical specifications of the medicament used, leading to a 'de facto' off-label delivery of the antibiotics. Registered antibacterials may have a single, two or multiple target fish species (sometimes including salmonid and non-salmonid species, freshwater and marine species) and usually with a single 'recommended' daily dose for all of them, regardless the relevant differences of the response to antibacterial administration amongst fish species (see section 3.1.1). In these circumstances, the knowledge, capacity and experience of the veterinarians is paramount to achieve good results of the treatment (see also Reg UE 06/2019 and 04/2019).

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

Antibacterial drugs	Recommended dosing schedule	Maximum residue Level, MRL ($\mu\text{g}/\text{kg}$)	Marker residue	Animal	Source	Registered in Mediterranean aquaculture	Commercial forms
<i>TETRACYCLINES</i>							
Oxytetracycline	75mg/kg 10d	100	Sum of parent drug and its 4-epimer	Fish	EMEA/MRL/023/95 (EMEA, 1995a)	Greece, Spain, Italy, Croatia	Oxyvet Vethellas 50% OTCAquacenOxitetracycline Acuimix 750 (Oxytetracycline 75%) Aquaculture Anprociclina 200 Ossitetracyclinacenavisa Anprociclina 200 Kyroxy 200 premix Ossibioticpremix Oxiter Oxifarm Ossitetra 200 premix Egocin 20%
Chlortetracycline	75mg/kg 10d	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	200mg/kg 5d	100-600	Parent drug	Porcine Poultry Bovine	EMEA/MRL/270/97-FINAL (EMEA, 1997a)	Not in fish	
<i>(FLUORO)QUINOLONES</i>							
Oxolinic acid	10-35mg/kg 5-7d	100	Parent drug	Fish	EMEA/MRL/41090/05-FINAL	Greece, Spain	Linacivet

							InoxylacideOxolinique 240 salmonides (oxolinic acid 25%) Oxomid 24%
Flumequine	12mg/kg 5d	600	Parent drug	Fish	EMEA/MRL/823/0 2-FINAL (EMEA, 2002)	Greece, Spain, Italy	Flumesya Colifarm 200 (flumequine 20%) Flumequine 50% Chinogel Flumequina 200 Naquilene 500 Colifarm
Sarafloxacin	10mg/kg 5d	30	Parent drug	Fish	EMEA/MRL/349/9 8-FINAL (EMEA, 1998)		
Enrofloxacin	10mg/kg 5d	100	Sum of enrofloxacin and ciproxacin	Bovine Ovine Porcine Poultry Rabbits	EMEA/MRL/820/0 2-FINAL (EMEA, 2002b)		
Danofloxacin	10mg/kg 5d	100	Parent drug	Fish	EMEA/MRL/818/0 2-FINAL (EMEA, 2002c)	Not in fish	
<i>SULFONAMIDES</i>							
Sulfadiazine Sulfamethazine	25mg/kg 5d	100	Parent drug	Bovine Ovine Caprine	EMEA/MRL/026/9 5 (EMEA, 1995b)	Greece, Spain, Italy	Sulfatrim Tribrissen Optiprim Doxatrim 15 PM (trimethoprim+sulfadiaz ine 15% Neopridimetorale Trivemet premix Doxatrim 15 PM

							Neopridimet
<i>DIAMINOPYRIMIDINES</i>							
Trimethoprim	5mg/kg 5d	50	Parent drug	Fish	EMEA/MRL/255/97-FINAL (EMEA, 1997b)	Greece, Spain	Sulfatrim Tribrissen Optiprim Doxatrim 15 PM (trimethoprim+sulfadiazine 15%)
<i>PENICILLINS-B LACTAM</i>							
Amoxicillin	80mg/kg 10d	50	Not determined	Not determined	Undated/updated 2008 (Revision1)(EMEA 2008)	Greece, Italy	Amoxicillin 100 - Colistin 250, Gammamix(PM)
Ampicillin							
<i>PHENICOLS</i>							
Florfenicol	10-15/kg 10d	1000	Sum of parent drug and its metabolites	Fish	EMEA/MRL/822/02-FINAL-(EMEA, 2002d)	Spain, Italy, Croatia	Frrocol AQUA Aquaflor 50% (florfenicol 50%)* Aquaflor 500, Floron Florocol
Thiamphenicol	15-40/kg 5d	50	Parent drug	Fish	EMEA/CVMP/162614/2006-FINAL (EMEA, 2006)		
<i>LINCOSAMIDES</i>							
Lincomycin	100/kg 5d	50-1500	Parent drug	Porcine Ovine Chicken	EMEA/MRL/749/00-FINAL-corr (EMEA, 2000)	Not in fish	
<i>AMINOCYCLITOLS</i>							



Spectinomycin	50/kg 5d	200-5000	Parent drug	Porcine Ovine Porcine Chicken	EMEA/MRL/826/0 2-FINAL (EMEA, 2002e)	Not in fish	
---------------	----------	----------	-------------	--	--	-------------	--

*Registered for salmonids

Table 3. Properties of important antibacterial groups for animal farming/aquaculture in EU/Mediterranean countries

Antibacterial class	Type	Mechanisms of action	Mechanisms of resistance	Evidence for resistance in fish pathogens	PK/PD interactions	Goal of therapy	Predictive indices of PK/PD	References
<i>TETRACYCLINES</i>	Bacteriostatic	Inhibitors of protein synthesis	Efflux, ribosomal protection, drug modification		(Co-dependent) Concentration & Time-dependent killing	Maximize amount of drug	AUC_{0-24}/MIC	(Burgess, Frei, Lewis, Fiebelkorn, Jorgensen, 2007)
<i>(FLUORO)QUINOLONES</i>	Bactericidal	Inhibitors of DNA gyrase	Altered target, decreased uptake		Concentration-dependent killing	Maximize concentration	C_{max}/MIC	(Dudley, 1991)
<i>SULFONAMIDES & DIAMINOPYRIMIDINES</i>	Bacteriostatic/in combination bactericidal	Inhibitors of folinic acid synthesis	Altered drug penetration, altered target enzyme, plasmid transfer		Time-dependent killing	Maximize duration of exposure	$T_c > MIC$	
<i>PENICILLINS</i>	Bactericidal	Inhibitors of cell wall synthesis	Enzymatic destruction, altered target, decreased uptake		Time-dependent killing	Maximizeduration of exposure		(McKellar, Sanchez, Jones, 2004)
<i>PHENICOLS</i>	Bacteriostatic	Inhibitors of protein synthesis	Plasmid-mediated resistance, reduced membrane permeability, mutation of the ribosomal subunit	High MIC	Time-dependent killing	Maximizeduration of exposure	$T_c > MIC$	(Burgess, Frei, Lewis, Fiebelkorn, Jorgensen, 2007)



<i>LINCOSAMIDES</i>	Bacteriostatic	Inhibitors of protein synthesis	Plasmid-mediated resistance, ribosomal modification, efflux, and drug inactivation	High MIC	Time-dependent killing	Maximizeduration of exposure	$T_c > MIC$	
<i>AMINOCYCLITOLS</i>	Bacteriostatic/ Bactericidal	Inhibitors of protein synthesis	Enzymatic modification, altered target, decreased uptake	High MIC	Mostly concentration-dependent killing	Maximize concentration	C_{max}/MIC	(McKellar, Sanchez, Jones, 2004)

AUC_{0-24} : area under the serum/plasma concentration curve at 24h; MIC: lowest concentration of an antibacterial which prevents visible growth of a bacterium; 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

3. Antibiotic selection, prescription and delivery: main criteria, technical aspects and constraints

The selection of the most adequate antibiotic therapy follows similar general criteria as in humans, terrestrial animals or other fish species. These are the main aspects to take into account:

- 1) The target: the affected fish stock
- 2) The problem: the disease and the causative agent: the pathogen or pathogens and the specific characteristics
- 3) The tools: antibacterials, main characteristics and pharmacokinetic properties
- 4) The delivery method
- 5) The therapeutic regimen / therapeutic strategy

In addition, finfish production presents another very relevant aspect that has not so many relevance in human or terrestrial animal farming antibiotherapy but in aquaculture and particularly in aquatic animal production presents a major relevance and constitutes the aspect

- 6) Environmental impact
- 7) Welfare issues

And last, but not least, another relevant aspect that should also be addressed is

- 8) the problems related to antibiotic resistance appearance

Although in this topic, Mediterranean fish farming present some particularities, this very relevant aspect for the 'One Health' concept should be considered in a holistic way together with the use of antibiotics for human and terrestrial and aquatic animal health.

However, the therapeutic antibiotic management in large stocks of aquatic finfish and particularly stocks of Mediterranean finfish species such as European sea bass and gilthead sea bream presents many relevant particularities.

3.1. The affected fish stock

This is the first and complex point that presents a number of relevant considerations. These considerations are based below.

3.1.1. Species

Gilthead seabream and European seabass are two completely different species, not only because they are taxonomically different teleost fish species (two different families: Moronidae, temperate basses in the case of the European sea bass and Sparidae, porgies), but also because they present many relevant morphological, physiological, metabolic and behavioural differences. One of the most frequent mistakes in the past in Mediterranean aquaculture was to consider both species in a similar way (in terms of husbandry and nutrition, as an example) or extrapolate

D3.3-Therapeutics for MMFF

available scientific information from salmonids as far as therapy is concerned. However, nowadays the current available scientific and technical knowledge on these species (Castells et al., 2000; della Rocca, et al., 2004a; Di Salvo, et al., 2013; Intorre et al, 2000; Malvisi et al., 1996; Rigos et al. , 1999; Rigos et al, 2006; Rigos, et al., 2002c; Rigos, et al., 2004b; Rigos, et al., 2002b; Rigos, et al., 2003a) clearly allows the development of very specific and detailed management and farming protocols for each species separately. However, in therapeutic antibiotic management this differentiation in some cases is not yet achieved or at least, considered.

3.1.2. Age/size/weight

Both species have long rearing periods (up to 2-3 years for large size fish) with very relevant metabolic changes according to the size, weight or age of the fish. Gilthead seabream and European seabass larvae are completely different organisms from fry, juveniles or adult fish from many different points of view and these differences may have direct implications for antibacterial treatments. However, these potential differences still remain unexplored.

3.1.3. Stock and rearing system

The target stocks for treatment can also present many relevant differences according to the different rearing scenarios. As it was introduced before, in Mediterranean aquaculture, reared stocks tend to be composed of large batches of fish -millions in larval tanks, hundreds of thousands in postlarvae and juveniles, and between tens of thousands (ponds, RAS) to hundreds of individuals in cages. The rearing units can also be diverse in Mediterranean aquacultures. 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 in flowthrough or recirculation systems while pre-on-growing is mainly based in tanks (with different volumes). All these aspects have relevant implication in issues such as the delivery method and the therapeutic regime but also in the environmental impact.

Several particular host factors must be taken into account for appropriate therapeutic management. Affected fish stocks should be seen as 'particular patients in particular conditions' with specific particularities. In addition to the previously described factors such as age or species, some other factors and circumstances can also be associated to the affected stock.

3.1.4. Water temperature

As in many other aspects, temperature plays a relevant role in the therapeutic approach. Water temperature is usually a factor not properly considered in finfish therapeutic approaches when these approaches are directly extrapolated from homeotherm terrestrial vertebrates. As water temperature strongly regulates fish metabolism, some aspects mainly related to the pharmacokinetic (PK) evolution of the different therapeutic molecules and their derivatives in the body of the fish are different 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 clearly influenced by water

D3.3-Therapeutics for MMFF

temperature (Rigos et al., 2002a; Rigos et al., 2002c). In many cases and due to different reasons, PK studies for different antibiotics and drugs are performed only at a single temperature and only very rarely these studies consider PK evolution at different temperatures. As a result, very frequently PK results for a specific active molecule at a specific temperature are recklessly extrapolated to other temperature scenarios, even if the range between both temperatures is not really relevant. As a consequence, most recommended therapeutic doses in fish are fixed doses, with no specific consideration of the rearing temperature of the fish. This scarcely considered aspect is probably causing relevant differences between the theoretical simple calculations made for finfish treatments and the real situation in the affected stocks. Another very relevant factor directly related to temperature and therapeutics in the particular case of medicated feeds delivery, are the temperature-related feeding rates. As feed pellets are used as the main carrier (vehicle) for the delivery of the medicine to the fish, dosage calculation of medicines/premixes to be added to the feed to prepare the prescribed medicated feeds requires the knowledge of the current and expected rearing temperature of the affected stock. Fish feeding rates are lower at lower temperatures and are higher at higher temperatures, so using a single therapeutic dose (mg of the selected active molecule/ Kg of fish), the same amount of medicine should be 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). The consequence of this fact is that the active compounds in medicated feeds to be delivered when fish are reared at low water temperatures, can be much more concentrated than at high temperatures, with potential consequences in the uptake of the medicated feed by the fish, palatability, and therefore, in the pharmacokinetic pattern of this treatment. In European seabass and gilthead seabream this has relevance in some wellknown different situations such as the antibacterial management in case of treatments against winter disease in gilthead sea bream or Pasteurellosis management in European sea bass outbreaks at 25°C.

3.1.5. Other physiologic and metabolic considerations

Most of the processes involved in the different compartments of PK can be influenced by differences in the physiology and metabolism related to other functions. Antibiotic PK can be modified if the digestive system activity is modified by factors such as feed conformation. Feed contents or feeding regime but also can be impaired at the presence of specific digestive diseases such as *Enteromyxum* or *Enterospora* infections and winter disease in gilthead sea bream or Coccidiosis in European seabass. Hepatic function is also involved in other PK compartments and changes in the hepatic antibiotic processing capacity can be found in certain conditions when the hepatic metabolism has been pushed to their limits (high energy diets, chronic stress, fatty liver) or is affected by chronic systemic diseases. In these particular situations, a more accurate selection of the antibiotic administration regime should be recommended.

3.1.6. Stock population

In field conditions, practical therapeutic approaches require some level of implication in order to facilitate the different calculations and logistics. Fish populations to be treated are generally treated as a 'single organism' by the total biomass estimation.

D3.3-Therapeutics for MMFF

This approach is also frequently used for feeding calculations but sometimes does not take into account some particularities relevant to the result of the treatment. Feeding calculations are normally based in highly homogeneous fish population in terms of the results of biometry or other biomass assessment methods. In very homogenous fish population 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 increasing. It also should be taken into account that dose calculations are usually done in healthy populations, with more or less predictable feeding behaviour. In real situations, the sick population is already affected by the outbreak and virtually in all the infectious diseases, appetite is strongly impaired or even abolished in a part of the stock. This is the reason why an accurate assessment of the evolution of the disease is also helpful to refine the therapeutic strategies.

3.1.7. Evolution of the disease in the stock

This factor is also strongly related to the disease typology and will be specifically addressed in the following section on pathogens. In Mediterranean aquaculture, the assessment of the precise timing in the evolution of the outbreak is a very relevant information for a decision-making. Typical bacterial outbreaks in gilthead seabream and European seabass are associated to *P. damsela* subsp. *piscicida*, *V. anguillarum* and other related bacterial species, usually display the pattern described in **Figure 1**. Some examples about specific disease evolution patterns can be frequently seen in gilthead sea bream and European sea bass production. Fast evolution of the disease is mainly observed in septicemic bacterial processes in gilthead seabream and European sea bass postlarvae and fry at nursery level. In these cases, Vibriosis in European sea bass and Pasteurellosis/Photobacteriosis outbreaks in European sea bass and gilthead sea bream develops particularly fast and antibiotic treatments are unsuccessful if they are not applied immediately after first signs of the disease are detected. Juveniles are even more sensitive to these and other bacterial problems (Vibriosis by other *Vibrio* species, Tenacibaculosis) and in these cases specific immunoprophylaxis, if available, cannot be applied until fry have developed a high level of immunocompetence (around 1 to 5 grams). Moreover, the development of a relevant level of protection is a process that can take some weeks after vaccination. For these reasons, in many cases, European sea bass and gilthead seabream fry present narrow time-windows, where antimicrobial treatments should be applied immediately.

Long chronic phases are particularly found in Pasteurellosis/Photobacteriosis outbreaks in both gilthead sea bream and European sea bass. In particular risk situations and mainly if the outbreaks appear at the beginning of the Pasteurellosis season (June-September according to the different geographical and temperature regimes), prolonged antibiotic treatments are advisable in the same stock but not if the previous antibiotic treatment failed.

The evolution of the mortality can be modelled in four different phases displayed in **Figure 1**:

D3.3-Therapeutics for MMFF

Phase 1: prodromic phase: the pathogen can be isolated from the stock but mortalities are still similar to the basal mortalities. No changes are detected in the behaviour of the fish.

Phase 2: acute phase: is characterised by a sudden increase of the mortality until mortalities reach a top. This phase is normally related to the acute part of the outbreak. The number of symptomatic fish (sick, not eating) that will die in the next 2-3 days is increasing. Fish dying in these days are usually the most susceptible ones (due to many different reasons: genetic background, stress, etc).

Phase 3: chronic phase: is characterised by a clear decreasing trend in the daily mortality rate but also the total population has substantially decreased and the remaining survivor stock is now composed by resistant fish (less susceptible individuals) that were not infected and developed any infection and also some survivors. In this scenario, the disease development is much more difficult because the total number of fish in the stock has decreased and also because the total survivor fish stock is now much less susceptible to the disease.

Phase 4: resolution phase: is achieved when mortalities are not high but still abnormal. Feeding behaviour is recovering but not completely normal. Mortality in this phase is mainly composed by chronically affected fish that finally succumb to the disease after being fighting with the disease for a long period.

In Phase 1 (prodromic) the fish stock is still intact and only very few fish are infected and display anorexia. In this case, nearly all the whole stock is still candidate for treatment using the medicated feed. In Phase 2, the number of affected fish rises very fast and in an exponential way. In very few days, the number of sick fish (and also with suppressed appetite) increases. It is possible to predict in this figure how many fish are anorectic in a certain day just counting the daily mortalities and estimating the mortality of the next three-four days. All these fish at this point are refractory to the treatment. The number of sick and dying fish is increasing progressively; the number of fish with reduced appetite is also increasing, decreasing therefore the efficiency of the treatment. In Phase 3 (chronic) the typology of the remaining fish in the stock has changed dramatically: in this case, most of the population is formed by naturally resistant fish survivors, antibiotic-protected fish and chronically affected fish. As the volume of the fish stock has substantially decreased, the number of fish that becomes infected and are sick for the first time in this outbreak is really low, so the fish that are eating the medicated feed in these third phase corresponds to naturally resistant fish and therefore, fish that do not require medication and antibiotic-protected fish, fish that still require a continuous supply of antibiotics. Fish that are still alive but chronically infected and still fighting against the disease are also displaying affected appetite so although the medicated feed is offered, they do not ingest it. Phase 4 (recovery) is characterized by a substantial reduction of the population at risk, formed mainly by naturally survivor fish, including survivor chronic fish that are not longer susceptible to the disease (natural immunoprophylaxis) and antibiotic-protected fish.

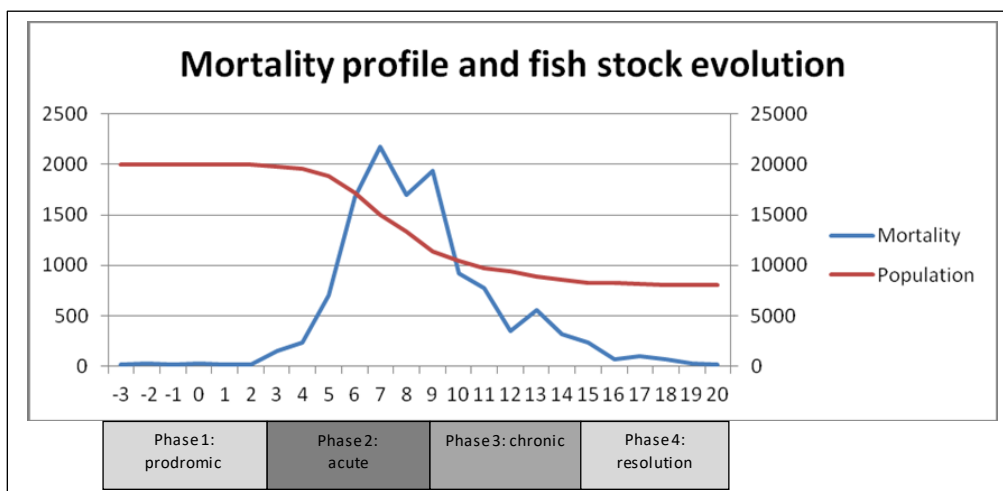


Figure 1: Simulation of the evolution of the daily mortality (scale in the left side, number of dead fish collected per day) and fish surviving population in the stock (scale in the right side, estimated number of surviving fish) in a typical severe outbreak (60% total mortality) associated to *P. damselae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

In this four-phases scenario, the role of the potential antibiotic treatment using medicated feeds should be carefully analyzed in each of the four phases. Phase 1 is the key phase when antibiotic administration has much sense and efficacy: all the stock is still eating and can become protected by the medication. Antibiotic 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 in few days if are not protected by an early medicated feed regime. As the disease progresses in phase 2 (evaluated by the rise of mortalities), the efficiency of the antibiotic treatment decreases dramatically. After reaching the plateau in phase 3 (chronic), only the susceptible but medicated fish requires a continuous supply of antibiotics. If the treatment was applied very early, the amount of susceptible-medicated fish will be high enough to justify the extension of the treatment. If the antibiotic treatment was applied late, then the therapeutical value of an extension of the treatment is very low. In phase 4 (recovery) the extension of the antibiotic treatment has only sense if the treatment was applied very early (phase 1 or early phase 2 stages) and if the epidemiological conditions of the fish stock or farm (temperature, high biomass, stress) indicate a relevant risk for resurgence of new outbreaks. These scenarios are schematized in the following drawings (Figure 2) following a hypothetical situation of bacterial outbreak.

In scenario 2, a description of the recommended theoretical dosages to be used according to each phase of the evolution of the mortality and also according to the changes on the real daily feed intake is given. Notice that using this scheme and using the modifications according to the mortality and adjusted feed intake, the amount of

D3.3-Therapeutics for MMFF

medicated feed is substantially different from the calculations made on the initial healthy stock and without taking into account the appetite changes in the different phases. These differences are expressed as following:

	Phase 1	Phase 2	Phase 3	Phase 4
General calculations	Stock: 10.000 Kg Feed: 100 Kg Antibiotic dose in the medicated feed: 2 g/Kg feed Total antibiotic used: 200 g per day	Stock: 10.000 Kg Feed: 100 Kg Antibiotic dose in the medicated feed: 2 g/Kg feed Total antibiotic used: 200 g per day	Stock: 10.000 Kg Feed: 100 Kg Antibiotic dose in the medicated feed: 2 g/Kg feed Total antibiotic used: 200 g per day	Stock: 10.000 Kg Feed: 100 Kg Antibiotic dose in the medicated feed: 2 g/Kg feed Total antibiotic used: 200 g per day
Specific realistic calculations	Stock: 10.000 Kg Feed: 100 Kg Antibiotic dose in the medicated feed: 2 g/Kg feed Total antibiotic used: 200 g per day	Stock: 7.000 Kg Feed: 35 Kg Antibiotic dose in the medicated feed: 2.8 g/Kg feed Total antibiotic used: 98 g per day	Stock: 6.000 Kg Feed: 42 Kg Antibiotic dose in the medicated feed: 2.4 g/Kg feed Total antibiotic used: 100 g per day	Stock: 5.000 Kg Feed: 50 Kg Antibiotic dose in the medicated feed: 2.2 g/Kg feed Total antibiotic used: 110 g per day

D3.3-Therapeutics for MMFF

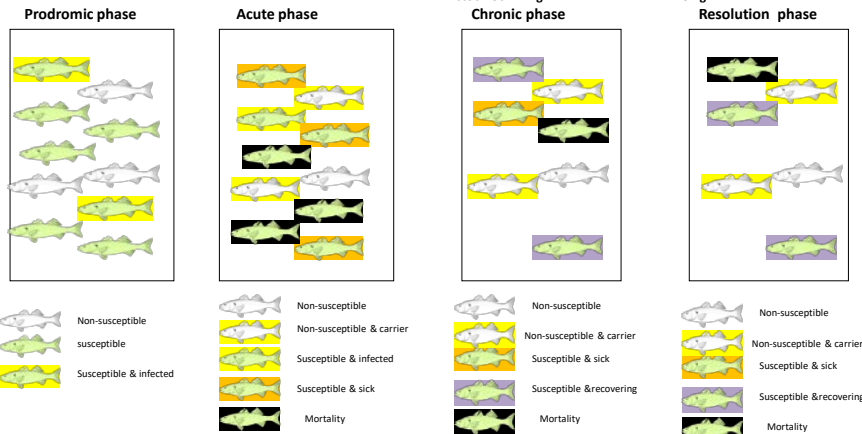
Scenario 1: European seabass stock, averaged 1 Kg, without any antibacterial treatment

Stock (100%) : 10.000 Kg
1 Kg fish
seabass normal feeding rate:
1%
Feeding: 100% normal rate:
100 Kg/day

Mortality: 3000 Kg (30%)
Fish still alive: 7000 Kg (70%)
Fish still "treatable and recoverable": 4000 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: +1000 Kg (10%)
Total mortality: 4000 Kg (40%)
Fish still alive: 6000 Kg (60%)
Fish still "treatable and recoverable": 5000 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: +1000 Kg (10%)
Total mortality: 5000 Kg (50%)
Fish still alive: 5000 Kg (50%)
Fish still "treatable and recoverable": 5000 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 deliver: 50 Kg
Real feed intake of the stock : 45 Kg



Scenario 2: European seabass stock, averaged 1 Kg, with antibacterial treatment

Stock (100%) : 10.000 Kg
seabass normal feeding rate:
1%
Feeding: 100% normal rate:
100 Kg/day
Antibiotic dose: 20 mg/Kg
WW/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: 28 - 42 Kg (35 Kg)
Fish still alive: 7000 Kg
Fish still "treatable and recoverable": 4000 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 mortality: **40%**
Reduced stock: theoretical feed to deliver: 60 Kg
Real feed intake :36 -42 Kg
Fish still alive: 6000 Kg
Fish still "treatable and recoverable": 5000 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 deliver: 50 Kg
Real feed intake: 45 Kg
Fish still alive: 5000 Kg
Fish still "treatable and recoverable": 5000 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

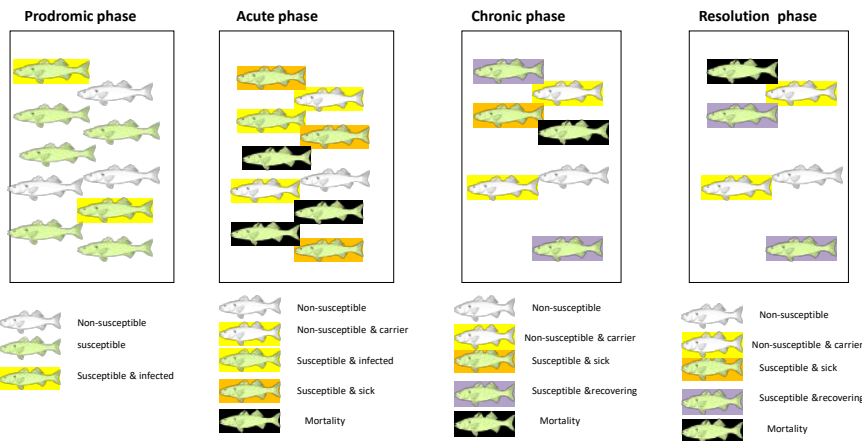


Figure 2: Initial population is separated in two groups: susceptible and not susceptible, as in all outbreaks there is always a part of the population (naturally not susceptible or naturally resistant) that not become "sick" during the process. Obviously, the percentage of susceptible fish in a population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

D3.3-Therapeutics for MMFF

The results displayed in this emulation clearly showed that a reduction higher than 50% of the total amount of medicated feed and antibiotic used is possible by simply implementing periodical corrections based on the current stock measurements and feeding rates estimations. Also notice that the theoretical therapeutic dose in this case is surprisingly quite stable (2.0-2.8 g/ Kg medicated feed) during all four phases. This emulation highlights the relevance of an accurate and precise evaluation of the outbreak follow-up and the use a precise day-to-day stock assessment and real feeding rateto refine the efficacy of the antibiotic treatments to use only the amount of required antibiotic and therefore, reduce the waste of medicated feeds and antibiotics and their release in the environment. These scenarios can be even much more dramatic (**Figure 3, Figure 4 and Figure 5**) if we compare the differences between good delivery strategies, with administration of the antibiotics in early stages (prodromic after efficient diagnostics) versus 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 considerably aid to realize the above scenarios and adjust medication dosing during bacterial epidemics (Parra, García, Sendra, Lloret, 2018).

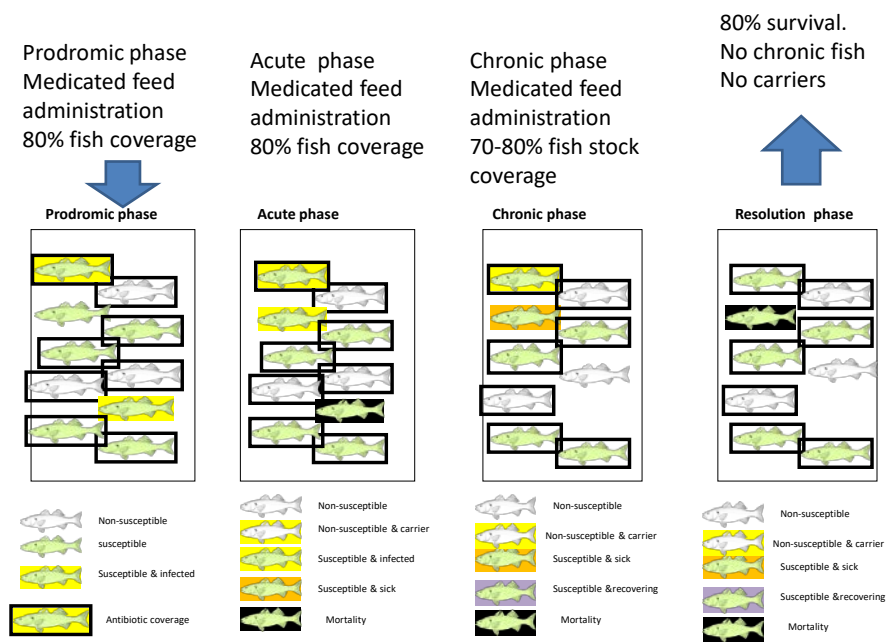


Figure 3: Early administration strategy: high antibiotic coverage is maintained during all the outbreak.

D3.3-Therapeutics for MMFF

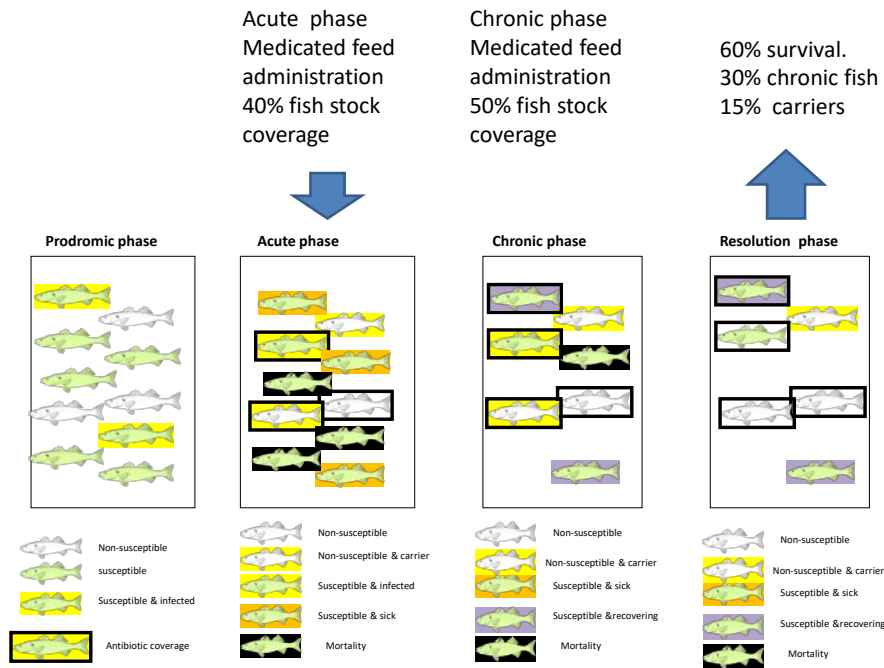


Figure 4: Late administration strategy: lower and more variable antibiotic coverage during the outbreak. This is the most frequent situation found in the field.

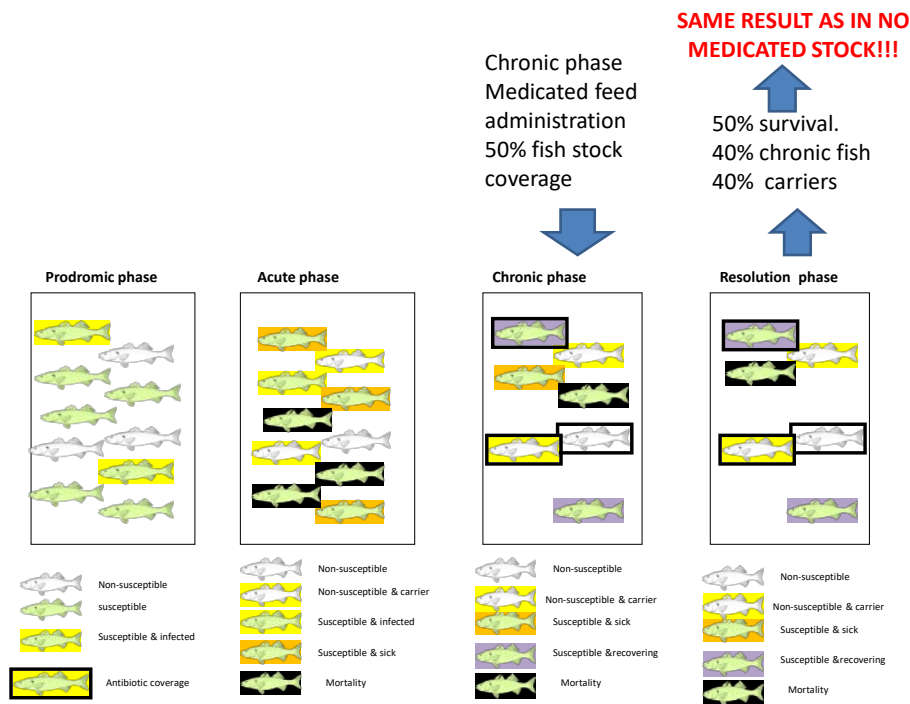


Figure 5: Very late administration: please notice that results in this case are the same to those obtained if fish do not receive any medication.

3.2. The disease and the causative agent: the pathogen or pathogens and the specific characteristics

The selection of the most suitable antibiotic should be done according to the specific characteristics of the disease and the pathogen. In finfish therapeutics, the most common criteria used are bacterial wall characteristics (gram positive or negative) and antibiotic sensitivity of the strain. Other criteria used, mainly in case that data on antibiotic sensitivity are not available, are historical records on the efficacy of the antibiotics against the same diseases and pathogen. The final selection is made according to these criteria but also to some other external constraints such as availability of the veterinary medicine and price. A more complete and detailed description about the current methods of evaluation of the antibiotic sensitivity in Mediterranean aquaculture are described in this report, in the section related to pharmacodynamics (PD) and MIC evaluation.

In addition to the laboratory results and the available information about breakpoints, epidemiological breakpoints (ECOFF) and/or MICs are also very important to take into account based on the history or previous records of antibiotic use in the same stock and also at farm level. Results of efficacy of the different antibiotic administration against the same pathogen as well as data about the evolution of the antibiotic sensitivity are very relevant to get predictive inputs about the potential efficacy of the antibiotic treatments. Surveillance of antimicrobial resistance at farm level has a direct benefit for the improvement of the efficacy of the treatments besides the importance of these data in the implementation of general national, transnational, European and global programmes on antimicrobial resistance assessment. As in the 3Rs principle in animal research, antibiotic treatments are a valuable tool, but they should be **R**eplaced by more efficient and powerful preventive measures and alternative treatment methods when possible, **R**educed in terms of quantity and frequency only where strictly necessary, and **R**efined in order to use the lowest amount of antibiotics for the highest efficacy in the treatments with the lowest impact for the environment and for the global concept of 'One Health'. This report, in many sections contributes with recommendations and strategies to improve these uses of antibiotics in antibacterial treatments in Mediterranean aquaculture.

3.3. The tools: antibiotics, main characteristics and pharmacokinetic properties

One of the most relevant parts in this report are the antibiotics that are used or can be used in Mediterranean aquaculture and particularly in gilthead sea bream and European sea bass production. A complete introduction to the legislation and antibacterial use has been previously described in this document, with a wide updated information of the different antibiotics that can be used in gilthead sea bream and European sea bass under the current EU and national legislation framework. The selection and use of these antibiotics in Mediterranean aquaculture requires also a complete update about the general and specific characteristics of each one of them. This knowledge of these characteristics is also very relevant for a complete

D3.3-Therapeutics for MMFF

understanding the pharmacokinetic (PK) and PD properties of these antibacterial compounds and how these compounds should be used for bacterial disease control with the best levels of efficacy, safety and responsibility.

3.3.1. Tetracyclines

Tetracyclines, discovered as early as in the 1940s, are a family of antibacterials that inhibit bacterial protein synthesis (mRNA translation) by binding to bacterial 30S ribosomal subunit of microbial 70S ribosomes. They are broad-spectrum low-cost bacteriostatic agents (Table 3), produced by the *Streptomyces* spp. fungi which exhibit a time-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 therapy of bacterial infections (Eliopoulos et al., 2003).

Oxytetracycline (OTC) is perhaps the most common tetracycline used worldwide for the treatment of bacterial fish diseases. The PK of OTC have been thoroughly investigated in European sea bass and gilthead sea bream (Malvisi et al., 1996; Rigos et al., 1999; Rigos et al., 2006; Rigos et al., 2002a; Rigos et al., 2004b; Rigos et al., 2003c; Rigos, et al., 2004a) (Table 4). The absorption of OTC in these two species is limited, with calculated bioavailabilities ($F\%$) values measured as low as 9-22% (Rigos et al., 2004b; Rigos et al., 2003c). 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 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 reduces solubility and consequently, its membrane permeability along the gastrointestinal tract, have been blamed for the low OTC absorption (Rigos et al., 2004a). It has to be considered that marine water contains high amounts of divalent cations that can increase the chelation effect of OTC in marine species. Some attempts to increase the OTC oral bioavailability through self emulsifying formulations for European sea bass were recently described (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 fish has been found to be 2.5-2.6 $\mu\text{g/ml}$ (Rigos et al., 2004b; Rigos et al., 2003c, Rigos et al., 2003a; 2004). 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; Rigos et al., 2003c) and in edible tissues of gilthead sea bream (Romero et al., 2010), suggesting perhaps 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 fish for 14 days at 19-28°C in gilthead sea bream, tissue levels can reach values of 7.7 and 14.7 $\mu\text{g/g}$ in skin and liver, respectively (Malvisi, et al., 1996). However, the same authors described lower and surprisingly decreasing levels in OTC muscle concentrations. Slow removal of OTC from the edible tissues after oral treatment was associated with longer withdrawal

D3.3-Therapeutics for MMFF

times(*WTs*) (20day period) in the same OTC-treated fish. Long *WTs* were also suggested (12 days) by Romero et al.(2010), who administered 30 mg/kg fish for 10 days at 14°C although the recommended *WTs* were as low as 2 days at 19.5°C.

Due to the relatively slow elimination of OTC in that species at least in medium/low water temperatures, a sequential 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.

Chlortetracycline (CTC) has limited use in fish disease treatments and even scarcely considered in fish farmingsince it is lacking authorization in aquaculture despite the factits 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 antibiotic in terrestrial animals and can be a promising alternative to oxytetracycline. It has been used empirically in fish farming (pangasius) in some southeastern Asian countries but very few scientific information is available regarding its use in aquaculture conditions. This is the reason why DOX was explored in Performfish. In our recent studies, DOX was tested in European sea bass plasma following a 5day oral administration of 100mg/kg fish (Table 4). The highest values approached around 0.7 mg/ml.

Since tetracyclines are bacteriostatic drugs with co-dependent action, but mostly concentration dependent killing, the Area under a curve (AUC)₀₋₂₄/MIC PK/PD index would be the most appropriate (Table 3).However, the AUC₀₋₂₄ of administered tetracyclines has not been calculated in most available studies in Mediterranean farmed fish, with only few exceptions. For DOX in particular, the AUC/MIC₉₀ was calculated to be more than 20 for the first days of treatment based on relatively low MIC values of important bacterial pathogens (Table 5). These promising *in vitro* values of DOX are reflected in lab trials where the compounds appeared very effective when orally delivered against heavy *V. harveyi* infections of European sea bass juveniles (Rigos et al., lab observations).

3.3.2. (Fluoro)quinolones

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

The kinetic profiles of some quinolones and fluoroquinolones have been widely investigated in European sea bass and gilthead sea bream (Table 4). Interestingly, OA absorption studies in European sea bass have revealed higher apparent digestibility values (64-92%) (Rigos, et al., 1999; Rigos et al., 2002c) compared with that calculated in gilthead sea bream (14-15%) (Rigos, et al., 2002b) indicating species-specific absorption differences. This is a very relevant aspect to take into account for specific

D3.3-Therapeutics for MMFF

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 than OA in gilthead sea bream (29%)(Rigos et al., 2003a). This might indicate that FLU is preferable to OA in diseased gilthead sea bream, assuming no differences in bacterial sensitivity to these two quinolones.

In agreement with that, plasma OA levels following single or multiple oral dosing in gilthead sea bream, were found to be $<1 \mu\text{g/ml}$ (Rigos et al., 2003b; Rigos et al., 2002b), while respective values for FLU after single dosing in gilthead sea bream were measured to be $1.7 \mu\text{g/ml}$ (Rigos et al. 2003a). This difference may be attributable to the greater *F* of FLU in gilthead sea bream.

Regarding the removal of OA in gilthead sea bream, it has been revealed 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 $100 \mu\text{g/kg}$ (EMEA, 2005a), as fast as 24 h after completion of treatment at 19°C (Rigos et al., 2003b). The rapid depletion of OA from the edible tissues of the same species was confirmed by Romero et al. (2010) who administered 30 mg/kg fish for 10 days at $14\text{-}19.5^\circ\text{C}$. 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 skin and vertebrae of gilthead sea bream act as reservoirs for FLU for prolonged periods even after cessation of treatment (12 mg/kg fish for 5 days), although drug levels remain below the MRL of $600 \mu\text{g/kg}$ (EMEA, 2002). The same study revealed consumer safe levels in edible tissues (muscle plus skin) even 24h post-treatment at $25\text{-}28^\circ\text{C}$. This finding is in agreement with Romero et al. (2010) who administered 30 mg/kg fish for 10 days at $14\text{-}19.5^\circ\text{C}$. On the contrary, higher FLU levels in the same tissues necessitated the calculation of *WTs* of 106 and 76h at $18\text{-}24^\circ\text{C}$, respectively (Tyrpenou et al., 2003).

Tissue distribution studies of SAR in gilthead sea bream have revealed accumulation in liver and vertebra, with vertebra acting as reservoir for the drug since levels persist after treatment has been terminated (Tyrpenou et al., 2002). In edible tissues, *WTs* were calculated to be 42 h at 25°C for a MRL of $30 \mu\text{g/kg}$ (EMEA, 1998). The rapid depletion of SAR from medicated fish tissues is a favorable characteristic allowing shorter *WT* softreated fish. However, distribution of SAR in fish circulation along with pharmacodynamic data is still required to obtain a complete picture of its possible efficacy against pathogens of European sea bass and gilthead sea bream.

Oral treatment (gavage) with 5 mg enrofloxacin (ENR) per kg fish resulted in C_{max} of $1.4 \mu\text{g/ml}$ in European sea bass plasma kept at 15°C (Intorre et al., 2000), while after IV injection or single oral administration of 2.5 and 10 mg/kg fish resulted in 3.8 and $1.2 \mu\text{g/ml}$, respectively in gilthead sea bream serum maintained at $25\text{-}27^\circ\text{C}$ (della Rocca et al., 2004a).

Danofloxacin was investigated in European sea bass after a multiple (5 days) in-feed administration (10 mg/kg) 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= $100 \mu\text{g/kg}$; EMEA 2002c). As in the case of SAR, distribution of DAN in fish circulation and pharmacodynamic data are lacking for euryhaline fish species.

D3.3-Therapeutics for MMFF

Rapid elimination of quinolones and fluoroquinolones (Table 4) from tissues of gilthead sea bream suggests that at least daily dosing (in specific cases 2 meals per day) 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 ($AUC_{0-24}/MIC, C_{max}/MIC$), dosages should be maximised wherever possible (Table 3). Finally, it also should be taken into account that quinolones are considered as antimicrobials critically important and with highest priority according WHO (2017), so probably their use in farm animals will start to be reduced and eventually banned.

3.3.3. Potentiated Sulfonamides

The potentiated sulfonamides (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 the DAP inhibition of tetrahydrofolic acid formation. Potentiated sulfa 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 sulfa drugs in veterinary medicine and they are widely used in fish medicine. The recommended dosage of potentiated sulfa in fish treatments is 25 and 5 mg kg⁻¹ fish (for 5-10 d) for SDZ and TRM, respectively (EMA, 1995b; 1997b).

There are several PK studies of sulfonamides /potentiated sulfonamides on gilthead sea bream but none in European sea bass (Table 4). Distribution of SDZ (25 mg/kg fish for 5 days at 24-26°C) in gilthead sea bream circulation was promising, reaching values as high as 2.9-3.2 µg/ml plasma in fish receiving fish or plant-oil based diets (Rigos et al., 2013). Withdrawal times to reach consumer safety levels (MRL of 100 µg/kg: (EMA, 1995b) were calculated to be 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 sea bream fillet. Depletion of both SDZ (30 mg/kg fish for 10 days) and TRM (30 mg/kg fish for 10 days) was reported to be rapid in gilthead sea bream 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 et al., 2010). Similarly, fast removal of SDM delivered in combination 5:1 with OMP at 50 mg/kg fish for 5 days, was apparent in gilthead sea bream kept at 26°C with drug levels dropping below MRLs (50 and 100 µg/kg MRLs for OMP and SDM, respectively: (EMA, 1995b; 1997b), 24h following treatment (Papapanagiotou et al., 2002). Longer WT_s (5-6 days) for SDZ and TRM were recommended by Zonaras et al. (2016) at 24-26°C.

Potentiated SFM drugs possess bactericidal effect with time-dependent killing profile ($T_{C>MIC}$) (Table 3). In this case, the dosing schedule must reach drug concentrations at sites of infection that exceed the MIC for the longest possible period. In consequence, the dosage regime must tend to divide the daily doses in two or more administrations.

3.3.4. Penicillin Derivatives

Penicillin derivatives (β -lactams), including amoxycillin (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. The usual dosage of β -lactams in fish treatments is 40-80 mg/kg 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 lack of registration for use in aquaculture in most Mediterranean countries and the shortage of relative PK fish studies. The kinetic profile and efficacy of AMP have not yet been investigated in euryhaline fish species. However, published pharmacodynamics for AMO indicate this drug is a promising antibacterial against important bacterial pathogens of euryhaline fish (Mazzolini et al., 1997). AMO displayed negligible bioavailability in gilthead sea bream (0.33%; (della Rocca et al., 2004a), questioning its use in this species, at least using oral administration. However, kinetic studies in European sea bass, may demonstrate improved AMO absorption, where it may be more appropriately used for antibacterial therapy.

As for SFM drugs, penicillin drugs possess bactericidal effect with time-dependent killing profile ($T_{C>MIC}$) (Table 3). The same conclusion as for potentiated sulpha drugs, must be taken for the dosage regimen in order to divide the daily dose during the day-time.

3.3.5. Phenicol

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, such as aplastic anaemia which has been seen with CAP use 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 fish for 10 and 5 days, respectively (EMA, 2002d; 2006).

The kinetic profile during treatment and efficacy of FLO have not yet been published in euryhaline fish species. However, preliminary trials on orally administered FLO in European sea bass revealed plasma concentrations around 1 μ g/ml following dosing of 10 mg/kg fish for 7 days (Rigos et al. in preparation). In the same work the WT in edible tissues were calculated to be less than 24 h in fish kept at 20°C. Similarly, in gilthead sea bream, FLO levels in muscle plus skin dropped below MRL (1000 μ g/kg; EMA, 2002d) on day 2 post-treatment after a dosing of 10 mg/kg fish for 10 days at 27°C (Di Salvo et al., 2013).

There are plenty studies however on the kinetics of THI in European sea bass (Castells et al., 2000; Intorre et al., 2002; Malvisi et al., 2002) and gilthead sea bream (Malvisi et al., 2002). Following oral THI administration via oral gavage, peak plasma THI concentration was measured to be as high as 5.6 and 9.4 μ g/ml in European sea bass, following 15 and 30 mg/kg dosing, respectively, indicating a dose-dependent

D3.3-Therapeutics for MMFF

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, respectively (Intorre et al., 2002). These differences may be due to the different routes of administration employed, and the influence of feed constitutes on THI absorption. The lower drug levels attained in treated fish in the latter study are more representative to 'at site' treatments where drugs are delivered via the feed indicating that absorption of THI is inhibited in the gut environment.

Regarding the distribution of THI in gilthead sea bream, it was found that it is well-distributed in the tissue compartments following a 5-day dosing at 40 mg/kg at 20-28°C (Malvisi et al., 2002). Intorre et al. (2002) suggested *WTs* of 120 and 144 h for a 5-day dosing at 15 and 30 mg/kg, respectively at 18-20°C considering an MRL of 50 ng/g (EMA, 2006). Similar *WTs* (80 and 89 h) were proposed for THI by the trial of Malvisi et al. (2002) in European sea bass and gilthead sea bream, respectively.

Phenicol are bacteriostatic compounds with time dependent action as in the case of SFM and penicillins (Table 3), so the same conclusions must be taken related to the dosage regimens.

3.3.6. Lincosamides

Lincomycin (LCM) is a natural antibacterial drug obtained from *Streptomyces lincolnensis*. It interferes in the protein synthesis binding to the 50s ribosomal subunit at the same place than 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 has been found in the accessible literature.

This antibacterial was also selected for evaluation in Performfish. Here, LCM displayed a promising distribution profile in European sea bass plasma following a 5 day oral administration of 100mg/kg fish (Table 4). The highest concentration reached values as high as 13 µg/ml. The $T_{C>MIC}$ has been suggested as the most appropriate for lincosamides (Table 3).

3.3.7. Aminocyclitols

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

SPE absorption was also included in Performfish. In this study, SPE displayed an adequate distribution profile in European sea bass plasma following 5 day oral administration of 50mg/kg fish (Table 4). The highest concentration reached values of 1.3mg/ml. The C_{max}/MIC ratio has been suggested as the most appropriate for aminocyclitols (Table 3).

Table 4. Selected pharmacokinetics of antibacterials in European sea bass and gilthead sea bream

Drug	Route	Dose (mg/kg)	Duration(d)	Weight(g)	Temp(C°)	t _{1/2β} (h)	F%	C _{max} (μg/ml)	WTs(h)	References
European sea bass										
OTC	IV	40		110	13	69				(Rigos, Alexis, Andriopoulou, Nengas, 2002a)
OTC	IV	40		110	22	10				(Rigos, Alexis, Andriopoulou, Nengas, 2002a)
OTC	OR-S	50		120	22		22	2.6		(Rigos, Nengas, Athanassopoulou, Alexis, 2004b)
DOX	OR-M	100	5	122	22			0.7		PerformFISH*
OA	IV	10		100	15	87				(Poher, Blanc, Loussouarn, 2003)
OA	IV	15		110	14	315				(Rigos, Alexis, Andriopoulou, Nengas, 2002c)
OA	IV	15		110	22	55				(Rigos, Alexis, Andriopoulou, Nengas, 2002c)
FLU	IV	10		120	18	11				(Rigos, Tyrpenou, Nengas, Alexis, 2002d)
ENR	OR-G	5		200-300	15			1.4		(Intorre, Cecchini, Bertini, Cognetti Varriale, Soldani, Mengozzi, 2000)
THI	OR-S	15-30		250-300				5.6-9.4		(Castells, Intorre, Bertini, Cristòfol, Soldani, Arboix, 2000)
THI	OR-M	15-30	5	250-350	18-20			0.9-1.3	144-120	(Intorre, Castells, CristoFol, Bertini, Soldani, Arboix, 2002)
THI	OR-M	40	5	128-150	20-28				89-80	(Malvisi, Della Rocca, Anfossi, Tomasi, Di Salvo, Zanchetta, Magni, Sello, Giorgetti, 2002)
DAN	OR-M	10	5	16-27	16.9-21.1				168-96	(Vardali, Kotzamanis, Tyrpenou, Samanidou, 2017)
LIN	OR-M	100	5	93	23			13		PerformFISH*
SPE	OR-M	50	5	152	26			1.3		PerformFISH*
Gilthead sea bream										
OTC	OR-M	75	14	50-70	19-28				480	(Malvisi, Rocca, Anfossi, Giorgetti, 1996)
OTC	IV	40		100	20	53				(Rigos, Nengas, Tyrpenou, Alexis, Troisi, 2003c)
OTC	OR-S	75		100	20		9	2.5		(Rigos, Nengas, Tyrpenou, Alexis, Troisi, 2003c)
OTC	OR-M	30	10	150-200	14-19.5				288-48	(Romero Gonzalez et al. 2010)
OTC	OR-M	37.5-75	7	75	18					(Rosa, Leston, Castro, Freitas, Barbosa, Pardal, Rema, Dias, Ramos, 2018)
OA	IV	20		100	20	12				(Rigos, Alexis, Tyrpenou, Nengas, Piper, Troisi, 2002b)
OA	OR-S	30		100	20		14	1.0		(Rigos, Alexis, Tyrpenou, Nengas, Piper, Troisi, 2002b)
OA	OR-M	30	10	120-170	19	13-19		0.9	<24	(Rigos, Nengas, Alexis, Tyrpenou, Troisi, 2003b)
OA	OR-M	30	10	150-200	14-19.5			24		(Romero Gonzales, Fernandez Fernandez, Vidal, Muros, Frenich, 2010)

D3.3-Therapeutics for MMFF

Drug	Route	Dose (mg/kg)	Duration(d)	Weight(g)	Temp(C°)	t _{1/2β} (h)	F%	Cmax(μg/ml)	WTs(h)	References
OA	OR-M	6-12	7	75	18					(Rosa, Leston, Castro, Freitas, Barbosa, Pardal, Rema, Dias, Ramos, 2018)
FLU	OR-M	12	5	60-80	25-28					(Malvisi, Rocca, Anfossi, Giorgetti, 1997)
FLU	IV	10		170	19	30				(Rigos, Athanassios, Ioannis, Maria, Maria, Maria, Gera, 2003a)
FLU	OR-S	20		170			29	1.7		(Rigos, Athanassios, Ioannis, Maria, Maria, Maria, Gera, 2003a)
FLU	OR-M	35	5	237-307	18-24	22.1-21.4			107-76	(Tyrpenou, Kotzamanis, Alexis, 2003)
FLU	OR-M	30	10	150-200	14-19.5				24	(Romero Gonzales, Fernandez Fernandez, Vidal, Muros, Frenich, 2010)
FLU	OR-M	6-12	7	75	18					(Rosa, Leston, Castro, Freitas, Barbosa, Pardal, Rema, Dias, Ramos, 2018)
SAR	OR-M	10	5	163-237	18-25	2.5-17.8			42	(Tyrpenou, Iossifidou, Psomas, Fotis, 2002)
ENR	OR-S	10		150	25-27			2.8		(della Rocca, Di Salvo, Malvisi, Sello, 2004a)
FLO	OR-M	10	10	150	27				96	(Di Salvo, della Rocca, Terzetti, Malvisi, 2013)
THI	OR-M	40	5	110-140	20-28				88-86	(Malvisi, Della Rocca, Anfossi, Tomasi, Di Salvo, Zanchetta, Magni, Sello, Giorgetti, 2002)
SDZ	OR-M	30	10	150-200	14-19.5				48	(Romero Gonzales, Fernandez Fernandez, Vidal, Muros, Frenich, 2010)
SDZ	OR-M	25	5	230	24-26			2.9-3.2	118-103	(Rigos, Zonaras, Nikoloudaki, Cotou, Henry, Varo, Alexis, 2013)
SDZ	OR-M	110-220	7	75	18					(Rosa, Leston, Castro, Freitas, Barbosa, Pardal, Rema, Dias, Ramos, 2018)
TRI	OR-M	30	10	150-200	14-19.5				24	(Romero Gonzales, Fernandez Fernandez, Vidal, Muros, Frenich, 2010)
TRI	OR-M	22-44	7	75	18					(Rosa, Leston, Castro, Freitas, Barbosa, Pardal, Rema, Dias, Ramos, 2018)
SDZ+TRI	OR-M	25+5	5	230	24-26				144-120	(Zonaras, Tyrpenou, Alexis, Koupparis, 2016)
SMX+OMP	OR-M	50	5		26					(Papapanagiotou, Batzias, Iossifidou, Psomas, 2002)
AMO	OR-S	80		120-160	22		0.3	1		(della Rocca, Zaghini, Zanoni, Sanguinetti, Zanchetta, Di Salvo, Malvisi, 2004b)
AMO	OR-M	80	10	50-180	22-26					(della Rocca, Zaghini, Zanoni, Sanguinetti, Zanchetta, Di Salvo, Malvisi, 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_{1/2β}: elimination half time; F: bioavailability; Cmax: maximum concentration after oral dosing (single or multiple); WTs: withdrawal times

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

* Compounds investigated in PerformFISH

3.3.8. PD of antibacterials against bacterial pathogens of European sea bass and gilthead sea bream

3.3.8.1. Minimum inhibitory concentrations(MIC)

MIC values against the target bacterium have, almost universally, been treated as the key PD parameter with respect to dose optimisation (Lees et al., 2006). Generally in medicine, there have been attempts to design treatment regimes that would allow for the treatment of bacterial infections that manifest less than full susceptibility. In these attempts, the MIC parameters that have been used are MIC₅₀ or MIC₉₀. If the design criteria for any therapy is that it 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 that allow the empirical identification of sensitive strains, except for *Aeromonas salmonicida* in salmonids (CLSI M-42 M-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 cut-off values (ECOFF) determined from the distribution of susceptibility measures made for a number of strains (Kahlmeter et al., 2003; Miller and Reimschuessel., 2006). When determined in this way, these fully susceptible strains are referred to as wild type (WT). However, the available information about MIC values and even ECOFF values for used antibiotics and relevant bacterial pathogens in gilthead sea bream and European sea bass or in general, for Mediterranean cultured marine finfish species, is really scarce. Most of the available data is from specific research studies and frequently, these studies only focus on specific antibiotics or bacteria. In contrast, there is potential background data in private companies, microbiology laboratories, fish health consultants and specialists as most of them perform antibiograms (Kirby-Bauer-disc diffusion method) as a complementary technique in the routine microbiological checks. Unfortunately, Kirby-Bauer-disc diffusion method has not yet been fully standardized for all the fish bacteria. Another limit in the use of data obtained by Kirby-Bauer is that usually results are of qualitative classification as 'resistant', 'sensitive' or 'intermediate' (without registering the size in mm of inhibition zone), based on the recommendations given by the company that supplies with commercial discs used in the diffusion tests. These recommendations establish different theoretical 'breakpoints' according to the inhibition diameter related to each antibiotic and concentration. However, it should be taken into account that most of this information is obtained from extrapolation of breakpoints in human and, sometimes, veterinary (with regard only to terrestrial animals) medicine and based on data from the European Committee on Antimicrobial Susceptibility Testing, EUCAST (www.eucast.org), BSAC (1991) or similar platforms and databases and not from specific databases based on microbiological, pharmacological and clinical data from fish and fish pathogens. This is one very relevant handicap for antibacterial fish disease treatment as these 'commercial breakpoints' does not take into account the differences between the human/terrestrial animals and fish bacterial pathogens and also the differences between PK and the therapeutical efficacy of the treatments. In

D3.3-Therapeutics for MMFF

some cases, clinical fish diseases practitioners have to use their own experience in the treatment success to empirically interpret these values for a more realistic situation.

Therefore, for these reasons the practical approach suggested by Bonev et al.(2008) concerning the use of regression analysis of the inhibition zone sizes plotted against the natural logarithm of antibiotic concentration to provide an estimate of the MICs is no longer considered applicable.

In light of these issues, as addressed in PerformFISH WP3 and particularly in the task 3.2 on diagnostics, the definition of MICs values obtained by standard methods is an essential tool to define breakpoint values, which are necessary in diagnostics for a correct and responsible antibiotic use in aquaculture. Furthermore, the definition of breakpoints is necessary also to set up a simple and fast antibiotic sensitivity test, as the system 'Perform-TEST' currently under development at UNIBO, which has the aim to give a reliable indication of the antibiotic of choice in a really short time. In this way, this new test will provide to perform a rapid and efficient treatment of the affected stock, avoiding suffering and mortality, and reducing the risk of antibiotic resistance (AMR) as a consequence of empirical treatments.

It is also important to bear in mind that the definition of MIC values in routine diagnostics also permits to monitor AMR trends in aquaculture in the production activities (in our case, Mediterranean Aquaculture). The rising of antibiotic resistance is one of the most relevant problems in WHO One Health 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 antibiotic resistances in aquaculture (Miranda et al., 2018) and also in Mediterranean aquaculture (Chelossi et al., 2003).

It is to be pointed out that currently there is only little MIC information reported in the literature about antibiotics against different bacterial pathogens of European sea bass and gilthead sea bream (Table 5). Moreover, the MICs are often referred only to a single antibiotic molecule or to few bacterial strains, and often data are not comparable to each other because the execution methods are not standardized, as regards mainly the culture media (e.g. Mueller Hinton, Cation-adjusted Mueller-Hinton broth, Nutrient broth, either unmodified or supplemented by the addition of various concentrations of NaCl), incubation time and temperature. For all these reasons, it is necessary to obtain MIC data on a huge number of fish bacterial strains isolated from European sea bass and gilthead sea bream during recent outbreaks or stored in bacterial collections following standardized methods and using several antibiotic molecules already authorized or potential candidates for their future application in aquaculture.

What is pretty apparent is the huge variation of MIC values (Table 5) 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 published studies (Lajnef, 2012) and in PerformFISH trials when tested against the above list of pathogens including also *T. maritimum*. Similar findings are evident in

D3.3-Therapeutics for MMFF

quinolone drugs such as OA, FLU and ENR as well in FLO, sulpha, potentiated sulpha and other antibacterials reviewed in [Table 5](#). In PerformFISH trials, MICs of LIN and SPE against *V. anguillarum*, *V. harveyi* and *P. damsela* subsp. *piscicida* were found to be relatively high as opposed to what was observed for *T. maritimum* in SPE.

Table 5. MICs values of several antibiotics recorded on bacterial pathogens from European sea bass and gilthead sea bream and other marine fish species available in international literature and also obtained from PerformFISH

Antimicrobial agents	Strains	Source	Year/ country	n° strains tested	Media / Reference	MIC (mg ^l ⁻¹) range	Mic ₅₀ (mg ^l ⁻¹)	MIC ₉₀ (mg ^l ⁻¹)	Breakpoint (mg ^l ⁻¹)	References
Oxytetracycline	<i>P. damsela</i> subsp. <i>piscicida</i>	<i>Seriola</i>	2002 Japan	74	Agar dilution method (NCCLS M31-A2, M7-A6) MH agar +2% NaCl 25°C for 48h	≤0.125-64	8	32	0.5	Kawanishi, 2006
Oxytetracycline	<i>P. damsela</i> subsp. <i>piscicida</i>	<i>S. aurata</i>	1996/2000 Spain	16	CSMHB 26°C±2 (Alderman & Smith 2001) 23±2°C for 48h	0.25-32				Martínez-Manzanares, 2008
Oxytetracycline	<i>P. damsela</i> subsp. <i>piscicida</i>	<i>Solea senegalensis</i>	2000/2005 Spain	23	CSMHB 26°C±2 (Alderman & Smith 2001) 23±2°C for 48h	0.25-32				Martínez-Manzanares, 2008
Oxytetracycline	<i>P. damsela</i> subsp. <i>piscicida</i>	<i>D. labrax</i> , grey mullet	Italy	10	Nutrient Broth +3%NaCl	62.5-250				Laganà, 2011
Oxytetracycline	<i>Vibrio</i> spp.	<i>Sparus aurata</i>	Italy	150	CAMHB+1%NaCl	0.12-128	1	4	<4->16	Scarano, 2014
Oxytetracycline	<i>V. alginolyticus</i>	<i>D. labrax</i>	Turkey	15	MH broth +1%NaCl+cation CLSI M49-A (22±2°C and 28±2°C)	4-≥16				Korun, 2008
Oxytetracycline	<i>V. alginolyticus</i>	<i>D. labrax</i> , <i>S. aurata</i>	Tunisia	17	MH broth +1%NaCl	0.48				Lajnef, 2012
Oxytetracycline	<i>V. anguillarum</i>		1983/1994 F-I-GR-DK-GP-C	30	MH agar + 1,5% NaCl	0.187-0.75	0.37	0.75		Guérin-Faublée, 1996

D3.3-Therapeutics for MMFF



Oxytetracycline	<i>Vibrio spp.</i>	<i>Seriola quinqueradiata</i>	2000 Japan	23	Nutrient broth W/O Mg ²⁺ and W Mg ²⁺	62.5 (W/O Mg ²⁺)- 125(W Mg ²⁺)				Kim, 2003
Oxytetracycline	<i>Vibrio spp.</i>	Flounder	2000-2002 Korea	23	Nutrient broth W/O Mg ²⁺ and W Mg ²⁺	<31.3 - 125(W/O Mg ²⁺)- <31.3>500 (W Mg ²⁺)				Kim, 2003
Oxytetracycline	<i>Vibrio spp.</i>	Rock fish, Rock bream, sea bass, striped mullet	2001-2002 Korea	8	Nutrient broth W/O Mg ²⁺ and W Mg ²⁺	<31.3 - 31.3(W/O Mg ²⁺)- <31.3- 125 (W Mg ²⁺)				Kim, 2003
Oxytetracycline	<i>Aeromonas spp.</i>	<i>S. aurata</i>	Italy	104	(CLSI,2011)	0.12-≥128	0.5	16	≤1-≥8	Scarano, 2014
Oxytetracycline	<i>Tenacibaculum dicentrarchi</i>	<i>Genypterus chilensis</i>	2015 Chile	4	CLSI 2014 Broth microdilution	2				Irgang, 2017
Tetracycline	<i>P. damsela subsp. piscicida</i>	<i>S. aurata</i>	1996/2000 Spain	16	CSMHB 26°C±2 (Alderman & Smith 2001) 23±2°C for 48h	0.25-32				Martínez-Manzanares, 2008
Tetracycline	<i>P. damsela subsp. piscicida</i>	<i>Solea senegalensis</i>	2000/2005 Spain	23	CSMHB 26°C±2 (Alderman & Smith 2001) 23±2°C for 48h	0.25-32				Martínez-Manzanares, 2008
Tetracycline	<i>P. damsela subsp. piscicida</i>		USA, Spain	1	MH agar +1%NaCl	<1.56				Ledo et al., 1987
Tetracycline	<i>P. damsela subsp. piscicida</i>	yellowtail	1984-1985	307	Japanese Society of Chemotherapy	0.20-50				Kusuda, 1988
Tetracycline	<i>V. anguillarum</i>		USA, Spain	7	MH agar +1%NaCl	<1.56				Ledo et al., 1987
Tetracycline	<i>Vibrio spp.</i>	<i>Sparus aurata</i>	Italy	150	CAMHB+1%NaCl	0.06-64	0.5	2	<4->16	Scarano, 2014

D3.3-Therapeutics for MMFF



Tetracycline	<i>V. harveyi</i>	<i>D. labrax</i> , <i>S. aurata</i>	Italy 2011-2018	30	CAMHB CLSI M07-A9, VET 04.A2	0.0625-0.125	0.25	0.25		Pretto, 2018
Tetracycline	<i>V. alginolyticus</i>	<i>D. labrax</i> , <i>S. aurata</i>	Tunisian	17	MH broth +1%NaCl	0.48-0.96				Lajnef, 2012
Tetracycline	<i>V. tubiashii</i>		USA, Spain	1	MH agar +1%NaCl	<1.56				Ledo et al., 1987
Tetracycline	<i>Aeromonas</i> spp.	<i>S. aurata</i>	Italy	104	(CLSI,2011)	0.12-≥128	0.5	4	≤1-≥8	Scarano, 2018
Tetracycline	<i>A. hydrophila</i> , <i>A. sobria</i> , <i>A. caviae</i>		USA, Spain	5	MH agar +1%NaCl	<1.56-25				Ledo et al., 1987
Chlortetracycline	<i>P. damsela</i> <i>subsp. piscicida</i>	yellowtail	1984-1985	307	Japanese Society of Chemotherapy	0.05-50				Kusuda, 1988
Doxycycline	<i>P. damsela</i> <i>subsp. piscicida</i>	yellowtail	1984-1985	307	Japanese Society of Chemotherapy	0.05-6.25				Kusuda, 1988
Doxycycline	<i>V. alginolyticus</i>	<i>D. labrax</i> , <i>S. aurata</i>	Tunisia	17	MH broth +1%NaCl	0.24-1				Lajnef, 2012
Doxycycline	<i>V. anguillarum</i>	<i>D. labrax</i>		11		0.125-0.5				Performfish
Doxycycline	<i>V. harveyi</i>	<i>D. labrax</i> , <i>S. aurata</i>		10		0.125-0.25				Performfish
Doxycycline	<i>P. damsela</i> <i>subsp. piscicida</i>	<i>D. labrax</i> , <i>S. aurata</i>		22		0.125-0.5				Performfish
Doxycycline	<i>T. maritimum</i>	<i>D. labrax</i> , <i>S. aurata</i>		7		0.25-0.5				Performfish
Flumequine	<i>P. damsela</i> <i>subsp. piscicida</i>	<i>Solea senegalensis</i>	2000/2005 Spain	23	CSMHB 26°C±2 (Alderman & Smith 2001) 23±2°C for 48h	0.25-8				Martínez-Manzanares, 2008

D3.3-Therapeutics for MMFF



Flumequine	<i>P. damsela</i> <i>subsp. piscicida</i>	<i>Seriola</i>	2002 Japan	74	Agar dilution method (NCCLS M31-A2, M7-A6) MH agar +2% NaCl 25°C for 48h	≤0.125-4	2	4	0.5	Kawanishi, 2006
Flumequine	<i>P. damsela</i> <i>subsp. piscicida</i>	<i>S. aurata</i>			MH broth +2%NaCl - MH broth+cation	0.3 (2%NaCl) or>38.25 (+cation)				Rigos, 2003a
Flumequine	<i>P. damsela</i> <i>subsp. piscicida</i>	<i>S. aurata</i>	1996/2000 Spagna	16	CSMHB 26°C±2 (Alderman & Smith 2001)	0.5-4				Martínez-Manzanares, 2008
Flumequine	<i>P. damsela</i> <i>subsp. piscicida</i>	<i>D. labrax</i> , greymullet	Italy	10	Nutrient Broth +3%NaCl	0.97-62.5				Laganà, 2011
Flumequine	<i>P. damsela</i> <i>subsp. piscicida</i>		USA, Spain	1	MH agar +1%NaCl	0.3				Ledo et al., 1987
Flumequine	<i>V. anguillarum</i> O1	<i>S. aurata</i> , <i>D. labrax</i>	2000-2001 Greece	1	MH broth +2%NaCl - MH broth+cation	0.15 (2%NaCl) or 4.78 (+cation)				Rigos, 2003a
Flumequine	<i>V. anguillarum</i> O1	<i>H. hippoglossus</i>		1	MH agar +2%NaCl Agar dilution method (Washington 1985) 48h at 20°C	0.06				Samuelsen, 1996
Flumequine	<i>V. anguillarum</i> O2	<i>H. hippoglossus</i>		1	MH agar +2%NaCl Agar dilution method (Washington 1985) 48h at 20°C	0.015				Samuelsen, 1996
Flumequine	<i>V. anguillarum</i>		USA, Spain	7	MH agar +1%NaCl	<0.075-0.3				Ledo et al., 1987

D3.3-Therapeutics for MMFF



Flumequine	<i>Vibrio</i> spp.	<i>Sparus aurata</i>	Italy	150	CAMHB+1%NaCl	0.06-128	0.5	4	<2->4	Scarano, 2014
Flumequine	<i>V. salmonicida</i>	<i>H. hippoglossus</i>		1	MH agar +2%NaCl Agar dilution method (Washington 1985) 48h at 20°C	0.03				Samuelsen, 1996
Flumequine	<i>V.splendidus</i>	<i>H. hippoglossus</i>		1	MH agar +2%NaCl Agar dilution method (Washington 1985) 48h at 20°C	1				Samuelsen, 1996
Flumequine	<i>V. alginolyticus</i>	<i>S. aurata, D. labrax</i>	2000-2001 Greece	1	MH broth +2%NaCl - MH broth+cation 22°C	1.2 (2%NaCl) or 38.25 (+cation)				Rigos, 2003a
Flumequine	<i>V. damsela</i>	<i>S. aurata, D. labrax</i>	2000-2001 Greece	1	MH broth +2%NaCl - MH broth+cation 22°C	0.019 (2%NaCl) or 0.15 (+cation)				Rigos, 2003a
Flumequine	<i>V. tubiashii</i>		USA, Spain	1	MH agar +1%NaCl	1				Ledo et al., 1987
Flumequine	<i>V. fluvialis</i>	<i>S. aurata, D. labrax</i>	2000-2001 Greece	1	MH broth +2%NaCl - MH broth+cation 22°C	0.15 (2%NaCl) or 4.78 (+cation)				Rigos, 2003a
Flumequine	<i>V. harveyi</i>	<i>D. labrax, S.aurata</i>	Italy 2011-2018	30	CAMHB CLSI M07-A9, VET 04.A2	<0.25-0.5	<0.25	0.5		Pretto, 2018
Flumequine	<i>Aeromonas</i> spp.	<i>S. aurata</i>	Italy	104	(CLSI,2011)	0.06-≥128	0.12	16	≤2-≥4	Scarano, 2018

D3.3-Therapeutics for MMFF



Flumequine	<i>A. hydrophila</i> , <i>A.sobria</i> , <i>A. caviae</i>		USA, Spain	5	MH agar +1%NaCl	<0.075-0.075				Ledo et al., 1987
Oxolinic acid	<i>P. damsela</i> <i>subsp. piscicida</i>	<i>S. aurata</i>	1996/2000 Spain	16	CSMHB 26°C±2 (Alderman & Smith 2001) 23±2°C for 48h	0.25-16				Martínez- Manzanares, 2008
Oxolinic acid	<i>P. damsela</i> <i>subsp. piscicida</i>	<i>Solea</i> <i>senegalensis</i>	2000/2005 Spain	23	CSMHB 26°C±2 (Alderman & Smith 2001) 23±2°C for 48h	0.25-16				Martínez- Manzanares, 2008
Oxolinic acid	<i>P. damsela</i> <i>subsp. piscicida</i>	<i>Seriola</i>	2002 Japan	74	Agar dilution method (NCCLS M31-A2, M7-A6) MH agar +2% NaCl 25°C for 48h	≤0.125-4	2	4	0.5	Kawanishi, 2006
Oxolinic acid	<i>P. damsela</i> <i>subsp. piscicida</i>	<i>D. labrax</i> , <i>greymullet</i>	Italy	10	Nutrient Broth +3%NaCl	3.9-62.5				Laganà, 2011
Oxolinic acid	<i>P. damsela</i> <i>subsp. piscicida</i>		USA, Spain	1	MH agar +1%NaCl	0.3				Ledo et al., 1987
Oxolinic acid	<i>P. damsela</i> <i>subsp. piscicida</i>	yellowtail	1984-1985	307	Japanese Society of Chemotherapy	0.015-12.5				Kusuda, 1988
Oxolinic acid	<i>V. anguillarum</i> O1	<i>H.</i> <i>hippoglossus</i>		1	MH agar +2%NaCl Agar dilution method (Washington 1985) 48h at 20°C	0.03				Samuelsen, 1996
Oxolinic acid	<i>V. anguillarum</i>		1983/1994 F-I-GR-DK- GP-C	30	MH agar +1.5%NaCl	0.023-0.75				Guérin-Faublée, 1996

D3.3-Therapeutics for MMFF



Oxolinic acid	<i>V. anguillarum</i> O2	<i>H. hippoglossus</i>		1	MH agar +2%NaCl Agar dilution method (Washington 1985) 48h at 20°C	0.015				Samuelsen, 1996
Oxolinic acid	<i>Vibrio</i> spp.	<i>Sparus aurata</i>	Italy	150	CAMHB+1%NaCl	0.06-32	0.25	4	<4->8	Scarano, 2014
Oxolinic acid	<i>V. anguillarum</i>		USA, Spain	7	MH agar +1%NaCl	<0.075-0.3				Ledoetal., 1987
Oxolinic acid	<i>V. salmonicida</i>	<i>H. hippoglossus</i>		1	MH agar +2%NaCl Agar dilution method (Washington 1985) 48h at 20°C	0.03				Samuelsen, 1996
Oxolinic acid	<i>V.splendidus</i>	<i>H. hippoglossus</i>		1	MH agar +2%NaCl Agar dilution method (Washington 1985) 48h at 20°C	0.5				Samuelsen, 1996
Oxolinic acid	<i>V. tubiashii</i>		USA, Spain	1	MH agar +1%NaCl	1				Ledo et al., 1987
Oxolinic acid	<i>A. hydrophila</i> , <i>A.sobria</i> , <i>A. caviae</i>		USA, Spain	5	MH agar +1%NaCl	0.075				Ledo et al., 1987
Oxolinic acid	<i>Aeromonas</i> sp	<i>S. aurata</i>	Italy	104	(CLSI,2011)	0.06-≥128	0.06	16	≤0.12-≥1	Scarano, 2018
Oxolinic acid	<i>Tenacibaculum</i> dic <i>entrarchi</i>	<i>Genypterus chilensis</i>	2015 Chile	4	CLSI 2014 Broth microdilution	4				Irgang, 2017
Enrofloxacin	<i>P. damsela</i> <i>subsp. piscicida</i>	<i>S. aurata</i>	1996/2000 Spain	16	CSMHB 26°C±2 (Alderman &	0.25-0.5				Martínez- Manzanares, 2008

D3.3-Therapeutics for MMFF



					Smith 2001) 23±2°C for 48h					
Enrofloxacin	<i>P. damsela</i> <i>subsp. piscicida</i>	<i>Solea</i> <i>senegalensis</i>	2000/2005 Spain	23	CSMHB 26°C±2 (Alderman & Smith 2001) 23±2°C for 48h	0.25-0.5				Martínez- Manzanares, 2008
Enrofloxacin	<i>P. damsela</i> <i>subsp. piscicida</i>	<i>Seriola</i>	2002 Japan	74	Agar dilution method (NCCLS M31-A2, M7-A6) MH agar +2% NaCl 25°C for 48h	≤0.125-0.25	≤0.125	0.25		Kawanishi, 2006
Enrofloxacin	<i>V. harveyi</i>	<i>D. labrax</i> , <i>S. aurata</i>	Italy 2011- 2018	30	CAMHB CLSI M07-A9, VET 04.A2	0.03125-0.25	0.125	0.25		Pretto, 2018
Enrofloxacin	<i>Tenacibaculum</i> <i>maritimum</i>	<i>Turbot</i> , <i>Sole</i> , <i>S. aurata</i> , <i>D.</i> <i>labrax</i>	Spain- Portugal 2003-2004	80	M31-A2	0.5-32				Avendaño- Herrera, 2008
Florfenicol	<i>P. damsela</i> <i>subsp. piscicida</i>	<i>Seriola</i>	2002 Japan	74	Agar dilution method (NCCLS M31-A2, M7-A6) MH agar +2% NaCl 25°C for 48h	0.25-0.5	0.5	0.5		Kawanishi, 2006
Florfenicol	<i>Vibrio</i> spp.	<i>Sparus</i> <i>aurata</i>	Italy	150	CAMHB+1%NaCl	0.06-32	0.5	4	<2->8	Scarano, 2014
Florfenicol	<i>V. harveyi</i>	<i>D. labrax</i> , <i>S. aurata</i>	Italy 2011- 2018	30	CAMHB CLSI M07-A9, VET 04.A2	<1-16	<1	<1		Pretto, 2018
Florfenicol	<i>Aeromonas</i> spp.	<i>S. aurata</i>	Italy	104	(CLSI,2011)	0.06-64	1	4	≤4-≥8	Scarano, 2018
Florfenicol	<i>Tenacibaculum</i> <i>dicentrarchi</i>	<i>Genypterus</i> <i>chilensis</i>	2015 Chile	4	CLSI 2014 Broth microdilution	2				Irgang, 2017

D3.3-Therapeutics for MMFF



Chloramphenicol	<i>P. damsela</i> <i>subsp. piscicida</i>	<i>Seriola</i>	2002 Japan	74	Agar dilution method (NCCLS M31-A2, M7-A6) MH agar +2% NaCl 25°C for 48h	0.5-64	32	64	4	Kawanishi, 2006
Chloramphenicol	<i>P. damsela</i> <i>subsp. piscicida</i>		USA, Spain	1	MH agar +1%NaCl	<1.56				Ledo et al., 1987
Chloramphenicol	<i>P. damsela</i> <i>subsp. piscicida</i>	yellowtail	1984-1985	307	Japanese Society of Chemotherapy	0.20-50				Kusuda, 1988
Chloramphenicol	<i>V. anguillarum</i>		1983/1994 F-I-GR-DK- GP-C	30	MH agar +1.5%NaCl	0.187-1.5				Guérin-Faubleé, 1996
Chloramphenicol	<i>V. anguillarum</i>		USA, Spain	7	MH agar +1%NaCl	<1.56				Ledo et al., 1987
Chloramphenicol	<i>Vibrio</i> spp.	<i>Sparus aurata</i>	Italy	150	CAMHB+1%NaCl	0.06->128	16	>128	<8->32	Scarano, 2014
Chloramphenicol	<i>V. alginolyticus</i>	<i>D. labrax</i> , <i>S.aurata</i>	Tunisia	17	MH broth +1%NaCl	0.48-0.96				Lajnef, 2012
Chloramphenicol	<i>V. tubiashii</i>		USA, Spain	1	MH agar +1%NaCl	<1.56				Ledoetal., 1987
Chloramphenicol	<i>Aeromonas</i> sp	<i>S. aurata</i>	Italy	104	(CLSI,2011)	0.25-64	0.5	4	≤8-≥32	Scarano, 2018
Chloramphenicol	<i>A. hydrophila</i> , <i>A.sobria</i> , <i>A. caviae</i>		USA, Spain	5	MH agar +1%NaCl	<1.56-3.12				Ledoetal., 1987
Thiamphenicol	<i>P. damsela</i> <i>subsp. piscicida</i>	yellowtail	1984-1985	307	Japanese Society of Chemotherapy	0.05->100				Kusuda, 1988
Trimethoprim	<i>P. damsela</i> <i>subsp. piscicida</i>	<i>Seriola</i>	2002 Japan	74	Agar dilution method (NCCLS M31-A2, M7-A6) MH agar +2%	≤0.125-512	1	256	8	Kawanishi, 2006

D3.3-Therapeutics for MMFF



					NaCl 25°C for 48h					
Trimethoprim	<i>Vibrio</i> spp.	<i>Sparus aurata</i>	Italy	150	CAMHB+1%NaCl	0.06-128	4	64	<8->16	Scarano, 2014
Trimethoprim	<i>V. alginolyticus</i>	<i>D. labrax</i>	Turkey	15	MH broth +1%NaCl+cation CLSI M49-A	≤8				Korun, 2008
Trimethoprim	<i>V. anguillarum</i>		1983/1994 F-I-GR-DK-GP-C	30	MH agar +1.5%NaCl	0.093-3	0,37	0,75		Guérin-Faublée, 1996
Trimethoprim	<i>V. alginolyticus</i>	<i>D. labrax, S.aurata</i>	Tunisia	17	MH broth +1%NaCl	0.48-16				Lajnef, 2012
Trimethoprim	<i>Aeromonas</i> spp.	<i>S. aurata</i>	Italy	104	(CLSI,2011)	0.12-≥128	32	64	≤8-≥16	Scarano, 2018
Sulfamonomethoxine	<i>P. damsela</i> subsp. <i>piscicida</i>	<i>Seriola</i>	2002 Japan	74	Agar dilution method (NCCLS M31-A2, M7-A6) MH agar +2% NaCl 25°C for 48h	16->512	>512	>512	32	Kawanishi, 2006
Sulfadimethoxine	<i>V. anguillarum</i>		1983/1994 F-I-GR-DK-GP-C	30	MH agar +1.5%NaCl	0.75-48	6	48		Guérin-Faublée, 1996
Sulfadiazine	<i>Vibrio</i> spp.	<i>S. aurata</i>	Italy	150	CAMHB+1%NaCl	0.12->512	>512	>512	<256->512	Scarano, 2014
Sulfadiazine	<i>Aeromonas</i> sp	<i>S. aurata</i>	Italy	104	(CLSI,2011)	0.12-≥256	≥256	≥256	≤38-≥76	Scarano, 2018
Trimethoprim: Sulfadimethoxine	<i>V. anguillarum</i>		1983/1994 F-I-GR-DK-GP-C	30	MH agar +1.5%NaCl	0.012-0.093	0,023	0,093		Guérin-Faublée, 1996
Trimethoprim: Sulfadimethoxine	<i>V. harveyi</i>	<i>D. labrax, S.aurata</i>	Italy 2011-2018	30	CAMHB CLSI M07-A9, VET 04.A2	<0.0625/1.187 5-1/19	<0.062 5/1.18 75	0.125/2. 375		Pretto, 2018
Amoxicillin	<i>Aeromonas</i> sp	<i>S. aurata</i>	Italy	104	(CLSI,2011)	0.06-≥128	16	≥128	≤8-≥32	Scarano, 2018

D3.3-Therapeutics for MMFF



Amoxicillin	<i>Vibrio</i> spp.	<i>S. aurata</i>	Italy	150	CAMHB+1%NaCl	0.06->128	>128	>128	<8->32	Scarano, 2014
Amoxicillin	<i>P. damsela</i> <i>subsp. piscicida</i>	<i>D. labrax</i> , <i>greymullet</i>	Italy	10	Nutrient Broth +3%NaCl	7.8-125				Laganà, 2011
Ampicillin	<i>P. damsela</i> <i>subsp. piscicida</i>	<i>Seriola</i>	2002 Japan	74	Agar dilution method (NCCLS M31-A2, M7-A6) MH agar +2% NaCl 25°C for 48h	≤0.125-16	≤0.125	≤0.125	0.25	Kawanishi, 2006
Ampicillin	<i>P. damsela</i> <i>subsp. piscicida</i>		USA, Spain	1	MH agar +1%NaCl	60				Ledo e tal., 1987
Ampicillin	<i>P. damsela</i> <i>subsp. piscicida</i>	yellowtail	1984-1985	307	Japanese Society of Chemotherapy	0.025->100				Kusuda, 1988
Ampicillin	<i>V. anguillarum</i>		USA, Spain	7	MH agar +1%NaCl	60->60				Ledo et al., 1987
Ampicillin	<i>Vibrio</i> spp.	<i>S. aurata</i>	Italy	150	CAMHB+1%NaCl	0.06->128	>128	>128	<8->32	Scarano, 2014
Ampicillin	<i>V. tubiashii</i>		USA, Spain	1	MH agar +1%NaCl	<3.75				Ledo et al., 1987
Ampicillin	<i>A. hydrophila</i> , <i>A. sobria</i> , <i>A. caviae</i>		USA, Spain	5	MH agar +1%NaCl	>60				Ledo et al., 1987
Ampicillin	<i>Aeromonas</i> sp	<i>S. aurata</i>	Italy	104	(CLSI,2011)	0.06-≥128	16	≥128	≤8-≥32	Scarano, 2018
Ampicillin	<i>V. alginolyticus</i>	<i>D. labrax</i> , <i>S. aurata</i>	Tunisia	17	MH broth +1%NaCl	128-256				Lajnef, 2012
Ampicillin	<i>V. harveyi</i>	<i>D. labrax</i> , <i>S. aurata</i>	Italy 2011- 2018	30	CAMHB CLSI M07-A9, VET O4.A2	32->32	>32	>32		Pretto, 2018
Streptomycin	<i>Vibriosp.</i>	<i>Sparus aurata</i>	Italy	150	CAMHB+1%NaCl	0.25->128	8	32	<6->25	Scarano, 2014
Streptomycin	<i>Aeromonas</i> spp.	<i>S. aurata</i>	Italy	104	(CLSI,2011)	1-≥128	16	64	≤6-≥25	Scarano, 2018
Erythromycin	<i>V. alginolyticus</i>	<i>D. labrax</i> , <i>S. aurata</i>	Tunisia	17	MH broth +1%NaCl	16-64				Lajnef, 2012

D3.3-Therapeutics for MMFF



Erythromycin	<i>Aeromonas</i> spp.	<i>S. aurata</i>	Italy	104	(CLSI,2011)	0.06-≥128	16	64	≤0.5-≥8	Scarano, 2018
Erythromycin	<i>Vibrio</i> spp.	<i>S. aurata</i>	Italy	150	CAMHB+1%NaCl	0.06->128	8	128	<0.5->8	Scarano, 2014
Gentamicin	<i>Vibrio</i> spp.	<i>S. aurata</i>	Italy	150	CAMHB+1%NaCl	0.06->128	2	4	<4->16	Scarano, 2014
Gentamicin	<i>V. harveyi</i>	<i>D. labrax</i> , <i>S.aurata</i>	Italy 2011-2018	30	CAMHB CLSI M07-A9, VET 04.A2	0.5-4	1	2		Pretto, 2018
Gentamicin	<i>Aeromonas</i> spp.	<i>S. aurata</i>	Italy	104	(CLSI,2011)	0.12-32	2	8	≤4-≥16	Scarano, 2018
Gentamicin	<i>P. damsela</i> <i>subsp. piscicida</i>	<i>Seriola</i>	2002 Japan	74	Agar dilution method (NCCLS M31-A2, M7-A6) MH agar +2% NaCl 25°C for 48h	0.5-4	1	2		Kawanishi, 2006
Kanamycin	<i>Vibrio</i> spp.	<i>S. aurata</i>	Italy	150	CAMHB+1%NaCl	0.25->128	8	16	<16->64	Scarano, 2014
Kanamycin	<i>Aeromonas</i> spp.	<i>S. aurata</i>	Italy	104	(CLSI,2011)	0.5-≥128	8	32	≤16-≥64	Scarano, 2018
Kanamycin	<i>V. alginolyticus</i>	<i>D. labrax</i> , <i>S.aurata</i>	Tunisia	17	MH broth +1%NaCl	32-64				Lajnef, 2012
Kanamycin	<i>P. damsela</i> <i>subsp. piscicida</i>	<i>Seriola</i>	2002 Japan	74	Agar dilution method (NCCLS M31-A2, M7-A6) MH agar +2% NaCl 25°C for 48h	4->512	>512	>512	64	Kawanishi, 2006
Cefotaxime	<i>V. alginolyticus</i>	<i>D. labrax</i> , <i>S.aurata</i>	Tunisia	17	MH broth +1%NaCl	0.48-0.96				Lajnef, 2012
Cephalotin	<i>Aeromonas</i> spp.	<i>S. aurata</i>	Italy	104	(CLSI,2011)	0.06-≥128	>128	>128	≤8-≥32	Scarano, 2018
Cephalotin	<i>Vibrio</i> spp.	<i>S. aurata</i>	Italy	150	CAMHB+1%NaCl	0.06->128	16	>128	<8->32	Scarano, 2014
Nalidixicacid	<i>V. alginolyticus</i>	<i>D. labrax</i> , <i>S.aurata</i>	Tunisia	17	MH broth +1%NaCl	0.48-0.96				Lajnef, 2012

D3.3-Therapeutics for MMFF



Nalidixicacid	<i>P. damsela</i> <i>subsp. piscicida</i>	yellowtail	1984-1985	307	Japanese Society of Chemotherapy	0.20-100				Kusuda, 1988
Bicozamycin	<i>P. damsela</i> <i>subsp. piscicida</i>	<i>Seriola</i>	2002 Japan	74	Agar dilution method (NCCLS M31-A2, M7-A6) MH agar +2% NaCl 25°C for 48h	2-4	2	2		Kawanishi, 2006
Dihydrostreptomycin	<i>P. damsela</i> <i>subsp. piscicida</i>	<i>Seriola</i>	2002 Japan	74	Agar dilution method (NCCLS M31-A2, M7-A6) MH agar +2% NaCl 25°C for 48h	2-128	4	32		Kawanishi, 2006
Fosfomycin	<i>P. damsela</i> <i>subsp. piscicida</i>	<i>Seriola</i>	2002 Japan	74	Agar dilution method (NCCLS M31-A2, M7-A6) MH agar +2% NaCl 25°C for 48h	0.5-8	1	2		Kawanishi, 2006
Sarafloxacin	<i>V. anguillarum</i>		1983/1994 F-I-GR-DK-GP-C	30	MH agar +1.5%NaCl	0.006-1.5				Guérin-Faubleé, 1996
Nitrofurantoin	<i>V. tubiashii</i>		USA, Spain	1	MH agar +1%NaCl	9.35				Ledo et al., 1987
Nitrofurantoin	<i>A. hydrophila</i> , <i>A. sobria</i> , <i>A. caviae</i>		USA, Spain	5	MH agar +1%NaCl	>60				Ledo et al., 1987
Nitrofurantoin	<i>V. anguillarum</i>		USA, Spain	7	MH agar +1%NaCl	9.35-18.75				Ledo et al., 1987

D3.3-Therapeutics for MMFF



Nitrofurazone	<i>V. tubiashii</i>		USA, Spain	1	MH agar +1%NaCl	9.35				Ledo et al., 1987
Nitrofurazone	<i>A. hydrophila</i> , <i>A. sobria</i> , <i>A. caviae</i>		USA, Spain	5	MH agar +1%NaCl	18.75-37.5				Ledo et al., 1987
Nitrofurazone	<i>V. anguillarum</i>		USA, Spain	7	MH agar +1%NaCl	9.35-18.75				Ledo et al., 1987
Colistin	<i>V. harveyi</i>	<i>D. labrax</i> , <i>S. aurata</i>	Italy 2011-2018	30	CAMHB CLSI M07-A9, VET 04.A2	0.25->8	>8	>8		Pretto, 2018
Apramycin	<i>V. harveyi</i>	<i>D. labrax</i> , <i>S. aurata</i>	Italy 2011-2018	30	CAMHB CLSI M07-A9, VET 04.A2	4-8	4	8		Pretto, 2018
Aminosidin	<i>V. harveyi</i>	<i>D. labrax</i> , <i>S. aurata</i>	Italy 2011-2018	30	CAMHB CLSI M07-A9, VET 04.A2	2-8	4	4		Pretto, 2018
Lincomycin	<i>V. anguillarum</i>	<i>D. labrax</i>		11		8-32				Performfish
Lincomycin	<i>V. harveyi</i>	<i>D. labrax</i> , <i>S. aurata</i>		10		32-128				Performfish
Lincomycin	<i>P. damsela</i> <i>subsp. piscicida</i>	<i>D. labrax</i> , <i>S. aurata</i>		22		4-8				Performfish
Lincomycin	<i>T. maritimum</i>	<i>D. labrax</i> , <i>S. aurata</i>		7		0.12-0.5				Performfish
Spectinomycin	<i>V. anguillarum</i>	<i>D. labrax</i>		11		8-16				Performfish
Spectinomycin	<i>V. harveyi</i>	<i>D. labrax</i> , <i>S. aurata</i>		10		14-64				Performfish
Spectinomycin	<i>P. damsela</i> <i>subsp. piscicida</i>	<i>D. labrax</i> , <i>S. aurata</i>		22		8-32				Performfish
Spectinomycin	<i>T. maritimum</i>	<i>D. labrax</i> , <i>S. aurata</i>		7		16-32				Performfish

3.3.8.2. *Optimisation of antibacterial treatments and application of PK/PD to the rational design of treatment regimen in fish*

Aquaculture activities are attempts to make profit by rearing aquatic products. Within this context, 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 optimise the reduction in loss due to infectious disease but also, and importantly, to do so in a cost-effective manner respecting environmental and social responsibility. There are also other issues which should be considered to the decision of the therapy such as the severity of the disease as well as the condition of the sick fish to withstand the treatment.

In contrast to human medicine and clinical veterinary medicine (eg. dogs, cats, horses), most treatments in aquaculture are '*sensu stricto*' metaphylactic in that they are administered to particularly large populations, that include some infected and some uninfected individuals. The concept of metaphylaxis is not always well understood as in some cases is mistakenly defined as mass treatments of a group of animals in advance of an expected outbreak of disease. Metaphylactic treatments should be always understood as a control treatment and should be clearly differentiated from therapeutic treatments (curative) and from prophylactic treatments (preventive). Metaphylactic treatment (control) is actually delivered to a group of animals that is developing a disease, while prophylactic treatment (preventive) is delivered to a group of animals that are still healthy but with a real risk to develop a disease. Lastly, 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 again to stress the fact that the main target in the metaphylactic treatments in aquaculture are not the affected or sick fish in a specific stock. The main objective of the treatments are those fish at very high risk when an infectious or parasitic disease outbreak has already triggered in the same stock, tank, cage, facility or farm. This is particularly relevant if we take into consideration that the main treatment delivery system used in aquaculture is oral treatment with medicated feeds and consequently, totally dependent on zootechnical concepts and variables such as temperature feeding ratios, distribution, appetite, feed acceptability, palatability or efficient medicated pellet ingestion. Treatment efficiency is in most of cases directly associated to the correct management of these variables in addition to the suitable medicine, dose and medicated feed production. Under these conditions, the control over the amount actually delivered to individuals is much more difficult to achieve. In contrast, in human and veterinary medicine where drugs are administered orally or by injection into large individuals there is a significant degree of control over the amount each individual receives.

Any attempt to place the dose regime used in oral antimicrobial therapy on sound, empirical and rational grounds requires the 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 that plasma concentrations must reach the MIC values during a large period of time or exceed the MIC values by a certain number. It has been previously claimed that it is the nonprotein-bound drug concentration at the site of bacterial infection, which determines the success of therapy (Drusano, 2004; Shojaee AliAbadi, Lees, 2000). Thus, although the amount of bound drug is occasionally considerable, it could be stressed that the free amount would appear to be the most relevant. This approach is totally valid for a therapeutic treatment scenario but presents some concerns in a metaphylactic treatment. As it is mentioned before, the target of the treatment in metaphylactic treatments are not the sick fish but the fish in the affected stock that most probably will be infected in the next hours and days. This means that in this situation there is still no infection so the paradigm and scenario for the efficiency (site of action) of the antimicrobial substances can be different as for the classic approach for therapeutic treatments. In metaphylactic treatments, plasmatic levels of the antimicrobial substances may also play a relevant role, but other protection mechanism should also be taken into account.

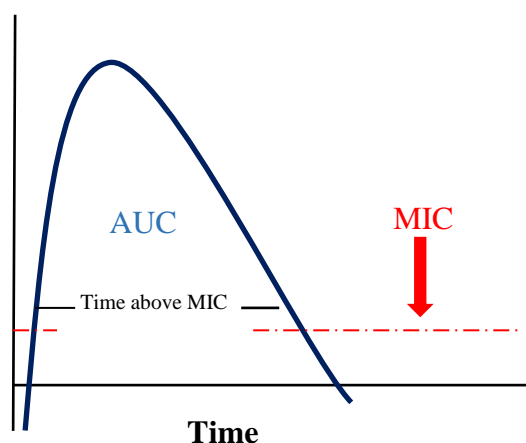


Figure 6: Pharmacokinetic/Pharmacodynamic parameters affecting antibiotic potency

3.3.8.3. MIC and PK/PD approaches

The efficacy of antibacterial treatments was in past evaluated solely by the integration of MIC values with maximum drug concentrations (C_{max} :MIC) in plasma (Figure 6)(Blaser et al., 1987; Stamm, 1989). The idea was that the *in vitro* MIC data can be used for *in vivo* application to predict the treatment efficacy of antibacterials (Bruun, et al. 2003). MICs however 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 (Branson, 2001; Smith et al., 1994). These are based on theoretical assessment of a drug's efficacy against a bacterial pathogen based on the requirement that the 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., 2004b; Coyne et al., 2004a) and have proposed otherwise in an attempt to particularise drug treatments generally in medicine (Craig, 2007; Shojaee AliAbadi & Lees, 2000). Actually, in the last years there have been very significant and empirically validated, advances in the application of PK/PD data to the setting of optimal dose regimen in human and veterinary medicine (Schmidt et al., 2008; Toutain et al., 2007).

Shojaee-AliAbadi and Lees (2000) initially proposed that an optimum dosage schedule should achieve drug concentrations at sites of infection in excess of MIC in the case of bacteriostatic and some bactericidal drugs (time-dependent effect) and drugs with action depending on high AUC or C_{max} :MIC ratios (concentration-dependent effect). Smith (2008) has however stressed that PK/PD approaches which have been developed for therapeutic administrations to individual large animals and particularly to humans have difficulties to be applied in aquaculture. He has suggested 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 are satisfactorily addressed it would not be possible to apply the PK/PD approaches that have been used to such advantage in human medicine, to the optimization of dose regime in aquaculture. It is possible, however, to examine the published literature on PK and PD of antibacterials in aquaculture in the light of the data requirements of the PK/PD approaches currently being developed for human therapies. A central component of these approaches has been the identification of which combination of PK and PD data (the PK/PD index) shows the best correlation with bacteriological and clinical outcomes.

In this regard, three PK/PD indices have been found to be of greatest value (Figure 6) (Craig, 2007). 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 is the ratio of the peak serum concentration (C_{max}) to the MIC (C_{max} /MIC). The third is the ratio of the area under the serum concentration curve at 24h (AUC_{0-24}) to the MIC (AUC_{0-24} /MIC). When 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

D3.3-Therapeutics for MMFF

guide to 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 is normally found to be the most appropriate. However, for those agents that show significant post antibiotic effects (PAE)(Craig & Gudmundsson, 1996) or sub-inhibitory effects (Odenholt, 2001), AUC_{0-24}/MIC may be more useful.

3.3.8.4. Potency

As it mentioned before, this is the PK/PD current approach from a theoretical basis, that should be taken into account in the design of the therapeutic strategy, but going back to Smith's concerns if these PK/PD approaches that are valid for individual therapeutic treatments in humans and large animals can be also valid for the aquaculture scenarios and consequently, for gilthead seabream and European seabass antimicrobial treatment strategies. As we said before, aquaculture treatments are mainly based on the metaphylaxis concept, this means, the protection of the fish at real risk (this is clearly different as the concept of preventive treatments) and not the sick fish. The reason for that is crystal clear: appetite is significantly decreased or abolished in sick fish or in other even more simple words: sick fish do not eat. Then, in this case the main paradigma of the MIC and antibiotic plasma levels is not longer valid in a metaphylactic scenario or at least is not valid for the sick fish in the stock. The real target for the treatment will be those fish, still healthy, still eating but in imminent jeopardy and at risk to die 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 using an appropriate therapeutic strategy. These fish are typically those still healthy, asymptomatic carriers or those during early incubation phases. In all these cases the bacteria (we are talking about antibiotics) are not still in septicaemic phase or significantly growing in the susceptible tissues or organs, so the main 'battlefield' is still not blood or plasma. In these cases, the main scenarios could be the main entrance portals, and amongst these portals, skin, gills and digestive system are widely recognised as the main ones in fish. In diseases where skin and gills play a relevant role as infectious route, such as infections by *T. maritimum* or some *Vibrio* species, antibiotic at the sufficient levels should reach these structures as fast as possible. Gills, due to their main role in respiration and osmoregulation are highly vascularised organs, so plasma values can be considered as a good indicator to protect against bacterial diseases affecting gills. Skin is also vascularised but present some anatomical and structural differences involving a differential distribution of the antibiotics compared with other organs such as liver or muscle. This has been widely demonstrated in European seabass and gilthead seabream in residue level studies. However, digestive system is, maybe not always, so well considered as potential portal of entry for diseases from the point of view of treatment, but is widely accepted as the basis of preventive methods using immunostimulation or probiotic strategies associated to the diets. The model of colonization of the intestine in the pathogenesis of *V. cholerae* infection in humans is well known, and also is well known the preference of many *Vibrio* species for the gut as a favourable habitat. The role of the intestine in other infectious diseases of European sea bass and gilthead sea bream caused by *Pseudomonas anguilliseptica*, *P.*

damselae subsp. *piscicida* and later *T. maritimum*, has not been so well described so far, but should not be disregarded. In this particular scenario, it should also be taken into account the local effect in the digestive system and in the intestinal microbiota of the antibiotic delivered as fish feeds as an indirect way to control bacterial pathogens in carrier fish or in early prodromic stages before the infection is spreading to the organism. In any case, the potential generation of antibiotic resistance in this specific site during antibiotic oral delivery should not be disregarded.

3.4. The delivery method

3.4.1. Use of medicated feeds in fish farms: best practices

Antibacterials in aquaculture can be delivered using three main routes of administration: in-water, in-feed, injection, topical application and gavage. The last three methods are delivery methods with a clear clinical approach, widely used in ornamental fish for individual fish cases, and only used in very specific and occasional cases in fish aquaculture such as broodstock management or research. Antibiotic in-water administration is used in ornamental fish treatments or in research but has a very limited use in aquaculture for different reasons (eg.hatcheries). Antibiotic bath treatments were described in the past as potential antibiotic delivery method for aquaculture (O'Grady et al., 1988), but nowadays this route of administration is no longer recommended. As large numbers of fish should be treated at the same time in aquaculture conditions, bath treatments require also large water volumes and consequently, large quantity of antibiotics. Antibiotic solubility, stability and availability for absorption in different water quality conditions (salinity, pH, temperature, hardness) can also be highly variable amongst the different antibiotics and unfeasible. Moreover, after the treatment, the residual water with the antibiotic used for the bath becomes a huge problem for waste management, as this water volume should not be released in the same way as normal farm sewage water due to environmental consequences. Antibiotic absorption via water bath decreases as fish increases size and weight so for this reason only larvae and small stocks of juveniles are occasionally treated using this system. This method is not commonly used in Mediterranean aquaculture it will not be described in this section.

Administration of antibacterials via the feed is clearly the antibiotic delivery system most frequently used in Mediterranean aquaculture. The methodology of oral treatments with antibiotic medicated feed is very similar to the methodology used in other technically-advanced finfish productions, such as salmon farming. The general principle of medicated feed is the use of a commercial food as a basis, mixing this food with the antibacterial (usually a commercial antibiotic premix). Medicated fish feed producers usually have their own special standard feed formulations for medicated feeds and for each species (in our case, European seabass and gilthead seabream) as this food formulation should also fulfil the nutritional requirements for the fish species and also have special characteristics to allow an appropriate physical and chemical properties for an optimal combination with the antibiotic premix.

3.4.2. 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 body weight (BW) fish daily (mg/kg BW/d). This dose is specific to the antimicrobial agent and is provided by the manufacturer of the premix. This recommended dose is based on the different studies performed by the manufacturer in order to get the license authorisation by the European (EMA) or by national authorities according to specific guidelines and procedures. However, these studies are frequently performed under certain experimental conditions (temperature, biomass per tank, 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), are simply referred as 'for fish' or even in worse cases, when cascade prescription is required, from terrestrial vertebrates. Thus, we suggest to use the 'recommended' dose only as a general reference and consider the possibility to modify the dosage according the different scenarios and the available knowledge.

Secondly, the calculation of the desired dosage of the premix in the feed, is performed in general, in grams of premix per tonne of feed. Four important factors are involved in this second calculation:

a) Concentration of active ingredient of the antimicrobial in the premix. In general, commercial compounds are not usually 100% pure premixes, so this has to be taking into account when calculating the dosage in the feed. Excipients used in the premixes (quantitative and qualitatively) are also relevant. Some excipients used in certain premixes cannot be specifically designed to bind to fish pellets efficiently. Lower concentrations of active antimicrobial substances may require the use of large amount of premix in the medicated feed under certain conditions and this can lead to severe technological manufacturing problems.

b) Daily feed intake is considered according to fish species, size and temperature. It is really important to have real data about how the fish are eating as it was stated before. For example, trout fingerlings can eat up to 2% of specific feeding ratio (SFR) in standard conditions; in comparison, the SFR of a 1 kg gilthead sea bream in winter time is really low, around 0.2 %. If the appetite is really compromised because of the diseases and/or the temperature is low, a higher dosage would be advisable.

c) Biomass to treat. The number and average weight of the fish have to be monitored on a regular basis, because the daily antimicrobial dose depends on the quantity of fish (kg) being treated. 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. Precision on this biomass assessments are consequently very relevant for the calculations. In addition, disease-affected stocks frequently present deviations from the norm due to the presence of mortalities or growth slowdown. If mortalities and growth reduction are not properly evaluated, then mistakes on the biomass assessment can lead to inefficient treatments or wastes and environmental impact due to uneaten medicated feed.

Example: 10000 European sea bass of average weight 200 g.

Standard theoretical treatment

Treatment against Vibriosis with Flumesyva (Flumequine 10 percent, Syva, S.A.) at 15 °C with a SFR of 0.5 percent.

- Fish biomass to treat: 10 000 fish x 200 g = 2 000 kg
- Flumequine dose: 12 mg/kg BW/d
- Flumequine needed/day: 12 x 2 000 = 24 000 mg = 24 g
- Flumesyva needed/day: 24 x (100/10) = 240 g
- Daily feed intake = 0.5% means 0.5 kg feed/100 kg BW
- Total daily feed: 2 000 x (0.5/100) = 10 kg
- Premix final dose: 240 g/10 kg = 24 g/kg = 24 kg/ton

Alternative realistic scenario

Theoretically 10000 European sea bass of average weight 200 g but after 4 days with 1% daily mortality and lower real average weight than expected (180 g) (Real biomass: 10.000 fish – 400 dead fish = 9.600 fish.

Affected stock with real feed intake of 0.4%

Treatment against Vibriosis with Flumesyva (Flumequine 10 percent, Syva, S.A.) at 15 °C with a SFR of 0.5 percent.

- Real Fish biomass to treat: 9 600 fish x 180 g = 1728 kg
- Flumequine dose: 12 mg/kg BW/d
- Flumequine needed/day: 12 x 1728 = 20736 mg = 20.7 g
- Flumesyva needed/day: 20,7 x (100/10) = 207 g
- Daily feed intake = 0.4% means 0.4 kg feed/100 kg BW
- Total daily feed: 1728 x (0.4/100) = 6,9 kg
- Premix final dose: 207 g/6,9 kg = 30 g/kg = 30 kg/tonne

In this case, required final dose should be 30 Kg/tonne and the theoretical calculation was 24 Kg/tonne. This means that fish stock will receive only 86% of the required dose and 3.1 Kg of medicated feed will be lost.

Also notice that ratios of premix/Kg feed (24-30 g/Kg feed) are very high and probably would cause problems in medicated feed manufacturing (see next section).

Depending on the daily feed intake (SFR), we will mix the quantity of premix in a greater or lesser quantity of feed. Feed suppliers should be able to prepare medicated feeds with different doses of the same premix to give the exact quantity of antimicrobial regardless of SFR variations.

3.4.3. Manufacturing of medicated feeds

Medicated fish feeds present a higher level of complexity compare 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 should keep and protect the pharmacological substances in the aqueous interface between its delivery and the ingestion by the fish adds another handicap in the fish medicated feed manufacturing.

- a) *Pelleted or extruded*: In the past, the medicated feed was made in the same line of manufacturing as the normal feed by adding the premix at the beginning of the process with 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 dueto the great loss resulting from the high temperatures and the pressure used during the manufacturing process. This is the reason why today medicated feeds are still made using the following two methods (b, c).
- b) *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.
- c) *Vacuum-coating*: The medicine premix is mixed with the oil, and the mixture is coated onto the pellet, with the help of vacuum process.

In both cases b) and c), a surface layer of medicine is created onto the feed. The medicated feed is produced in a specific and dedicated line of the feed factory. It has been demonstrated that oil-coated medicated fish diets suffered leaching to a greater degree compared with pelleted or extruded pellets and may also create higher palatability problems to finfish (Rigos et al 1999; Xu and Rogers, 1994). Both these factors may result in a reduction of total drug amount available within the treated population. Quality of the binding properties and stability of the coating is therefore very relevant to reduce the losses due to leaching and this is also a relevant issue to be taken into account in the selection of the medicated feed supplier. Premixes should be close to 100% pure (or at least with high concentration of active ingredients) and made of fine-size particles in order to use low doses and to avoid problems related to homogeneity and palatability. However, in general they are not 100% pure and coarse (rough particles), a situation that is very typical when we have to use by exceptional prescription a non-fish premix. Then, we can face serious problems of homogeneity and palatability. Selection of specific premix formulations for fish (when available) using specific forms of the different active substances and excipients (in terms of faster and more efficient availability and absorption) are always highly advisable.

Sometimes good homogeneity of the finished medicated feeds can be complicated to achieve, depending on the quality and dosage used of the premix. A coefficient of variation not above 10% is suggested as quality criterion. Training of the staff responsible for the manufacturing process and continuous monitoring of the antimicrobial level in the finished feed are essential to have a good homogeneity.

Palatability of the medicated feed can be also an issue, causing dramatic reduction of the feed intake because of the bad taste of the premix. Moreover, if fish do not eat the medicated feed quickly or reject it, the loss of antimicrobial agent into the water via leaching might be important. 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 an 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 during the manufacturing process. Careful medicated fish feed delivery is highly recommended.

3.5. The therapeutic regime / therapeutic strategy

3.5.1. Medicated feed administration

Fish can be fasted for 12 to 24 h to increase their appetite and without inducing any welfare issues; this could be coordinated with the time of arrival of the medicated feed to the farm. In any case, the fasting period must be authorized by the farm veterinarian, taking into account fish size, environmental conditions (e.g. water temperature, oxygen levels), appetite and palatability of medicated feed and other welfare recommendations for the species. 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. In distributing the feed by hand, the feeders are forced to take care of procedures and to monitor closely that sick fish are actually eating the medicated feed. This principle is even more relevant in 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 in the farms are also highly recommended. A careful supervision of the medicated fish distribution is the best choice to assure a 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 from the feeding behaviour after each treatment (products, dose, conditions, feed rejection, feed delivered vs feed non delivered to the stock) are very relevant information that should be registered and considered for the next and future treatments.

3.5.2. Therapeutic procedures: medicated feed delivery guidelines

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

Concentration of all the daily recommended dose of the medicament in a single meal intake or distribution of all daily recommended dose in all the feed given during all the day? This strategy is advisable in certain conditions: high temperatures and high feed intake when specific PKs are implemented.

3.5.3. Pharmacokinetics at different temperatures

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

3.5.4. Duration of treatment

The duration of treatment is recommended by the premix manufacturer and also can be prescribed by the veterinarian according the current knowledge and practical experience with the antibiotic and the diseases. Typically antibiotic treatments will be around 5 to 12 days, but duration of the antibiotic treatments is still under debate in human and also animal medicine. Sometimes, in chronic disorders, such as furunculosis in turbot or bacterial kidney disease in rainbow trout, it can be longer. From field experience, if after 15 days of treatment, there is no improvement of the situation, it is time to stop the treatment and analyse the fish again; maybe there was a wrong diagnosis at the beginning of the process. Some new studies in human medicine based on clinical results demonstrate that in certain diseases, shorter courses of therapy are as efficient as longer 7-14 days (Spellberg, 2016). Long therapeutic strategies are also classically associated to lower risk of development of microbial resistances reducing the number of viable bacteria that can develop resistances but recent studies seems to indicate that expanded treatments in the time increases the induction of resistances as other relevant and diverse microbial populations (digestive tract) can be in contact with the antibiotic and generate these resistances. As antibiotics are mainly delivered by oral route in fish, this possibility should not be underestimated. In any case, trends on the remission of the signs of the disease and reduction of the mortality are relevant information to recommend a shorter or longer treatment. For example, Vibriosis tend to present faster recovery after treatment than Pasteurelosis, so Vibriosis could be good candidates to shorter medication periods

3.5.5. Main mistakes in the use of medicated feeds

- Inadequate medicine selection. Due to empiric treatments without proper diagnosis and without taking into account the historical data
- Low premix dose selection. Due to (1) failure to accurately estimate the biomass to be treated; (2) lower than expected feed intake; and (3) failure to consider the concentration of antimicrobial agent in the premix
- Wrong duration of treatment. When therapy initiation is early, mortality is sometimes reduced dramatically after five to six days of treatment, and then

D3.3-Therapeutics for MMFF

frequently the farmer stops the treatment. That is the easiest way to have relapses. Completion of the prescribed course of treatment is strongly recommended

- Use of antimicrobials as prophylactics. It is not justified to prevent the outbreaks. This practice is not recommended because outbreaks are generally not prevented, but perhaps delayed. Such practise is blamed to create bacterial resistance in all animal farming industries
- Application of antimicrobials in viral infections (e.g. VNN) is considered of course misuse. It can be justified only if there are combined secondary bacterial infections and after accurate diagnostic and clinical evaluation of the outbreak
- Repeated use of the same antimicrobial agents. Generally, there is availability of a few licensed antimicrobial agents, and this sometimes leads to the use of the same ones continuously. In order to avoid resistance, it should be advisable to closely monitor the sensitivity of the isolates we obtained. Resistance does not develop from one day to the next; if there is evidence of a slight reduction of sensitivity, it would be time to rotate antibiotics.

3.5.6. Suitability of the treatments and treatment efficacy evaluation

Other relevant issues but frequently underated on the whole process of the medication practices in Mediterranean aquaculture are the evaluation of the suitability of the treatments and the evaluation of the efficacy of the treatments applied. In the particular case of the antibacterial treatments, both concepts (suitability and efficacy) should also be revised as they are also very relevant for the improvement of the therapeutic adjustment and the global sustainability of treatments.

The nature of the disease is also a factor which could be underestimated. For example, infections with slow progression or asymptomatic infections which 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. damsela* subsp. *piscicida*. It is known that this pathogen can be persistent in asymptomatic fish. This fact can be related to the particular ability of this bacterium to survive and multiply within macrophages (Elkamel et al., 2003). As chronic forms in Pasteurelosis are very common and recurrence of the infection are frequently described, particular long-term therapeutic strategies and adequate antibiotic dosage should be taken into account in these cases. Similar approaches should also be taken in other common or occasional persisting bacterial infections in Mediterranean aquaculture such as Nocardiosis, Epiteliocystis or Mycobacteriosis.

3.5.7. Antibiotic overprescription

Antibiotic over prescription is an universal problem in human and veterinary medicine and also in aquatic medicine. This problem is particularly critical in large groups of food animals and aquaculture finfish production is maybe the most extreme representative. In general, the control of bacterial diseases using antibiotics can be done following different approaches: therapeutic treatment, metaphylaxis, prophylaxis and continuous routine control. The concept of therapeutic treatment is the most widely known and is based in the use of medicines (antibiotics in our case)

in animals that are still sick. As we will see later on, this concept is not strictly used in aquaculture fish farming through medicated feed delivery. Prophylactic treatments are related to prevention but always in absence of infection. It should be highlighted that the new regulations 4/2019 and 6/2019 do not allow the use of medicated feed for prophylactic purposes. They could be applied following a wide range of criteria, from the presence of strong, clear and objective risks or to weak, unclear and subjective risks. The most extreme case is the continuous routine control using subtherapeutic doses, a totally non-desirable practice used in the past in several terrestrial farming activities and closely related to the concept of antibiotics as growth promoters.

The concept of metaphylaxis is much more controversial as this word is used to describe two apparently similar, but significantly different, concepts. Some authors describe metaphylaxis associated to the concept of risk factor, this is, as the use of medicines/antibiotics when one or several risk factors are present in a specific population. Other authors, however, describe metaphylaxis associated to population at real risk, this is, as the use of medicines/antibiotics when a certain population stock is experiencing or has already been diagnosed a certain (usually early) level of bacterial disease and an overt disease outbreak is highly probable. In this case, the target of the medicines is not the already sick animals (fish, in our case) but the real target is the still-not-infected, but the close-to-be-sick fish that are in the same cage, tank or pond of the sick fish or even are in cages, tanks or the vicinity of the affected stock (depending on the isolation between rearing units).

The differences between the two definitions are particularly relevant as 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 the risk-metaphylaxis should be understood much closer to prevention. In Mediterranean aquaculture, antibiotic treatments are always applied under the concept of metaphylaxis associated to populations at real risk, as always treatments are applied after the clinical identification of a bacterial disease in the population at risk. However, in aquaculture there is an additional and also very relevant and differential issue in these treatments concerning other species and is related to the fact that these treatments rely on delivery of the antibiotics through the medicated feeds and also the fact that the feed ingestion is fully suppressed in sick fish. These two facts cause that the supplied antibiotic only reaches the part of the stock still not affected, but not the affected one. This particular point is absolutely relevant to understand the real mechanism on how the medicated feeds act to control a bacterial outbreak in a fish population and has also very relevant implications for the efficient and sustainable use of antibiotic-medicated feeds. Some of these implications are summarized here:

- 1) In a antibiotic treatment using medicated fish feeds, the amount of feed and antibiotic 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. This estimation can be done 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 the feeding behaviour is highly recommended in this situation

- 2) As medicated feed will be delivered to still healthy fish, antibiotic plasma values are still relevant as a reference, but are not as relevant as the classical therapeutical approach in sick fish, where bacteria can also be found in the fish 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” (Bousquet-Melou et al, 2010). As medicated feeds are processed in the digestive system before the antibiotic absorption in the intestine and in some cases, systemic bacterial infections such as vibriosis can be originated in the intestine, the specific effect of the antibiotic concentrations in the special intestinal microenvironment should not be disregarded.

The decision to medicate/treat with antibiotics a tank, pond or cage is normally taken (or should be taken) after receiving enough evidence that a certain bacterial pathogen is causing mortality or morbidity in a fish stock. However, the ‘evidence’ concept is not always so clear and some times decisions are made simply based on ‘suspicion’ or weak evidence (risk-metaphylaxis of prophylactic treatments). In these cases, better diagnostic tools and diagnostic protocols in terms of accuracy, availability cost, swiftness are required. When the diagnosis of a primary bacterial disease is clear enough, then the selected therapeutic strategies will have a strong basis. Diagnostics and diagnostic protocol improvement has been also addressed in Performfish Task 3.2 so we expect a relevant improvement in the future in this aspect.

However, in some cases, despite robust diagnostics and evidences are clear enough, that bacteria are not the primary problem, some fish health veterinarian practitioners tend to include antibacterial substances as a part of their therapeutic recommendations even if the use of antibiotics is not strictly necessary. Some reasons can explain this trend:

- i) scarcity of other efficient therapeutic measures: as there are very few therapeutic alternatives, antibiotics are sometimes seen as relatively safe alternative ‘to do something’.
- ii) secondary bacterial infections frequency: for some viral (nodavirus) and parasitic diseases (monogeneal, external protozoan parasites, internal parasites) or skin diseases (petequeal rash), other concomitant (accompanying) bacterial problems or secondary bacterial problems (*T. maritimum*, *V. harveyi*, *V. alginolyticus*) can be associated to the primary problem during the development of the outbreak. As these bacteria can be also easily isolated as contaminants during the sample collection and processing for diagnostic (bacteriological, molecular), the precise implication of this accompanying or secondary bacterial diseases is not always properly identified.
- iii) risk criteria: health risk evaluation in Mediterranean finfish aquaculture is still based on personal experiences and only very few recommendations or

standards on how to proceed are available. In case of high levels of uncertainty, precaution tends to prevail even at risk of excessive caution. In this cases, the focus of the problem in the affected fish stock is usually much more important that more general problems such as the effects of the antibiotics in the environment and the risk of antibiotic resistance generation.

Another relevant issue to take into account is what happens when medicated feeds are distributed to the fish. The overall evaluation of the dynamics of the disease in the affected fish stocks and the environmental and rearing conditions affecting these stocks are absolutely paramount to design the most suitable therapeutic strategy. Best efficacy rates of the treatments against bacterial diseases are normally achieved when antibiotic treatment is delivered in the optimal conditions and using the most suitable delivery protocols.

A real evaluation of the proper efficacy of the treatments is another relevant handicap in Mediterranean aquaculture. Very frequently, the only way to evaluate the apparent efficacy of the treatment is the estimation of the reduction of the mortalities or signs of the diseases, including appetite. In only very few cases it is possible to assess with a high level of confidence if the recovery of the stock is due to the effects of the antimicrobial substance or if it is simply a part of the natural recovery after an infectious disease outbreak. In very few cases in field conditions, is possible to have a comparable non-treated control group of fish or different groups affected at the same level and at the same time. This is a very important difference from the antibiotic efficacy studies 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 reinforce the relevance of the extensive (in amount of batches examined in different condition) field efficiency monitoring. Another very frequent handicap for Mediterranean finfish aquaculture are the problems associated to the very complex logistics of the medication with medicated feeds, including diagnostics, prescription, production of the medicated feeds, transport to the farms and distribution to the affected stocks. We already discussed some aspects of the problems associated to diagnostics and prescriptions but also the production of the medicated feeds, transport and distribution is still one of the most relevant problems. As it was said before, fast delivery of the medicated feeds immediately after the decision to medicate is paramount in the success of the treatment. However, in the Mediterranean industry scenario, this delivery can be slow down due to many different reasons and setbacks:

- 1) Issue of the compulsory veterinary prescription and shipment to the medicated feed producer
- 2) Amount of medicated feed
- 3) Authorised on-site medicated feed production or supplied by a fish feed company or another authorised external supplier
- 4) Availability of premixes (type and amount)
- 5) Production time

- 6) Delivery: logistics associated to geographical situation of medicated feed production centers and farms. Connection by roads/transport by ferries, arrival to the farm
- 7) Problems associated to storage and delivery of regular feed and medicated feeds.

Other indirect problems related to the use of medicated feeds should not be underestimated. These problems are mainly associated to what should we do with the surplus medicated feeds?. This is a very frequent problem as sometimes medicated feed orders tend to be bigger than strictly necessary ('order some more medicated feed, just in case'), mortalities are bigger and faster than expected, reducing the biomass to feed or the disease suddenly disappears from the stock and it was decided to withdraw the treatment.

3.6. 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 in human health (Alderman & Hastings, 1998) and also for the environment. The concern of the implications in human health is mainly related to the misuse of antimicrobial medicines in intensive terrestrial animal agriculture and aquaculture and the emergence of antibiotic resistances. This problem, therefore, should be seen as a global problem, not only related to aquaculture. Concerning the environmental impact of antibacterial substances, these effects have implications in human health but also seen from an ecotoxicological point of view. This problem was already addressed by FAO some years ago (FAO, 2005) and nowadays is a controversial topic and one of the most discussed negative aspects of aquaculture and frequently highlighted by the media. These impacts in the aquatic environment are unquestionable, but the level of these impacts may also vary greatly amongst places and activities and in many cases are mixed with the huge impacts also in the aquatic bodies of antibacterials from human origin and terrestrial animal farming. It is well known that advanced aquaculture systems like salmon aquaculture in Norway have successfully minimised these impact, but the situation in other geographical areas with lower technological development and with less advanced regulation and supervision policies remains alerting. The EU policies in this sense have also set high standards and are implemented through the Marine Strategy Framework Directive (MSFD), 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 specific potential environmental impacts of antibacterials in Mediterranean aquaculture were already identified and commented by Rigos and Troisi (2005). In this work, the environment releases mechanisms of the antibacterial substances (exemplified in OTC) were very clearly exemplified in a scheme (Figure 7).

Antibacterial substances (and their metabolites) released in the environment are mainly found in these diments, redistributed in the water column and/or can be

D3.3-Therapeutics for MMFF

transferred to other organisms, causing three different types of potential problems, as Rigos and Troisi (2005) have described. This process may cause:

- 1) generation of antibiotic resistances
- 2) accumulation of antibiotics in other organisms
- 3) toxic effects in several organisms.

The presence of all the three types of environmental problems has also been described related to the Mediterranean Aquaculture in scientific and technical papers or in specific studies (Chelossi et al., 2003; Di Cesare et al. 2013;Colombo et al 2016) and recently is widely addressed in the H2020 project TAPAS (<http://tapas-h2020.eu/>). However, as it was stressed previously, the presence of antibiotics 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 for the future is the reduction and minimisation of the use of antibiotics and their strategies are focused in this same direction (IUCN, 2007). A part of this strategy will also mean the rationalisation of the treatments that is one of the main objectives of this document.

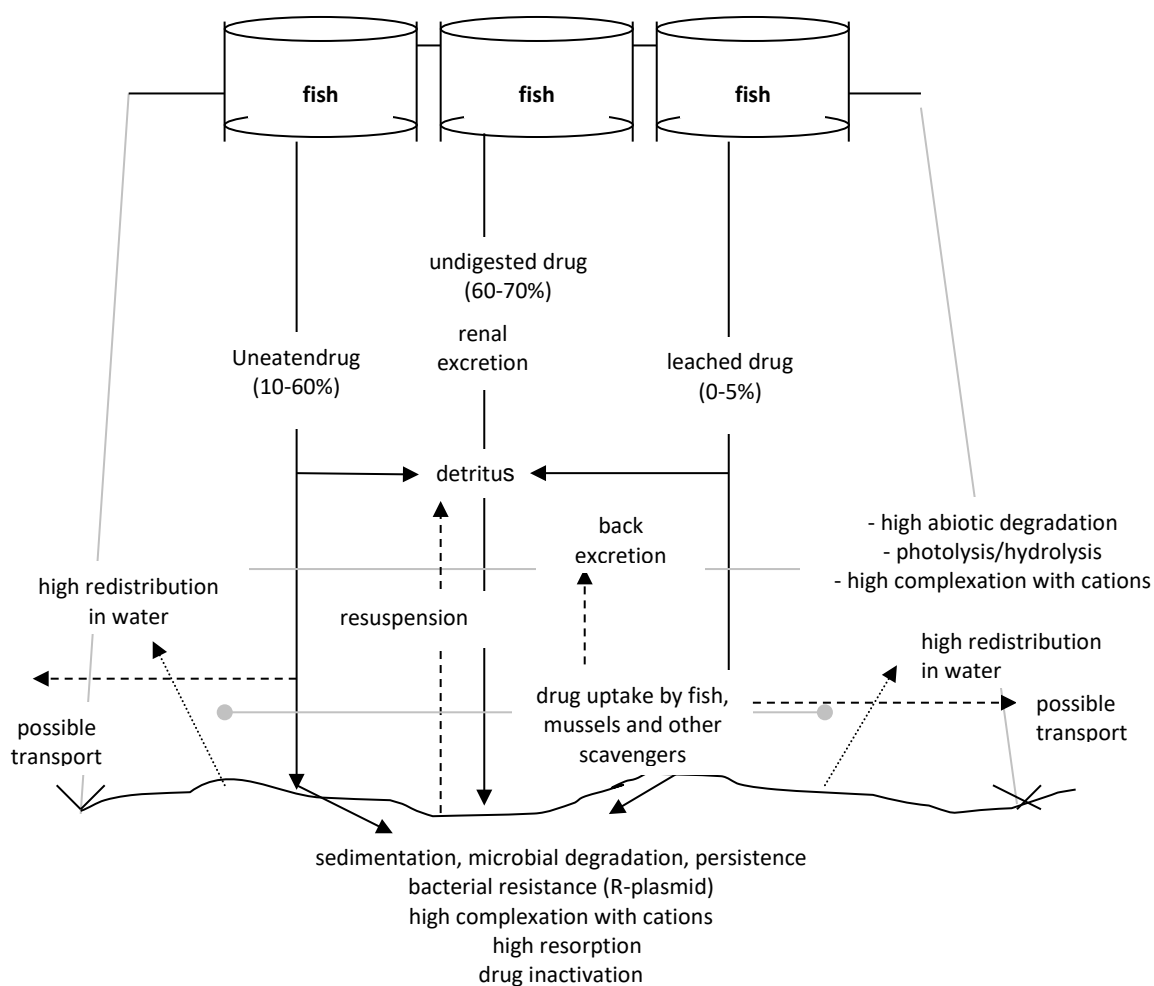


Figure 7: Environmental fate of orally administered antibacterials (OTC) in euryhaline fish farming sites; adapted and modified from Rigos and Troisi (2005)

4. Conclusions & recommendations for best practises

Proper and prompt diagnosis is essential for a successful treatment and the first primary step for confronting the bacterial pathogen given that sick fish will inevitably show reduced appetite and responded time is crucial for the preparation and delivery of the medicated feed to the farm. The handling stress and the cost associated with a particular treatment must be balanced with the expected benefits before decision for therapy to be applied is made. It is likely that in some disease cases the cost of treatment might exceed the value. Moreover, in some occasions, the severity or nature of the disease does not warrant treatment because infection will remain low during the incidence (empirical evaluation) or on the other hand, very progressive and sick fish may not withstand handling stress and treatment. Thus, in both cases, application of therapy should be carefully evaluated and perhaps rejected.

It is understandable that empiric administration of medicines in full should be avoided. Medicine selection will be based on sensitivity test (disk diffusion, MIC) and if initiation of the treatment is urgent, selection should be based for the initiation of the therapy on historical data from the farm and/or treatment response in neighbour farms and corrected if necessary after the *in vitro* results. The MRL of the target /or of other fish species should be taking into account in the design of the treatment schedule considering also an adequate WTs if fish are to be consumed soon.

Tetracyclines, mainly represented by OTC, have received wide attention as reflected by the large number of published work in Mediterranean farmed fish. Its slow elimination may suggest that a sequential dosing schedule is needed as a more prudent and economical treatment strategy, at least in medium/low water temperatures. Interestingly, DOX appears as a promising alternative considering its adequate circulatory levels in European sea bass, the low MIC values and the encouraging clinical outcome mainly based on small lab trials.

Although some quinolones/fluroquinolones showed promising PK profiles (mainly FLU over OA) based on the plenty published data, this group is of highest priority according WHO and CIA (Critical Importance Antimicrobials) (2017), so probably their use for farm animals will start to be reduced and eventually banned. Anyhow, considering their drug type coupled with their rapid elimination from tissues of Mediterranean farmed marine fish, daily or twice-daily dosing is suggested, especially at higher water temperatures where depletion is faster, to maintain maximum tissue concentrations. There is missing information regarding absorption of ENR, SAR and DAN in the circulation of Mediterranean fish species.

Concerning sulfonamides /potentiated sulfonamides, while plenty PK-depletion but limited absorption data exists for gilthead sea bream, surprisingly such information is lacking for European sea bass.

Penicillin derivatives have little (experimental) use in euryhaline fish farming, probably due to the lack of registration for use in aquaculture. PK studies of AMP are missing in euryhaline fish species. AMO displayed negligible bioavailability in gilthead sea bream, although its MIC values are rather promising. Further PK studies in European sea bass, may demonstrate improved AMO absorption, where it may be more appropriately used for antibacterial therapy.

D3.3-Therapeutics for MMFF

With respect to phenicols, preliminary trials with FLO in European sea bass revealed promising findings regarding absorption, removal and clinical outcome. More research is obviously needed to obtain a complete picture of FLO PK value in both European sea bass and gilthead sea bream. On the other hand, there are plenty PK studies of THI in both species.

Other new tested compounds in PerformFISH belonging to lincosamides and aminocyclitols such as LCM and SPE, respectively, showed adequate absorption profile (especially LCM) but relatively high MIC values against important bacteria of European sea bass and gilthead sea bream. Evaluation of PK/PD models followed by clinical trials will provide more data concerning the potential value of these two compounds.

Consideration of species dependent differences are recommended when applying/adopting dosing schedules after selection of the appropriate antibacterial. Special attention should be also given to other important factors such as fish size, fish density, growing environment and especially water temperature which appears to totally alter the PKs 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 planning is recommended based on the concept of metaphylactic treatment and the anticipated inappetence of the sick population.

CHAPTER TWO: use of antiparasitics and parasite control strategies

1. Introduction

Pathogens are among the main limiting factors of the aquaculture industry, as they can produce financial losses estimated to be about 20% of the total production value. Among them, it is estimated that the world annual grow-out loss due to parasites in finfish farming ranges from 1% to 10% of harvest size, with an annual cost that can reach up to \$9.58 billion (Shin et al., 2015).

The main objective of this document is to present the current knowledge on therapeutic and control measures for the most relevant parasitic diseases in European sea bass and gilthead sea bream. This study covers the knowledge on basic preliminary information from limited laboratory experiments in these two fish species, information from other similar fish species or from differing fish species (mainly from salmon) harbouring parasites of similar taxonomic groups, that can be applied to Mediterranean fish farming. Current parasite therapeutic and control practices is also presented. The final aim is to provide the Mediterranean fish farming industry with updated information, and also with relevant information about potential and available parasite control strategies, each one with details about their strengths, problems, applicability and concerns and also future clues, so the industry specialists can run a benchmarking exercise in order to evaluate the most suitable methods and strategies to be implemented in the future. These future selected strategies can be considered as “new tools” for parasite control and can be implemented and eventually validated in the PerformFISH WP6: Testing and Validation: Combining promising solutions, technologies and testing in industrial settings.

2. Overview of the main parasitic diseases

The number of parasite species described in gilthead sea bream and European sea bass (in wild and farmed fish) is large and has been referred in many scientific publications (see Sitjà-Bobadilla et al. 2014, for European sea bass 2014, and Colorni&Padrós, 2011 for gilthead sea bream). In **Table 6** and **Table 7**, some of the most frequently described endo- and ectoparasites in farmed conditions are summarized.

Table 6. Main ectoparasites found in farmed gilthead sea bream (GSB) and European sea bass (ESB). Impact is related to the prevalence, frequency of the parasitosis, its pathological effect and economic costs in aquaculture. VH= very high, H= high, M= moderate, L = low, O = Occasional U= Unclear

Group/Species	Hostsite	Impact	Host	References
MONOGENEA				
<i>Diplectanumaequans</i>	Gills	H	ESB	González-lanza et al., 1991; Ogut & Ozum, 2014; Öktener et al., 2008; Fioravanti et al., 2006
<i>Diplectanum laubieri</i>	Gills	L	ESB	González-lanza et al., 1991
<i>Furnestinia echeneis</i>	Gills	H/M	GSB	Antonelli et al., 2010; Fioravanti et al., 2006
<i>Neobenedenia melleni</i>	Skin, fins, eyes	L	GSB	Colorni & Diamant, 2005
<i>Sparicotyle chrysophrii</i>	Gills	VH	GSB	Sitjà-Bobadilla et al., 2010; Fioravanti et al., 2006
CRUSTACEA				
Isopodes				
<i>nylocraphysodes</i>	Gills, skin	L	ESB	AlvarezPellitero, 2004
		L	GSB	AlvarezPellitero, 2004
<i>Ceratothoa estroides</i>	Oral cavity	VH	ESB	Horton & Okamura, 2001, 2003; Mladineo, 2002; Vagianou et al., 2006b
		L	GSB	Horton & Okamura, 2001; Vagianou et al., 2006b
<i>Ceratothoa paralella</i>	Oral cavity	M	GSB	Papapanagiotou & Trilles, 2001
<i>Emetha audouini</i>	Oral cavity	L	ESB	<u>Papapanagiotou et al., 1999</u>
<i>Nerocilaorbingnyi</i>	Gills, skin	L	ESB	Bragoni et al., 1984; Horton & Okamura, 2001
Copepods				
<i>Caligus</i> spp.	Oral cavity, skin	L	ESB	Ragias et al., 2004; Vagianou et al., 2006a; Fioravanti et al., 2006
<i>Lernanthropus kroyeri</i>	Gills	VH	ESB	Antonelli et al., 2012; Manera&Dezfulli, 2003
PROTOZOA				
<i>Amyloodinium ocellatum</i>	Skin, gills	VH	ESB	Paperna, 1980; Fioravanti et al., 2006
		U	GSB	Fioravanti et al., 2006; Pereira et al., 2011
<i>Cryptobia</i> spp.	Gills	L	ESB,	Alvarez-Pellitero et al., 1993; Fioravanti et al., 2006
		O	GSB	Alvarez-Pelliteroetal., 1995
<i>Cryptocaryon irritans</i>	Gills	VH	ESB	Colorni & Burguess, 1997; Fioravanti et al., 2006
		H	GSB	idem
<i>Ichthyobodo</i> sp.		O	GSB	Alvarez-Pelliteroetal., 1995
<i>Neoparamoeba</i> spp.	Gills	L	ESB,	Dykováetal. 2000
		L	GSB	
<i>Philasterides dicentrarchi</i>	Systemic	L	ESB	Dragesco et al., 1995; Santos et al., 2010
<i>Trichodina</i> spp.	Gills	L	ESB,	Alvarez-Pellitero et al., 1993; Fioravanti et al., 2006
		L	GSB	Alvarez-Pellitero et al., 1995; Fioravanti et al., 2006

Table 7. Main endoparasites found in farmed gilthead sea bream (GSB) and European sea bass (ESB). Impact is related to the frequency of the parasitosis, its pathological effect and economic costs in aquaculture. VH= very high, H= high, M= moderate, L = low, O = Occasional. U = Unclear

Group/Species	Hostsite	Impact	Host	References
DIGENEA				
<i>Cardicola aurata</i>	Gills	M	GSB	Holzer et al., 2008; Fioravanti et al., 2006
APICOMPLEXA				
<i>Cryptosporidium molnari</i>	Stomach	M	GSB	Sitjà-Bobadilla et al., 2005
		O	ESB	Sitjà-Bobadilla et al., 2005
<i>Eimeria bouixi</i>	Intestine	L	ESB	Fioravanti et al., 2006
<i>Eimeria dicentrarchi</i>	Intestine	M	ESB	Sitjà-Bobadilla, et al., 2014; Gjurcevic et al., 2017
<i>Eimeria sparix</i>	Intestine	L	GSB	Sitjà-Bobadilla et al., 1996
<i>Goussia sparix</i>	Intestine	L	GSB	Sitjà-Bobadilla et al., 1996
MICROSPORIDIA				
<i>Enterosporea nucleophila</i>	Intestine	VH	GSB	Palenzuela et al., 2014
<i>Glugea</i> sp.	Muscle	O	GSB	Mathieu-Daude et al., 1992
<i>Loma</i> sp.	Intestine	L	ESB	Caffara et al., 2010
<i>Pleistophora</i> sp.	Muscle	O	GSB	Athanasopoulou, 1998
MYXOZOA				
<i>Ceratomyxa auratae</i>	G. bladder	O	GSB	Rocha et al., 2015
<i>Ceratomyxa labracis</i>	G. bladder	O	ESB	Fioravanti et al., 2006
<i>Ceratomyxa diplodae</i>	G. bladder	L	ESB	Alvarez-Pellitero & Sitjà-Bobadilla, 1993; Fioravanti et al., 2006
<i>Ceratomyxa sparusaaurati</i>	G. bladder	L	GSB	Sitjà-Bobadilla et al., 1995; Palenzuela et al., 1997; Fioravanti et al., 2006
<i>Enteromyxum leei</i>	Intestine	H	GSB	Sitjà-Bobadilla & Palenzuela, 2012
		O	ESB	Sitjà-Bobadilla & Palenzuela, 2012
<i>Kudoa</i> sp.	Intestine	O	ESB	Rigos et al., 1999
<i>Kudoa iwatai</i>	Systemic	L	GSB, ESB	Diamant et al., 2005
<i>Ortholinea auratae</i>	U. bladder	O	GSB	Rangel et al., 2014
<i>Sphaerospora dicentrarchi</i>	Systemic	M	ESB	Sitjà-Bobadilla & Alvarez-Pellitero, 1993; Fioravanti et al., 2006
<i>Sphaerospora</i> (ex. <i>Leptotheca</i>) <i>sparidarum</i>	Trunk kidney	L	GSB	Sitjà-Bobadilla & Alvarez-Pellitero, 2001; Fioravanti et al., 2006
<i>Sphaerospora</i> (ex. <i>Polysporoplasma</i>) <i>sparix</i>	Trunk kidney	M	GSB	Palenzuela et al., 1999; Fioravanti et al., 2006
<i>Sphaerospora testicularis</i>	Testis	H	ESB	Sitjà-Bobadilla & Alvarez-Pellitero, 1993; Fioravanti et al., 2006

A large number of parasites have been found in farmed fish but only some of them have been described to cause pathologies and real impact under current Mediterranean farming conditions. Most of these parasites and parasitic diseases have also been widely addressed in the H2020 project **ParaFishControl**, (<http://www.parafishcontrol.eu/>). In fact, the approaches on parasites and parasitic diseases in PerformFISH, as it is indicated in its DoA, were designed to complement and implement this project into the particular environment of Mediterranean finfish aquaculture. So, a relevant cooperative effort between these two H2020 projects has been deployed in order to reinforce and complement their own objectives.

In **PerformFISH**, an early internal preliminary assessment amongst the members of the consortium and companies was set up in order to record the most relevant disease problems in Mediterranean marine fish farms. In this preliminary assessment, copepod and isopod infections by *L. kroyeri* and *C. oestroides* in European sea bass and *S. chrysophii* in gilthead sea bream were identified as the main parasitic diseases with higher impact in the performance of these two fish species. These species were included in many tasks of the different work packages besides WP3. Other relevant diseases such as microsporidians and myxosporean intestinal parasites are also addressed in some of the tasks.

3. Parasitic diseases and health management and control of the European sea bass and gilthead sea bream in Mediterranean aquaculture

In parasite control and in contrast to bacterial diseases, there is not always a clear border between prevention and treatment. For most parasitic infections and in some rearing systems (mainly on-growing phase), the mere presence of the parasite in the farm or the facilities does not necessarily imply the presence of a problem or a disease. In these cases, the problems are mainly associated to the infection levels (parasitic load, developmental stages of the parasites) and also to the specific conditions of the affected stock (age, weight, physiological conditions, rearing system, etc.). Only when infection levels go beyond a certain level or fish susceptibility increases due to farming conditions, first disease signs arise. Therefore, parasites can be frequently found in subclinical stages under the current Mediterranean marine farming conditions. The total elimination or eradication of parasites from these farming sites is almost impossible in most cases. Thus, control strategies would be addressed to routine preventive measures, which are in some cases the same used as treatments. The only difference would be that 'preventive measures' are referred to when no evident signs of disease are detected, whereas 'therapeutic measures' to when signs of disease are detected. This is the reason why in this document 'control measures' is more appropriately used to refer to both situations. Finally, measures targeting the fish and designed to increase their resilience to parasites can also be included into an open wide concept of 'control' and therefore will also be reviewed in this document.

Different control measures and strategies have been described for some parasites and parasitic diseases affecting European sea bass and gilthead sea bream. Some of them were summarized ten years ago in a review of the treatments for parasitic disease in

D3.3-Therapeutics for MMFF

Mediterranean aquaculture (Athanasopoulou et al. 2009). However, for many parasites these treatments, measures and strategies are described only at laboratory research scale and only very few are applied routinely. Moreover, many of these control strategies display only a limited efficiency in their capacity to control or eliminate the parasites.

Different aspects should be taken into account when broaching parasite control. The first aspect is the **targeted parasites**. Some treatments may be very parasite species-specific, whereas others can be useful against a parasite group. The second aspect is **the delivery method**, which may vary depending on the parasite location in the host. Ectoparasites such as monogeneans, ciliates, flagellates, crustaceans and isopoda (**Table 6**) are located in external parts of the fish, such as skin and fins (Trichodinids), gills (*Amyloodinium ocellatum*, *Cryptocaryon irritans*, *S. chrysophrii*, *Diplectanum aequans*, *L. kroyeri*) or oral cavity (*C. oestroides*). By contrast, endoparasites (**Table 7**) are found in different internal organs such as those of the digestive tract (*E. leei*, *E. nucleophila*). Substances used to control ectoparasites can be delivered as **bath** treatments or **orally**, whereas endoparasites are treated mainly orally. Other delivery methods, such as injection, are only very occasionally used when few animals are to be treated (broodstock or research). A third and specific aspect concerning parasite control to be taken into account is **the aquatic environment**. In many parasitic diseases, environment plays a major role in the development of parasites and severity of diseases. Parasite life cycles frequently involve development stages (eggs, swimming, larval or resistance stages) that are free in the water bodies or attached to different substrates (seabed, pond bottoms, nets, tanks, biofouling, intermediary hosts, etc.), which are very relevant targets for control measures. Thus, the management of the environment is as important as that of the fish. Furthermore, bath treatments are frequently applied together to the environment and fish, when fish cannot be removed from this environment. This aquatic condition makes the difference when comparing with terrestrial animals in which host can be treated separately easily.

4. Antiparasitic substances

Antiparasitic substances for parasite control are also a relevant aspect to consider in this section. First of all, it should be strengthened the huge gap between the antiparasitic active molecules considered by the current EU legislation (EMA's substances indicated for food-producing species), the commercial products available in EU or products specifically licensed in each EU country (**Table 8**), and the antiparasitic substances described and tested at laboratory level (**Table 9**). This big gap has been circumvented partially with some mechanisms (medicament prescribing cascade) and specific considerations for aquaculture problems (MUMS) that mitigate to some extent these needs, but still are hampered by other administrative issues, such as the exceptionality and the time-lasting administration procedures of the importation processes. This problem has mainly an administrative root, is acting as a relevant bottleneck, and solutions should include all the levels previously described. In **Figure 8** we try to

schematically present how PerformFish is addressing these problems. Basic research on parasite treatments is also being conducted in ParaFishControl project.

Such registered compounds are critically very limited in Mediterranean marine fish farming. Currently, only formalin baths have recently reached a legal status in several Mediterranean countries including Greece & Spain (Table 8). In other areas (Scotland, Ireland) and for Atlantic salmon, other substances are available via diet or bath (Table 8). Application of some of them to European sea bass or gilthead seabream is possible (cascade system, special import), but complicated. Unfortunately, there is a great possibility that formalin will be banned, stressing the urgent need for new effective antiparasitic alternatives.

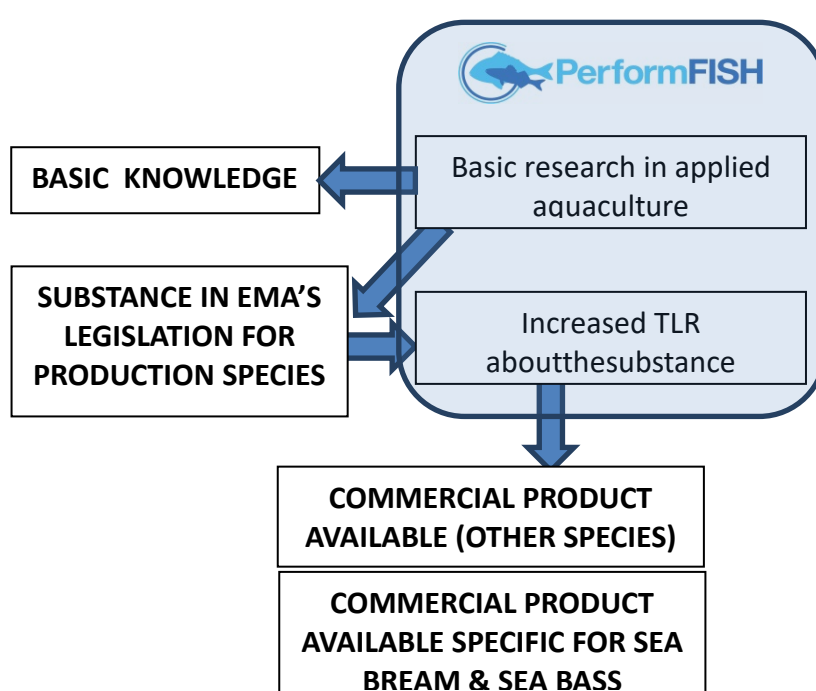


Figure 8: PerformFISH contribution to fighting parasites

Many different **chemical substances** have been experimentally tested to control different Mediterranean marine fish parasites with variable results (e.g. Athanassoupoulou et al. 2009) (Table 9). Some of them are medicaments *sensu stricto* and most are designed to interfere with parasites' metabolism, to kill them or reduce their capacity of movement, growth, multiplication or reproduction (benzoylureas, such as Diflubenzuron). More recently, some **natural substances** and compounds have also been tested as alternative to chemical substances, with promising, but also questionable results. These substances may also affect parasites through the modification of the tissue environment of the fish (epithelium, mucus...), or by reducing their attachment capability to the target fish tissue or their viability. However, most available information is based on *in vitro* experiments or on limited laboratory-scale trials. Furthermore, only very few of them are really used in the current practice due to different and strict regulatory framework. In some cases,

treatments are described for similar parasites but in other species and under different environmental conditions (mainly in Atlantic salmon and other salmonids), but with only occasional specific references to gilthead sea bream and European sea bass. In these cases, although cascade prescription on exceptional import mechanisms can be claimed, the scarcity of previous data and experiences in the use of these medicines in gilthead sea bream and European sea bass can be a relevant hurdle for the veterinarian prescribers.

Table 8. List of antiparasitic substances against parasites of European sea bass and gilthead sea bream under EMA consideration for use in fish and commercial products (medicines) specifically licenced for salmonids and/or European sea bass and gilthead seabream

Antiparasitic substance	Administration route	Dosage	Target pathogens	Country	MRL ($\mu\text{g}/\text{kg}$)	References
Formalin	Bath	100-250ppm 1 h	ciliates monogeneans	Greece, Spain	0	AquacenFormal dehidro (Cenavisa)
Emamectin benzoate	Oral	50 $\mu\text{g}/\text{kg}$ /day, 7days	Parasitic crustaceans	Salmonids	100	EMEA/MRL/863 /03-FINAL
Diflubenzuron	Oral	3mg/kg/day, 14days	Parasitic crustaceans	Salmonids	10	EMA/CVMP/15 3976/2018 (salmonids)
Lufenuron	Oral	10mg/kg /day, 7 days	Parasitic crustaceans	Salmonids	1350	EMEA/V/MRL/0 03749/FULL/00 01
Azamethiphos	Bath	0.1-0.2ppm, 60min	Parasitic crustaceans	Salmonids	No	Procedure no EU/11/185/FVG
Hexaflumuron	Bath	2ppm, 60- 120min	Parasitic crustaceans	Salmonids	500	EMA/CVMP/34 7671/2014
Deltamethrin	Bath	2-10ppm, 30min	Parasitic crustaceans	Salmonids	10	EMEA/MRL/731 /00-FINAL

To sum up, although there is a relevant body of literature on the use of active substances in fish, the final number of products available in the current regulated farming practice is much lower. Similarly, the number of scientific and technical papers dealing with parasite treatment of gilthead sea bream and European sea bass is relatively high, but only a very limited number of substances is registered (**Table 8**). The reasons behind this limited availability are summarized below:

- Limited or partial efficacy of some of the tested substances due to specific parasite characteristics (life cycles)
- High toxicity of some substances
- Difficulties in their application (baths at large scale)
- Cost of the active substances
- Relevant environmental issues
- Regulatory issues (efficacy, safety, residues & food safety)
- Cost of the licensing administrative procedures
- Relatively limited market in European sea bass and gilthead sea bream production.

Table 9. Chemical substances experimentally used against parasites of European sea bass, gilthead sea bream and other sparids

Substance	Delivery method	Concentration/ duration	Best dose	Type of assay	Target parasite	Efficacy	Reference
Deltamethrin	Bath	0.1µg-10mg/L 0.5-48h	0.05mg/L 2h	<i>In vitro</i>	<i>C. oestroides</i>	High	Athanasopoulou, et al., 2001
Deltamethrin	Bath			<i>In vivo</i>	<i>C. oestroides</i>	Medium	Çolak et al., 2018
Deltamethrin	Bath	0.05mg/L, 30 min		<i>In vitro</i> , <i>In vivo</i>	<i>C. oestroides</i>	High	Vagianou et al., 2017
Diflubenzuron	Diet	3mg/kg 14d		<i>In vivo</i>	<i>C. oestroides</i>	High	Bouboulis et al., 2004
Teflubenzuron	Diet	10-20 mg/Kg BW		<i>In vivo</i>	<i>L. kroyeri</i>	No effect	Tokşen et al., 2009
Trichlorfon	Diet	50 mg/Kg BW, 7 days		<i>In vivo</i>	<i>L. kroyeri</i>	High	Tokşen et al., 2010
Trichlorfon	Diet	50 mg/Kg BW, 5-7 days		<i>In vivo</i>	<i>D. aequans</i>	Medium - High	Toksen et al., 2012 Toksen et al., 2013
Azamethiphos	Bath	1 ppm/2h		<i>In vivo</i>	<i>D. aequans</i>	High	Toksen, 2007
Azamethiphos	Diet	2 mg/Kg BW, 5 days		<i>In vivo</i>	<i>D. aequans</i>	Medium	Toksen et al., 2013
Ivermectin					<i>L. kroyeri</i>		Athanasopoulou, et al., 2008
Emamectin benzoate	Oral	10-100µg/kg 7d	100µg/kg	<i>In vivo</i>	<i>L. kroyeri</i>	High	Toksen et al., 2006
Praziquantel	Oral Bath	400mg/kg 6d 50 ppm – 30 min		<i>In vivo</i> <i>In vitro</i>	<i>S. chrysophrii</i>	Low Low	Sitjà-Bobadilla et al., 2006
Hydrogen peroxide	Bath	200 ppm – 30 min		<i>In vitro</i>	<i>S. chrysophrii</i>	High	Sitjà-Bobadilla et al., 2006
Hydrogen peroxide	Bath	200 ppm – 30 min		<i>In vitro</i>	<i>C. oestroides</i>	Low, easier manual removal	Rigos, Lab trials
Hydrogen peroxide	Bath	100-200 ppm for 30 min, repeat 6 days later	200 ppm, twice	<i>In vivo</i>	<i>A. ocellatum</i>	High	Seoud et al., 2017
Formalin	Bath	350 ppm – 30 min		<i>in vivo</i>	<i>S. chrysophrii</i>	High	Sitjà-Bobadilla et al., 2006
Formalin	Bath	200 ppm for 1 h		<i>In vivo</i>	<i>Microcotyle</i> /* <i>Pagrus pagrus</i>	High	Katharios et al., 2006

D3.3-Therapeutics for MMFF

Formalin	Bath	350 ppm		<i>In vivo</i>	<i>D. aequans</i>	High	Giavenni, 2012
Formalin	Bath	100 ppm for 1 h		<i>In vivo</i>	<i>C. irritans</i>	Low	Rigos et al., 2001
Formalin	Bath	150 ppm for 1 h		<i>In vivo</i>	<i>C. oestroides</i>	High	Vagianou et al., 2017
Copper sulphate	Bath	(0.5 - 0.7 ppm, 5 times at 2 d intervals		<i>In vivo</i>	<i>C. irritans</i>	Low	Rigos et al., 2001
Hyposalinity	Bath	8-10 psu, for 3 h at 3 d intervals		<i>In vivo</i>	<i>C. irritans</i>	High	Rigos et al., 2001
Hyposalinity* (control measure, not treatment sensu stricto)	Incubation of <i>E. leei</i> stages	1 h, 34-0 ‰	8 ‰	<i>In vitro</i>	<i>E. leei</i>	Moderate	Yokoyama & Shirakashi, 2007
Salinomycin & Amprolium	Diet	0.1 g & 0.1 g/kg, 30 d		<i>In vivo</i>	<i>Myxobolus sp.</i> <i>*Puntazzo puntazzo</i>	High	Athanasopoulou et al., 2004b
Salinomycin & Amprolium	Diet	Salinomycin (12%) + amprolium (50%): 70+100 mg/Kg BW 56 d.		<i>In vivo</i>	<i>E. leei</i>	High	Golomazou, et al., 2006
Salinomycin & Amprolium	Diet	Salinomycin (12%): 70mg/Kg BW Salinomycin (12%) + amprolium (50%): 60+100 mg/Kg BW		<i>In vivo</i>	<i>Polysporoplasma spariss</i>	High	Athanasopoulou et al., 2004a
Fumagillin	Diet	15mg/kg 21 d		<i>In vivo</i>	<i>Pleistophora sp.</i>	Medium	Athanasopoulou, 1998
Fumagillin	Diet	6mk/kg, 3-6 wks		<i>In vivo</i>	<i>Myxobolus sp.</i>	High	Athanasopoulou et al., 2004b
Fumagillin	Diet	6mk/kg, 56 d.		<i>In vivo</i>	<i>E. leei</i>	Medium-Low	Golomazou et al., 2006
Fumagillin	Diet	2-25 mg/Kg BW. 3-6 wks		<i>In vivo</i>	<i>Polysporoplasma spariss</i>	average	Athanasopoulou et al., 2004a
Fumagillin	Diet	0.1 % 8 wks, 44, 91 days		<i>In vivo</i>	<i>Sphaerospora testicularis</i>	Limited, long term	Sitjà-Bobadilla & Alvarez-Pellitero, 1992
Mebendazole	Bath	400 ppm for 1 h		<i>In vivo</i>	<i>Microcotyle sp.*</i> in <i>Pagrus pagrus</i>	Low	Katharios et al., 2006

D3.3-Therapeutics for MMFF



Mebendazole	Diet	200 mg/kg, 1 dosing	PK study	<i>In vivo</i>			PerformFISH
Toltrazuril	Diet	0.6 ml/kg, 2 times (repeat 5d)		<i>In vivo*</i> <i>in Puntazzo puntazzo</i>	<i>Myxobolus sp.</i>	Low	Athanasopoulou et al., 2004b
Toltrazuril	Diet	10 mg/kg, 1 dosing	PK study	<i>In vivo</i>			PerformFISH

5. PerformFISH: New developments: Mebendazol & Toltrazuril

As previously indicated, PerformFISH new developments in antiparasitic treatments were selected and designed in order to increase the TRL (Technology Readiness Level) of some of these active substances for specific use in European sea bass and gilthead sea bream, the main target species of this project. Oral mebendazole delivery was selected as a good candidate for *Sparicotyle* treatment due to its proved efficacy in terrestrial animals and in fish (Schmahl et al., 1989). Only partial works on mebendazol efficacy were specifically done in sparids (Katharios et al., 2006) and surprisingly only until very recently some pharmacokinetic studies (oral and intravascular administration) have been made in fish (Xu et al., 2016). A very similar situation was found for toltrazuril for the myxosporean and microsporidian treatment, with only partial studies in Sparidae species (Athanasopoulou et al., 2004) and turbot (Bermúdez et al., 2006), being the basic information on pharmacokinetics in fish that is even more scarce than for mebendazole.

Results on pharmacokinetics of mebendazole and toltrazuril after oral delivery as well as technical details on procedures are separately presented in Annex 4 and Annex 5. These studies at this moment are limited in their value as they were designed as preliminary PK studies and will require future data on clinical efficacy using challenges with pathogens. Currently, these challenge models have already been developed for *Sparicotyle chrysophrii* (model available at HCMR; Dr. Rigos) and *Enteromyxum leei* (models available at IATS-CSIC; Dr. Sitjà-Bobadilla) and can be the perfect complement for researchers or companies interested to expand these studies in order to escalate the knowledge on these substances and potentially develop commercial products.

6. Treatments: delivery method

Two main delivery/application systems are usually described in parasite control. Each one has advantages and drawbacks that should be considered when selecting the most suitable method for each parasitic disease and the specific circumstances of each facility.

6.1. Oral treatments / in-feed administration

This route is suitable mainly for parasites located in digestive tract, other endoparasites, and ectoparasites that feed on fish blood. For the parasites of the gastrointestinal tract, the oral treatment is particularly efficient against those living in the lumen of the digestive tract (several adult forms of nematodes and trematodes) as the active substances are directly delivered to the parasite microenvironment (intestinal lumen). However, these worms are mainly found in wild fish, but not under aquaculture conditions. Some chemical and natural substances and feed additives may play certain role in controlling other parasites, such as Myxozoa, Microsporidia, Apicomplexa and some flagellates that undergo certain developmental stages within the digestive lumen, either fighting them or reducing their infectivity.

For parasites located mainly within the intestinal mucosa, the treatment strategies are different, as the intestinal mucosa is a very particular and complex tissue, with selective absorption and transport of substances to circulation and certain excretion

D3.3-Therapeutics for MMFF

activities. Absorption can be related to the chemical properties of the active molecules used and to other characteristics of the intestinal microenvironment (pH). However, the particular location of the different parasitic stages (e.g. oocysts, merogonic, gamogonic phases in coccidian parasites, intracytoplasmic and intranuclear stages of *E. nucleophila*, and paracellular location of *E. leei*) makes even more complicated the evaluation of the access of these substances to the parasites. Once absorbed and released into fish circulation, these substances can reach other endoparasites in the fish blood and internal tissues (*Cardicolaaurata*, other myxozoans and microsporidians), and some ectoparasites (*S. chrysophrii* other monogeneans) that feed on blood, gill and skin epithelial cells. The dose of the active substance present in these tissues may vary according to the compound, its absorption, distribution and further metabolism (mainly hepatic). This is the reason why pharmacological and particularly pharmacokinetic (PK) studies are so relevant for the evaluation of the therapeutic efficacy of these substances.

Several active substances have been described for in-feed medication against parasites in European sea bass and gilthead sea bream (**Table 9**), however, none of them are currently used for therapeutic treatment in normal farming activities. These compounds are mainly targeted against parasitic crustaceans (copepods and isopods), monogeneans and intestinal myxosporean parasites. For crustacean parasites, the use of diflubenzuron has been described for *C. oestroides* control and emamectin benzoate for *L.kroyeri*. Praziquantel and mebendazole has been described for the treatment of *S.chrysophrii* infections. Salinomycin + amprolium, fumagilin and toltrazuril have been described for the control of myxosporeans (including *E. leei*) and microsporidians. Most studies were based on previous records about treatment efficacy in other fish species (mainly in salmonids) and using similar doses and delivery protocols. The resulting efficacy is quite varied. Benzoyliureas were particularly promising in some cases. In diflubenzuron treatments against *C. oestroides* in European sea bass, preliminary very positive results were obtained using a medicated feed (Bouboulis et al., 2004). However, trials with teflubenzuron against *L.kroyeri* in European sea bass with a dose range of 10-20 mg/ Kg BW were not effective in the control of juveniles and adults of this copepod. Since then, no further studies on the efficacy of this compound have been published for Mediterranean fish species. More recently, lufenuron, another benzoylurea widely used for crop protection and flea control in domestic animals, has been tested in Atlantic salmon (Poley et al. 2018). This compound was also successfully used in the control of *G. maxilaris* (parasitic isopod) in an exhibition aquarium with a single delivery of 10 mg/Kg BW in feed once per month, with progressive reduction on the parasite number and complete eradication of the parasite after six months (Hispano, 2016). Some avermectins were also tested with variable results. Ivermectin administered orally to European sea bass at 0.2-0.5 mg/Kg BW had a good efficacy (Athanassopoulou et al., 2001), though these doses were close to the toxicity range. Emamectin benzoate efficacy was tested in European sea bass against *L.kroyeri* at 10-100 µg/KG BW, resulting in a partial reduction of parasite load (Toksen et al., 2006).

For the control of monogeneans and particularly *S.chrysophrii*, praziquantel was tested (Sitjà-Bobadilla et al., 2006) using doses between 40 and 400 mg/Kg BW

D3.3-Therapeutics for MMFF

displaying partial results in the reduction of the infection prevalence in higher doses but also displaying relevant problems of palatability. As Benzimidazoles were also described in the treatment of gill flukes in fish. In particular, this active substance was described as very efficient in the control of another Microcotylid species: *Microcotylesebastis*, in rockfish, *Sebastes schelegi*. (Kim et al, 1998). Furthermore, its efficacy against *Microcotyle (S.chrysophrii)* in bath treatments was demonstrated in cultured red porgy *Pagrus pagrus* in the Mediterranean (Katharios et al. 2006). As no specific challenges in oral delivery were performed in gilthead sea bream, mebendazole was selected as a candidate for evaluation in PerformFISH WP3. As a previous step for efficacy and safety tests and in order to have a more detailed knowledge on the pharmacokinetics of this substance. Results on pharmacokinetics of Mebendazole detailed in the specific document complementary to this one (see section 5).

In-feed medications against Myxozoa and Microsporea in gilthead sea bream have also been tested. Salinomycin (12%) alone or combined with amprolium (50%) was tested at 70mg/Kg BW and 60+100 mg/Kg BW with apparently positive results in the treatment of sharpsnout sea bream (*Puntazzo puntazzo*) against *E. leei* (Athanassopoulou et al., 2004b) and in gilthead sea bream against *P. sparis* and *E. leei* (Athanassopoulou et al., 2004b). Toltrazuril was also used against *Myxobolus* sp. in sharpsnout sea bream (Athanassopoulou et al., 2004b) with 2 dosages of 0.6 ml/kg every 5 days, but with only partial positive results. No specific studies have been done using fumagillin against *E. leei* in gilthead sea bream, but this active substance was tested against another *Enteromyxum* sp., *E. scophthalmi*, a close-related species affecting turbot (*Scophthalmus maximus*) (Bermúdez et al., 2006). In the latter study, some potential effects of the treatment were claimed based on the parasite morphology using 10 mg/kg BW for 5 days before and 5 days after exposure to the parasite. However, this treatment schedule did not ameliorate the clinical progress of the disease.

To sum up, the available information for in-feed treatments for parasite control of gilthead sea bream and European sea bass is fragmented and based on a limited number of experiments. Most of them are based in small-scale preliminary trials on naturally-infected fish, using commercial products that are added to the feed and using different basic methodologies to assess data on efficacy and safety of these treatments. Only very few ones have been performed under highly controlled experimental laboratory conditions, and with a complete pharmacological follow-up. Therefore, the available information obtained till now is relevant, but should be taken only as preliminary and indicative. These studies have to be implemented under controlled consistent conditions, with standardized challenge procedures and with a wide monitoring of all the aspects of the trials (including pharmacological approach) in order to confirm these previous results. In a next step, large-scale field trials should also be necessary for knowledge escalation until commercial application.

6.2. Bath treatments / In-water medication

This route is suitable mainly for the control of ectoparasites. Although certain degree of absorption of some substances through the skin, gills or even through the intestine should not be disregarded, most antiparasitic substances used by bath (as with antibacterials) have poor or negligible absorption rates. Thus, in this delivery system active substances target directly the parasitic forms on fish surfaces in contact with water (skin, fins, gills and oral cavity), but also, any potential parasitic form present in the water body treated. Bath treatments are very laborious and may have considerable environmental implications and serious applicability difficulties in large sea cages (use of tarpaulins). In some cases, however, fish can be treated in specific separate bath treatment containers (tanks or even large containers such as barges), or also “*in situ*” treatment, when the substance is delivered to all the water body of the rearing unit (or a significant part of it). In the separate bath treatment, only the fish (and the parasites attached to the fish) are exposed to the product, whereas in the unit treatment, other potential parasitic forms in the system can also be exposed to the substance. Each method presents advantages and disadvantages that are summarized in **Table 10**.

The selection of the method may be related to many different factors. In general, *in situ* baths are preferred because the fish management required is lower (except baths in sea cages) and the cost of the therapeutic product used is relatively low.

Table 10. Advantages and disadvantages of different bath applications

Method	Direct control of the fish	Contingency measures in case of intoxication signs	Amount of product to be used	Fish management required (fish netting, pumping, transport)	Risk of stress associated to management	Environmental issues
Separate bath	Easier	Easier (bring the fish back to the tank)	Low	Higher	High	Lower
In situ bath	More difficult	Much more difficult	High	Lower (tanks) Average (cages)	Lower (tanks) High (cages)	Higher

6.2.1. Baths: treatments, preventive treatments, water conditioning and water disinfection, water sanitization: the different sides of the same polyhedron

Although this section is mainly related to treatments, water quality is at the core of nearly all the aspects related to aquaculture. The quality of the water in the aquaculture facilities may depend on the specific environmental conditions where this activity is performed (mainly in extensive systems or cage farming) and/or the specific production characteristics and how water is managed (filtration, RAS). In many fish farming systems (and also in Mediterranean gilthead sea bream and European sea bass production) water quality can be managed in different ways. Physical methods

D3.3-Therapeutics for MMFF

(filtration, UV) are the most widely used methods, but also water quality can be managed using chemical methods. At first sight, maybe the word 'chemical management' does not fit very well with the idealised concept of 'water purity' used in aquaculture. However, in many other scopes of human activities directly related with water, chemicals are very frequently used. This is particularly the case of drinking water (use of chlorine and ozone), water disinfection in poultry industry (Chlorine dioxide, organic acids) or swimming pool conditioners and sanitisers (chlorinated disinfection products, hydrogen peroxide, ozone, copper sulphate, benzalkonium chloride, and flocculants). Aquaculture may use similar approaches to keep water quality in the best range for fish and also guarantee hygienic conditions for fish and humans. However, sometimes the use of these substances has been questioned, mainly due to the direct extrapolation of the regulations used in terrestrial animal farming and also due to lack of awareness and knowledge of the reality of the aquaculture technology.

In aquaculture, the difference between the use of substances for a disease treatment, for prophylactic use and for water conditioning can not be so clear, as the application method can be the same or very similar in all three situations. A good example is the control of protozoan ciliates in Mediterranean aquaculture. In routine practice, protozoan ciliates tend to appear and increase in numbers due to their feeding activity on detritus, bacteria and algae. Therefore, it is necessary to apply routinely some 'control application' in the tanks or ponds (usually using a low dose of formalin) to keep the ciliate protozoan populations under control. In many cases, Trichodinids are part of these ciliates. Sometimes, when the ciliate / Trichodinid populations exceed the limits or there are some indications that the number of ciliates is too high, then an extra application of the 'control treatment' is recommended. Although the dose and the way of application in both cases is exactly the same, we can refer to that as treatment, control bath or simply as a ciliate hygienization. However, nowadays there is still a big difference on the requirements and availability of substances used to control parasites depending on their consideration as treatments (medicines), disinfectants or simply water conditioning substances. This particular situation leads to very different criteria for the use of the same substance and delivery system depending on it is considered.

Some of the bath control methods are based on simple physical methods that change water characteristics. Freshwater and saltwater baths are the most clear and direct example of parasite control, mainly for ectoparasites, and usually work well for protozoans, which are killed or weakened through a simple osmotic shock. For *A. ocellatum* (Bessat and Fadel, 2018), *C. irritans* (Colorni, 1985), Trichodinids, *Cryptobia* sp. or external amoebae, freshwater baths can be therefore considered as part of the therapeutic and control strategies in gilthead sea bream and European sea bass. However, these methods are not specific and may also have detrimental effects on the fish, since marine fish have to cope with the change in salinity for the duration of the treatment. Thus, these treatments should only be applied to fish populations in good health condition or slightly parasitized capable to cope with the physiological impact of the treatment. European sea bass is a particularly interesting species for freshwater treatments as it is a euryhaline species and theoretically more adapted to

D3.3-Therapeutics for MMFF

low water salinity conditions. Gilthead sea bream can also be considered as an euryhaline species and it can adapt to brackish water conditions under certain conditions (time, salinity level).

Although freshwater seems a very easy, cheap and available resource for treatment, this is not the case for the Mediterranean aquaculture. Freshwater is usually available as tap water but the regular tap water supply in aquaculture facilities is normally designed for the day-to-day activities. When freshwater is required for antiparasitic baths in inland facilities, large freshwater volumes and specifically devoted large water storage tanks or freshwater wells are required. For marine cages, very large volume of freshwater is required and in the Mediterranean area this resource is normally scarce, expensive and difficult to find close to the production sites. Special caution should also be taken when tap water is used as it is frequently chlorinated as water disinfectant. Freshwater hardness may also affect the efficacy of the treatment.

Water temperature has been used in salmon farming industry to control sea lice (Thermolicer, Optilice). Warm water (30-34°C) short delousing flushes are being applied, but some detrimental effects have also been observed in the treated stocks, from increased mortality and susceptibility to other diseases, to lethargy and haemorrhages. Theoretically, a similar strategy could be used in the control of European sea bass and gilthead sea bream crustacean ectoparasites, but it should be noticed that the normal thermal tolerance range of these two species (and their parasites) is higher than that of salmon. Welfare issues should also be taken into account.

Filtration, UV and ultrasound are also potential physical methods that can be helpful to control parasitic diseases under certain conditions (mainly in hatchery and in RAS), reducing the amount of free parasitic forms (dinospores, theronts, infective stages, oncomiracidia, swimming larvae, etc.) in the system. In any case, filtration capacity and UV doses should be adapted to the specific characteristics of each parasite to achieve the highest disinfecting efficacy.

6.2.2. General chemical bath methods

General chemical bath methods include the addition of some active chemical substances in the water. Two main chemicals/molecules: formalin and oxygen peroxide have been described in the literature for the treatment of ectoparasites, mainly *S. chrysophrii* and *C. irritans* (Table 9).

6.2.2.1. Formalin

Formalin is the most widely used substance in aquaculture treatments for many years, not only for the treatment of ectoparasites, but also for its bactericidal activity (Leal et al., 2016). As hydrogen peroxide, formalin activity against parasites is related to the high chemical reactivity of formaldehyde (the main active molecule in formalin solutions) against biological molecules such as proteins, carbohydrates and nucleic acids. This high reactivity is unspecific and tends to alter the chemical configuration of these biological molecules and impairing their properties and activity. Nowadays is used in hatchery and nursery mainly to control protozoan ciliates proliferation in tanks and also to control trichodinids and *C. irritans* infections in tanks and ponds. It is also

widely used to control *S. chrysophrii* in tanks and mainly in sea cages. Sensitivity of eggs, larvae and adults of *S. chrysophrii* to exposure of 300 ppm formalin for 30-60 minutes was tested *in vitro* (Sitjà-Bobadilla et al., 2006) and these doses are very close to those currently used in fish farms for the control of this monogenean. Similar results were obtained in eggs of *D. aequans* (Cecchini and Cognetti-Varriale, 2003). Doses and exposure times may vary depending on the rearing systems, fish age and targeted disease, but the values provided in **Table 11** can be used as a reference.

It is very important to consider that the doses are based on dilutions from the commercial formalin (37-40%) and not in the total formaldehyde content of the solutions. Under unfavourable conditions (high organic load), the activity of formalin can be reduced and free formalin level after 15-20 minutes should be determined to adjust the treatment. As in many other applications of active substances, formalin also can provoke behavioural changes in fish during treatments, such as early excitement and swimming activity immediately after the administration of the diluted formalin and some minutes later, fish start to display a more lethargic behaviour. However, this activity can be different when fish are crowded (sea cages). Stress related to formalin use has also been described (Yavuzcan and Ergonul, 2010).

Table 11. Suggested doses and exposure times of formalin according to rearing systems.

Parasitic disease	System/fish stage	Dosage	Available references
Trichodinids	Hatchery	70 ppm initial dose (open dynamic system)	Authors, personal observations
	Nursery	150 ppm /1 h	Authors, personal observations
	Juveniles	150-200 ppm / 1 h	Authors, personal observations
<i>C. irritans</i>	Tanks, ponds/ Juveniles	100 ppm (3-4 h, mainly at night for 4-5 consecutive nights)	Colorni and Padrós (2010)
<i>A. ocellatum</i>	Tanks/Juveniles, adults	50 ppm 1 h or 4 ppm 7 h 100-200 ppm 6-9 h, repeated treatments	Paperna (1984)
<i>S. chrysophrii</i>	Ongrowing ponds/ Juveniles	150-200 ppm 1 h, repeated treatments) 200-300 ppm (1 h)	Colorni and Padros (2010) Aquacen technical leaflet

		250 ml Aquacen/ 1000 L (which is 95 g formaldehyde (1000 L)	
<i>S. chrysophrii</i>	Ongrowing cages/ Juveniles	250 ml Aquacen/ 1000 L (which is 95 g formaldehyde (1000 L 200-300 ml formaldehyde 37-40% / 1 h	Aquacen technical leaflet
<i>D. aequans</i>		300 ppm (1 h): parasite egg control	Cecchini and Cognetti-Varriale (2003)
<i>D. aequans</i>	Earth ponds/ Adults	375 ppm (1 h)	Giavenni, R (1983)

6.2.2.2. Hydrogen peroxide (H₂O₂)

Hydrogen peroxide (H₂O₂) is also a widely used chemical substance against ectoparasites and also to control bacteria and virus and as disinfectant in aquaculture, but also in medical and other uses. Oxygen peroxide activity is related to its high oxidative activity, oxidizing in an unspecific way many different molecules, and breaking down into oxygen and water, without any other by-products. This absence of residues and the natural degradation make this substance particularly attractive for its use in aquaculture. However, this unspecific high oxidizing activity poses also potential harmful effects in the fish, from irritation to toxicity. Another relevant issue is its stability and activity in water depending on water temperature and organic load. This effect is well known in Atlantic salmon, as treatments with hydrogen peroxide against sea lice performed at warmer temperatures, especially above 14°C, tend to increase fish mortality during and after treatment. Recently, the reduction of this mortality has been described if hydrogen peroxide is used together with a substantial reduction of temperature (Overton et al., 2018). This effect is due to the chemical characteristics of hydrogen peroxide, a thermodynamically unstable molecule that increases its catalytic degradation as temperature and pH increases. Thus, activity is lower at low temperature and low pH. Therefore, a trade-off between activity against the parasite and potential toxicity for the fish should be assessed in each treatment scenario.

Concentrated hydrogen peroxide industrial formulations can normally be found in 35-40 % weight/weight (or active ingredient) as general-purpose disinfectants. However, only few formulations have been specifically designed for Aquaculture. Asperix® (Vet Evonik) and Paramove® (Aqua Pharma) have been licenced for salmon treatment in some North European Countries and Ox-Aquaculture® (Ox-Aqua) for the prevention of several marine fish diseases (including *S. chrysophrii* infections).

For European sea bass and gilthead sea bream, hydrogen peroxide has been tested both for *in vitro* and *in vivo* for *A. ocellatum* and *S. chrysophrii*. For *A. ocellatum* 75-150 mg/L for 30min with a second treatment after 6 days has been recommended (Beraldo, personal communication). These doses are similar to those recommended

D3.3-Therapeutics for MMFF

under rearing conditions in other cultured fish species such as *Polydactylus sexfilis* (Montgomery-Brock et al., 2007). *S. chrysophrii* oncomiracidia larvae were sensitive to direct exposure to hydrogen peroxide (50-200 ppm, 30 minutes) (Sitjà-Bobadilla et al., 2006), whereas adults were much more resistant and only high doses (200 ppm, 30 minutes) eradicated 100 %. Similar doses are recommended for field treatment (Colorni and Padrós, 2010). Eggs were completely resistant to these doses and exposure times. However, field application is much more difficult probably due to the stability and oxidative activity of H₂O₂ at the normal high temperatures in the Mediterranean. Before reliable information on the efficacy of the treatment and also on the safety levels for fish at different temperatures is available, the application of H₂O₂ to control gilthead sea bream and also European sea bass is still risky.

Copper sulphate has also been described mainly for *A. ocellatum* and *C. irritans* control. For the particular case of the control of *A. ocellatum* doses of 0.75-1 g/m³ in ponds/tanks as prolonged immersion are one of the most frequently recorded methods for all marine species. The basis of the treatment is the effect of copper ion, as it has a strong algicidal activity and copper ion levels should be strictly maintained around 0.12-0.20 mg/l as copper has a very narrow margin between therapeutic level and toxicity and also is only effective against dinospores, not against trophonts. This method is used in aquaria but not currently used in Mediterranean aquaculture as there are not licensed commercial products and also due to the safety therapeutical margin and environmental issues.

6.2.2.3. Specific chemical substances

Specific chemical substances: other specific substances have been used in bath to control parasites (mainly monogenean and crustacean ectoparasites) of gilthead sea bream and European sea bass. Some of these specific substances have already been used in salmon industry mainly to control sea lice. These substances include organophosphates (**azamethiphos**) and pyrethroids (**deltamethrin**) for parasitic crustaceans, and benzimidazoles (**mebendazol**) mainly for *S. chrysophrii*.

Organophosphates are used as pesticides, herbicides and also in the parasite control of terrestrial and aquatic animals. Trichlorfon and diclorvos were two of the main organophosphate pesticides used in the past to control crustacean and monogenean parasitic diseases, but now their producers have voluntarily cancelled all food, feed and field crop registrations. Azamethiphos has been more recently used in the control of sea lice in Atlantic salmon, together with deltamethrin and emamectin benzoate. It has been licensed as a commercial product (Salmosan[®]), but its efficacy is nowadays disputed to the high level of resistance displayed to this molecule after some years or continuous use in the sea lice control, and also due to environmental impacts associated to the use of azamethiphos in sea cages. As organophosphate insecticide, azamethiphos is active against invertebrates including crustacean parasites, so its potential effect against parasite invertebrates can include crustacean and monogenean parasites. The activity against monogeneans was tested in European sea bass with *D. aequans* natural infections (Toksen, 2007).

Deltamethrin is another active substance used in the control of sea lice in Atlantic salmon that has also been tested in European sea bass for the control of *C. ostreides*

D3.3-Therapeutics for MMFF

(Athanasopoulou et al., 2001; Colak et al., 2018) with variable results. In Colak et al. (2019), a single treatment with 0.35 ml/m³ for one hour using a commercial presentation used in salmon (AphaMax®) was able to reduce significantly the prevalence of the infection and also to reduce the dynamics of the parasite population at farm level, mainly controlling the number of sexually mature females.

Benzimidazoles such as albendazole and mebendazole, and other similar active substances have been used in baths (and also in in-feed treatments) to control monogenean parasites in marine and freshwater fish with varied results depending on fish species and rearing conditions. There are no references of the efficacy of the use of mebendazole in bath in gilthead sea bream or European sea bass, but there is a single description of no success in the control of *S. chrysophrii* in red porgy using 400 ppm of mebendazole for one hour. In the same study, the authors describe good results using a formalin bath (200 ppm, 1 h) (Katharios et al., 2006).

6.2.3. Treatment delivery

The degree of difficulty in the implementation of bath treatments is also highly related to the type of system used, the size of the units and the number of units to be treated.

6.2.3.1. Antiparasitic bath treatments in tanks

Bath treatments in tanks can be done using **static baths** or **dynamic baths**. In static baths, the substance is directly added to the tank and no water renewal is applied. In the second case, water renewal is maintained together with constant addition of a certain amount of the substance in the inlet water flow or in the tank. This second system is only used under certain conditions (permanent bath when water renewal is compulsory, effluent disinfection) as it requires a higher amount of product and may have important environmental implications. Most treatments for gilthead sea bream and European sea bass under farming conditions are based on static baths and may involve short timed baths (dips), mid-term and long-term baths.

6.2.3.1.1 Short-timed baths

Short-timed baths (dips) are usually performed as separate baths, using high doses of the substance with low exposure times (seconds/few minutes) in small-sized tanks or containers (less than 200 litres). This system is suitable for nursery, with fish from 0.5-5 g, as the basis is similar to other management procedures (grading, swim bladder triage, vaccination, etc.) where fry are normally collected using nets. Dips can be performed using specifically designed nets (calibrated for a certain amount or weight of fish) and immersed in specific containers. The system is quite similar to that used for fry bath vaccination (a relatively large number of fish can be treated each time). When treated, fry can be rinsed with clean water to dilute or remove the substance and transferred to a new tank with clean water. As the weight of the fish increases, this system becomes less practical. In dip baths, time control, oxygen supply and periodic substitution of the water/substance in the treatment tank are paramount.

- 1) Prepare the container and add oxygen supply and measure system
- 2) Add water with the right dose of product. Check oxygen levels
- 3) Fish collection (calibrated net)

- 4) Fish immersion with time control. Checking oxygen and fish reactions
- 5) Fish net removal
- 6) Periodical evaluation of the quality of the dip bath used (organic matter, measure the level of substance. Change for new treatment solution when necessary.

Proceed again to point 3

6.2.3.1.2 Standard baths

Standard baths are performed in the same tank for a limited time (usually between 10 and 60 minutes) and can be used for larvae fry or bigger fish. The operation should start by increasing the water renewal and reducing the amount of faeces and organic matter present in the tank, to reduce any potential interference with the treatment. Specific oxygen supply and control should also to be provided. Then, the oxygen supply is switched on and the water volume level of the tank is lowered until fish are concentrated at a certain level (usually at the same short-time concentration levels used in other management procedures), in order to minimise the amount of product to be used. This strategy also allows a progressive reduction of the concentration of the dose just switching on again the water supply in the tank. It is very important to gauge the exact amount of water in the tank for the calculation of the total amount of product to be used. Before the release to the tanks, the total amount of product to be used should be previously pre-diluted in a separate container with clean water. This will reduce the possibility of the generation of 'clouds' of high concentration of the product when released into the treatment tank. It is also highly recommended to distribute the content of the pre-diluted treatment in several points of the tank. Once poured, it is important to check the response of the fish for the first 5-10 minutes in order to detect any unexpected reactions, and it is preferable to keep the surveillance until the treatment has finished. When finished, water supply and flow are resumed to reduce progressively the concentration of the substance until it is completely eliminated. If necessary, effluents with the diluted substance can be diverted from the usual effluent system using specific pipes.

6.2.3.1.3 Antiparasitic bath treatments in cages

Antiparasitic bath treatments are particularly difficult in standard floating sea cages, which is the predominant grow-out system in the Mediterranean aquaculture, due to different reasons:

- Size of the cages
- Complex logistics
- High dependency on weather and sea conditions
- Volume of water in the baths
- Risks of the operations
- Amount of therapeutic substances to be used
- Total cost

D3.3-Therapeutics for MMFF

Bath treatments in cages can be done using two different methods: transferring the fish to specific bath barges or floating containers or *in situ* treatments using tarpaulins. The first method is frequently used to control amoebic gill disease (AGD) in salmon in several countries (Australia, Scotland) a preventive measure to control AGD and requires relevant infrastructures (barges, containers dedicated to this specific purpose, and specific technical staff). This is the choice method because salmon aquaculture is usually based in areas protected from bad weather conditions. This could also be implemented in particular farms in the Mediterranean, but not in the majority of farms, as they are offshore and less protected from strong winds and sea currents (high energy farms). This is the reason why most of the bath treatments are done using tarpaulins. Tarpaulins can use the simple skirt technique or use closed tarpaulins, depending on the experience and skills in each farm.

Open skirt technique requires less technical labour than closed tarpaulins. Normal operations require hoisting up the bottom of net until it is at the same level of the skirt. This involves the concentration of the fish stock close to the water surface, with the subsequent stress. Then, tarpaulins are arranged around the cage forming a skirt that acts as a barrier and stops the normal water renewal through the nets, keeping the chemical (formalin, hydrogen peroxide, etc.) or medicament inside the net. However, this water renewal blockade also affects the normal water flow and the normal oxygen supply. In addition, the temporary increase of the biomass due to the concentration of the fish, and the increase in the metabolic oxygen requirements associated to crowding stress, makes oxygen a limiting factor very fast. Therefore, oxygen levels should be checked constantly, emergency oxygen supply should be at hand and applied if necessary. Once skirts are displayed around the cage, the treatment can be added at different points of the cage, preferably using pre-diluted large water volumes for a more homogeneous distribution of the product. A fast distribution of the product into the whole cage can also be enhanced using vigorous air or oxygen bubbling or using pumps and perforated distribution tubes in the cage. Monitoring the concentration of the active product in different points and depths (as the skirt system is a semi-open system, part of the product can be lost under strong water currents) and careful surveillance of fish reactions are paramount to reduce the risk of toxicity or hypereaction problems. In this case, skirts should be removed immediately to reactivate the normal water flow into the cage and dilute the product.

Closed tarpaulin treatments require an extra effort as the bottom of the cage should also be closed. The tarpaulin is nearly watertight and the leak of product during treatment is significantly reduced, and this has a major repercussion on the treatment dosage control and environmental issues. Closed tarpaulin bags have recently been designed for salmon sea lice control (Nilsen et al, 2017). In this case, tarpaulins forms a bag that completely covers the nets, similar to a treatment cage and has a water outlet system that can be used to pump the water with the chemicals, avoiding the release into the environment and facilitating their disposal or even their reuse.

7. Disease remediation through nutritional intervention

As in other terrestrial and aquatic species, alternative and complementary strategies to control fish diseases in general and particularly parasites have recently been launched. One of these strategies is based on increasing the resilience of the hosts using feed additives that contain immunostimulants that reinforce the immunological status of the fish. Their potential effect in specific diseases can be tested through pathogen challenges or through the evaluation of different immunological parameters. The effects of these diets on the immunological status of fish has been widely published (Vallejos-Vidal et al, 2016). Other novel strategies include the use of specific natural molecules addressed to modify the biochemical profile of the parasite, affecting certain parasite structures. Finally, plant derived extracts and compounds have also been on focus as an alternative to standard medicines and chemical products, mainly due their ability to control pathogens and parasites and their status out of the strict legislation on medicines and biocides. All these strategies have been explored and tested in gilthead sea bream and European sea bass in the control some parasitic diseases.

Different feed additives, some of them already commercialized, have been shown to reduce the disease outcome in *E. leei*-infected gilthead sea bream in the context of the ParaFishControl project (Palenzuela et al., 2017; Piazzon et al., 2017) (Table 7). The benefits, in terms of reduction of the intensity of infection by *S. chrysophrii* in gilthead sea bream through the addition of caprylic acid alone (Rigos et al., 2013), or combined with organic iron and oligomanans as immunostimulants (Rigos et al., 2016) in the diet have recently been published. This is a good example of a synergistic strategy, adding the effects of caprylic acid to an immunostimulant and a mineral (iron) to cope with the anaemic condition typically induced by sparicotylosis. Origanum essential oils were also tested in gilthead sea bream against several parasites (Athanasopoulou et al., 2004a; Athanasopoulou et al., 2004b; Yiagnisis et al., 2016) with varied success (Table 12). In addition, other studies (Pérez-Sánchez et al., 2015; Piazzon et al., 2016) have provided information on the immune response of gilthead sea bream at intestinal level, after different dietary interventions, which could be of potential interest in parasitic intestinal infections such as *E. leei* or *E. nucleophila*.

Table 12. Alternative/natural compounds used against parasites of European sea bass & gilthead sea bream and other sparids

Substance	Delivery	Concentration/ duration	Environment	Target pathogen	Efficacy	References
Caprylic acid	Oral	200mg/kg fish, 60d	<i>In vivo</i>	<i>S. chrysophrii</i>	Low	(Rigos et al., 2016; Rigos et al., 2013)
Sanacore	Oral	0.2-0.4% diet	<i>In vivo</i>	<i>E. leei</i>	Mid	Palenzuela et al., 2017

D3.3-Therapeutics for MMFF



Origanum essential oils	Oral	8-12ml/kg	<i>In vivo</i>	<i>Myxobolus</i> sp.	Low	Athanasopoulou et al., 2004b
Origanum essential oils	Oral			<i>P. sparis</i>		Athanasopoulou et al., 2004a
Origanum essential oils	Oral	1.6- 2.4 ml/kg	<i>In vivo</i>	<i>E. leei</i>	High	Yiagnis et al., 2016
Sodium butyrate	Oral	0.4 % diet	<i>In vivo</i>	<i>E. leei</i>	Mid	Piazzon et al., 2017

8. Parasitic disease management through environmental control

Many parasites affecting Mediterranean aquaculture have one or several developmental stages in their life cycle out of the fish host, either in the water bodies or other farm structures in contact with fish. The environmental control of these stages is also relevant for controlling these diseases and has been widely adopted in the control of crop pests and terrestrial plagues.

The 'environmental stages' that should be considered are:

- *Amyloodinium*: free dinospores, tomons
- *Cryptocaryon*: cysts (tomonts) and theronts
- *Enteromyxum*: trophozoites as infective stages, plus actinospores in other putative hosts involved in a possible diheteroxeneous cycle
- *Enterospora*: free spores
- *Sparicotyle*: eggs and swimming miracidia
- *Ceratomyxa*: manca stages in the plankton
- *Lernanthropus*: copepodit stages

A. ocellatum infections in gilthead sea bream or European sea bass are mainly found in fish kept in closed systems such as aquaria, tanks or ponds. *A. ocellatum* infection is not found in open sea cages, as the concentration of developmental stages seen in closed systems is unlikely in open sea due to water dynamics. Dinospores are released to the water body and water currents easily disperse them, in the same way as water currents disperse microalgae in the sea. However, in closed systems, water renewal is reduced and dinospores, hatched in large numbers from tomonts, are not dispersed, increasing their capacity and probability to re-infect the fish stock. Furthermore, tomonts encyst in the substratum (bottom of tanks and ponds) and remain in the vicinity of the fish stock, beginning again the life cycle. The very fast (few days and temperature-related) and exponential (64-128 new infective stages are released in each cycle) life cycle of this dinoflagellate, facilitates the fast and massive reinfection of the fish. Thus, *A. ocellatum* control strategies should take into account not only the treatment of the trophonts in the fish, but also other stages potentially present in the system.

The ciliate *C. irritans* has a similar life cycle to that of *A. ocellatum* and therefore similar approaches should be considered. In addition, the trophont stage is found under the skin and gill epithelium of the fish, making the external treatments against this form less efficient than in *A. ocellatum* infections. Cysts are also very difficult to treat and only theronts are sensitive to bath treatments. In fact, cysts are very resistant to any kind of disinfectant (and doses) used in the tanks holding fish, and the most efficient way to fight against them in flow-through systems is to move the infected stock to another clean tank and clean and disinfect the empty tank. Indeed, much more efficient disinfection protocols, including desiccation or heat treatments, or higher

D3.3-Therapeutics for MMFF

doses of biocides can be applied to empty tanks to completely eliminate cysts. The same process is applied again in the tank with the infected fish (according to water temperatures), as trophonts in the fish will be transformed in a new wave of cysts (tomonts) and will be found again on the floor and walls of the tanks. In this case, the number of tomonts will be much lower than in the previous change. After 3-4 tank changes and fish bath treatments (formalin) to eliminate theronts, the infection can be kept under control or eliminated from the system. In recirculation systems, biofilters, pipes and other structures can be a source of cysts, a sanitary break with full disinfection of the system should be applied. For ponds, a similar strategy to that of tanks should be applied: transferring infected fish to clean ponds, desiccating infected ponds and disinfecting them with quicklime or similar products.

The knowledge on the infection management of the microsporidian *E. nucleophile* is far from that of other parasite groups, mainly due to the fact that it is a recently described species and no trials have been done, yet. Since spores are the main environmental stage, tank changes and water disinfection strategies similar to the other parasites can be applied. However, it should be considered that these spores are very microscopic (1.5 μm) and can easily be retained and that the involvement of an invertebrate host in the cycle, as in other Enterocytozoonidae (Freeman & Sommerville, 2009) has not been discarded, yet. Furthermore, some egg surface disinfectants presently used in zebrafish laboratories are ineffective against spores of the microsporidian *Pseudolomaneuropilia* (Sanders et al., 2012).

For *E. leei*, infective trophozoites can be found free in the water, in faeces, in organic material (excreted intestinal mucosa casts) or in the intestine of dead fish. Periodic and systematic mortality removal is one of the most efficient and simple methods to keep the infection level under control in all types of rearing systems, as the cannibalistic behaviour of gilthead sea bream favours that infected dead fish enter into the system again. Fast and efficient removal of organic wastes potentially containing trophozoites can also reduce the impact of the disease. Water currents in cages may play this role on site (though water currents may also favour the dispersal of the disease to wild fish and other cages or farms in the vicinity). An increase in the filtration and waste removal (protein skimmers) and the implementation of water disinfection systems (UV, ozone, electrooxidation) may increase the efficiency of the system in terms of trophozoite removal from the system (see Sitjà-Bobadilla & Palenzuela, 2012).

For *Sparicotyle*, eggs are the main and fastidious environmental stage of the parasite. Eggs realised from gravid adults are ovoid and possess an elongated “tendrill-like” filament on each side. The end of the distal filament is provided with a hook with two barbs (Antonelli et al., 2010). These filaments act as Velcro® straps, allowing the eggs to attach on the epithelium between the fish lamellae, but also to other filamentous substrates and structures (ropes, nets, filters, fouling) (Roubal, 1994) of sea cages or tanks. Without these substrates, eggs cannot remain attached and can be removed more easily from the system. Therefore, the main measure to control sparicotylosis would be to remove such structures/substrates. In sea cages, this aspect is paramount, as eggs can become easily attached if nets and ropes are frayed. The use of fraying-resistant nets can reduce the attachment capacity. Biofouling is another

D3.3-Therapeutics for MMFF

relevant risk for the attachment of *Sparicotyle* eggs, as many organisms (macro & microfouling) may have filamentous structures that allowing their attachment. Periodic net changes coupled with efficient net cleaning and disinfection procedures is also another very efficient measure to reduce the number of eggs present in the cages. However, before using again the cleaned and disinfected nets, we suggest a more careful evaluation of the fraying level of the nets. “Egg traps” (structures very rich on small fibres, like synthetic wool or other similar fabrics) can be placed in the sea cages and periodically removed, cleaned and disinfected or replaced by new clean traps). Swimming miracidia have a relatively short survival time. They can be treated in tanks and ponds with formalin or hydrogen peroxide using normal doses for parasites, but also alternative strategies (potential phototropism) can be evaluated.

For *Ceratothoa*, eggs are maintained in the female’s marsupium until they hatch and develop into pullus II stage, and then can be released from the female. Then, pulli II (also referred as manca larvae) swim freely in the environment until a host (normally a juvenile fish, in our case European sea bass juveniles) is detected and attach to the fish skin of the tail or trunk, and actively progress towards the anterior part of the fish, reaching the gills and orobranchial cavity. In some cases (e.g. death of the host) the manca larvae can detach from the fish body and swim again to find another host. Thus, pulli II larvae can be found as a planktonic stage. In the host, the pulli mould into other stages (pullus III, pullus IV) and finally develop into juvenile or pre-adult stages, losing the capacity to swim and starting the development into a sessile parasite definitively attached to the host. Under cage culture conditions, due to the relatively high density of fish hosts, it is expected the pulli II larvae to be free swimming for only a short time. There is no information whether hatching rates are related to any kind of biological rhythm or the larvae develop phototropism or chemotropic behaviour towards the host. These characteristics could be exploitable as alternative control strategies (light traps, chemotropic disruption substances).

Lernanthropus, as other copepods, typically presents two nauplii, one infective copepodid, four fixed copepodids and one preadult. This life cycle has similarities with the well-known cycle of the sea louse *Lepeophtheirus salmonis* and therefore, similar strategies can also be adopted. Environmental control could be applied to swimming forms: naupli and the infective copepodid. Naupli stages, like eggs released from females, drift passively and do not swim directionally, so they can be considered as fully planktonic. Control at this stage is difficult as planktonic layer movements are mainly related to surface water currents. However, vertical distribution of eggs and naupli may display a particular pattern, as for many others planktonic organisms. Copepod naupli tend to be present in the upper layer of the water surface, so strategies designed to avoid the direct contact of the swimming forms (infective copepodids, mainly) with the hosts (European sea bass) could be an efficient way to reduce their attachment to the hosts. Skirts, submerged cages or the introduction of a surface net that keeps the fish into deeper waters are strategies already implemented against sea lice in Atlantic salmon that could be potential solutions. Naupli and copepodid also may display a phototactic and day/night vertical migration behaviour and also may be responsive for water accelerations relate to the swimming activity of the fish, as it is also described in sea lice. This can also be used to establish

strategies in order to separate fish from the water layer with highest intensity of free-swimming forms.

Other environmental control strategies can also be considered for crustacean and monogenean parasites. In salmon industry, biological control of sea lice infection through cleaner fish is nowadays a successful and widely used strategy. However, the species used in salmon farming for this biological control (wrasses and lumpfish) cannot be used in Mediterranean aquaculture due to very different temperature regimes and thermal tolerance of these species. Cleaning behaviour in some local Mediterranean species has been described. Species such as *Symphodus melanocercus*, *Ctenolabrus rupestrisor* *Coris julis* juveniles have been recently considered in a preliminary study (Riera, 2018) and can be exploited as local species for biological control at mid-term.

9. Specific management and integrated control strategies

As many other parasitic diseases in terrestrial and aquatic organisms, parasitic diseases in European sea bass and gilthead sea bream require multifocal approaches for their control. In most cases (mainly in on-growing systems with high interchange with the external environment), parasites cannot be eradicated from their environment, as they are part of the aquatic ecosystem. In this scenario, the best option is to work towards the control of the parasite populations in order to reduce or minimize their impact as diseases. Multidisciplinary and integrative approaches have been proved to have the highest efficacy in the control of parasitic diseases. Together with the therapeutic and prophylactic measures, some other strategies such as genetics and nutritional management can be used. Genetics of disease resistance in fish is nowadays a major issue for many species and has been particularly addressed in the salmon industry and also recently addressed in projects such as FishBoost, ParaFishControl and PerformFISH.

The nutritional approach is also a relevant issue in this multidisciplinary approach. This aspect is already developed in section 7 and is also implemented in PerformFISH in WP3 (Task 3.4.4: bioactive substances from Marine Natural Products (MNP)) and in WP4 (Task 4.2) Task 4.2: Development of Sustainable and Cost-Effective Feeds using Commercially Available Novel Raw Materials of Low Ecological Footprint that Support Enhanced Growth Performance, Robustness and Welfare of Farmed Fish.

Vaccination, as the basis of immunoprophylaxis can also be considered, but compared with vaccines and vaccination for bacterial and viral diseases, parasite vaccines are much more complex and difficult to develop in fish, as well as in humans and terrestrial animals. This is the reason why vaccines and vaccination against parasites may have a lower immediate relevance in the disease control compared with viral and bacterial diseases. However, some relevant advances have been recently made in ParaFishControl project. Acquired protection has been described in gilthead sea bream surviving *E. leei* infections (Picard-Sánchez et al., 2019) and further studies are now being aimed at deciphering the protective antigens to develop a vaccine within the ParaFishControl project. In addition, successful and promising good results on

D3.3-Therapeutics for MMFF

Amyloodinium vaccines have been obtained by the team of the University of Udine (UNIUD). The same team and researchers (UNIUD) are also partners in PerformFISH, so their developments in ParaFishControl, if agreed, can also be tested and validated in the PerformFISH WP6.

Then we summarise some integrative potential approaches on specific parasitic diseases

***Sparicotyle* infections:**

- Combined formalin bath treatment + net change
 - Step one: *Sparicotyle* infection level assessment (scoring system): Task 3.2: diagnostics and decision-making. Assessment at cage/ farm level
 - Step two: Cage treatment + *Sparicotyle* infection level post-treatment assessment
 - Step three: periodicity: repeat assessment each X months according the situation of the farm, season, water temperature and others
- Therapeutic and control measures (oral): mebendazole recommended when commercial products could be available
- Nutritional management
 - Nutritional strategies: parasite modification through host nutritional profile (caprilic acid)
 - General nutritional palliative strategies: blood (iron), epithelial cell replacement (fatty acids, vitamins), healing reinforcement (vitamins)
- Immune system reinforcement through diet (general health diets)
- Reduction of the egg attachment capacity and egg traps (flow-through tanks and recirculation):
 - Oncomiracidia control (tanks): bath treatments.
 - Mucus modification
 - Genetic selection

***L. kroyeri* infections:**

- Naupli/copepodit management: measures to reduce the exposure/contact of juveniles and adult European sea bass (adapted from the sea lice control in Atlantic salmon).
 - Presence in plankton follow-up: geographical areas of the Mediterranean with lower impact of *L.kroyeri*, water currents.
 - Skirts: it is necessary to evaluate the ecological behaviour of *L.kroyeri* copepodits and check if they tend to accumulate in the sea surface.
 - Immersed cages or adapted cages were European sea bass are kept under a certain depth, avoiding the upper layer. Snorkels are not necessary as sea bass

D3.3-Therapeutics for MMFF

does not need to reach the surface for swim bladder regulations as salmon does.

- Production in tanks, ponds or RAS instead of cages.
- Copepodit traps: studies on copepodit ecology are necessary. Copepodits can be attracted to traps by light, pheromones or other attractants.
- Cleaner fish: there are some Mediterranean fish species (mainly labrids) that display delousing behaviour in the wild but also under laboratory conditions (Padrós, personal observations). This could be an alternative to explore.
- Other measures used or studied in salmon (flush, thermolicer, lasers, ultrasounds) can also be adapted to European sea bass.
- Bath treatment of *L.kroyeri* juveniles and adult in fish:
 - Hydrogen peroxide baths: Used and legal in salmon, to be evaluated in the European sea bass.
 - Piretrines, etc: legal problems.
- In-feed treatments: azamethyphos, Lufenuron: to be explored.
- Nutritional management (same approach as for Sparicotyle): Nothing has been done, but options are available from sea lice and Caligus some plant extracts have been tested.
- Genetic selection.

Isopoda (*Ceratothoa*) infections:

- Adult (male and female) management: manual removal (while grading or vaccination). Check whether some anaesthetics play also a role in detaching the parasite
- Manca (pullus II) management: Manca traps: ex: praniza larvae of *Gnathia* present positive phototropism
- Bath treatment of *Ceratothoa* juveniles and adult in the fish:
 - Hydrogen peroxide baths: Used and legal in salmon, to be evaluated in the European sea bass.
 - Piretrines, etc: legal problems
- In-feed treatments: azamethyphos, Lufenuron
- Nutritional management (same approach as Sparicotyle. Nothing has been done, but alternatives are available from sea lice and caligus some plant extracts has been tested
- - Genetic resistance

Enteromyxum infections:

- Mortality removal: decreasing new infection by cannibalistic behaviour and reduction of the presence of infective forms in the infected decomposing dead fish.
- Faecal matter removal: water currents in cages (but dissemination!) and increasing water renewal in tank/ponds. Disinfection in RAS.
- Oral treatments: Toltrazuril (to be tested), natural substances
- Nutritional management: remediation diets reinforcing intestinal regeneration, reducing digestion effort (high digestibility diets) and reinforcing immune system. Sanacore as example.
- Genetic selection: possible in the future. Thus far, results obtained within a Spanish project (PROGENSA-III), showed that some gilthead sea bream families selected for better growth performance, have a significantly lower intensity of infection when experimentally challenged with *E. leei*, but no differences were found in terms of prevalence (data not published, Sitjà-Bobadilla personal communication).

10. Potential quantitative environmental pollution of different antiparasitic approaches

Fish farming activities without control can impact the environment as a result of organic and inorganic nutrient loss, discharge of products from treatments, genetic contamination, etc. The potential environmental impact of different active substances used in parasitic diseases treatments and control in European sea bass and gilthead sea bream should also be evaluated and minimized when possible. The impact of the treatments may vary depending on different aspects:

- Type of substance
- Amount of substance
- Release into the environment / pre-treatment available
- Impacted area and impacted environment

The environmental impact of the treatments depends on the specific substance. Hydrogen peroxide used in baths has a low level of environmental impact in terms of fast degradation and production of zero-impact metabolites post-degradation. Formalin has more complex interactions with the aquatic environment (Leal et al., 2016) and they may vary from dissolved oxygen depletion to biocidal effects as algicide and bactericide (related to effects on bacteria of RAS biofilters or in local environmental microbiota). However, it should also be stressed that formaldehyde is also subjected to strong biodegradation activity in the aquatic environment. This is also well demonstrated in RAS systems, where formaldehyde is progressively

D3.3-Therapeutics for MMFF

degraded by the microbiota (mainly by methylotrophic bacteria) present in the system (water, biofilms, biofilters). In some cases, formaldehyde can become completely degraded in 24-48 h. Moreover, its half-life time under natural environmental conditions is estimated to be 36 h (Leal et al., 2016). This degradation process is normally accelerated at higher temperature.

Formalin release can be easily remediated by oxidation with ozone or hydrogen peroxide. In both processes (biodegradation or oxidation), formaldehyde is converted to formic acid, which is also considered as a highly biodegradable and with a low environmental impact. Leal et al. (2016) also recommend the use of advanced oxidation processes (AOPs) (e.g. UV/H₂O₂, photo-Fenton) for the pre-treatment of high concentration of formaldehyde solutions. Thus, in aqueous solution formaldehyde seems to have a relatively low environmental impact and the main concerns are related to the amount (concentration) of the substance released into the environment. As it is also explained by Leal et al (2016), the FDA recommendation for formaldehyde release into environment should not exceed 1 mg l⁻¹ and also other specific limit values range between 0.74 and 2.4 mg l⁻¹.

In copper sulphate treatments, copper levels and total amount released into the environment should be evaluated. Heavy metals are considered harmful to the environment. Some of them present a high toxicity at low levels, whereas others are toxic only if taken in excess. This could be the situation of copper, that is a naturally occurring element present in terrestrial and aquatic environments and it is also essential for humans, animals and plants. Thus, the environmental concernedness related to the use of copper sulphate should be related to the amount of this molecule used and the possibilities for fast and wide dilution into the environment.

Other substances like diflubenzuron, azamethiphos or permethrins are in fact pesticides very similar to other pesticides used in agriculture and for the control of terrestrial animal pests. Their environmental impact in the aquatic environment requires a joint analysis, as their use in both (agriculture/aquaculture) cases can end up in the aquatic ecosystems, mainly in the local invertebrate fauna.

The treatment method should also be considered in the evaluation of the environmental impact. Differences in pollution volume between bath with chemicals vs oral administration of chemicals/natural compounds are also extremely apparent (Table 13). As can be calculated, a typical 2-month summer treatment regimen against a *S. chrysophii* outbreak in gilthead sea bream may easily result in the release of several formalin tons in the environment. On the other hand, several kg of active chemical compound/s are released when an oral medication schedule is applied, whereas administration of natural compounds/functional feeds are relatively harmless to the environment. However, the efficacy of formalin treatment still cannot be reached by oral administration of any compound (chemicals/non-chemicals) against this particular parasite, and until an alternative method to formalin is developed, modified treatment strategies should be adopted such as less frequent formalin treatments in combination with feed-administered antiparasitic compounds.

Table 13. Environmental pollution schemes from different treatment approaches against *S. chrysophii* in gilthead sea bream; hypothetical therapy model of 20,000 m³ total net volume /100,000 kg fish, during 60 d in summer

Treatment type	Dosing schedule	Conditions	Effectiveness	Issues	Released amount
Formalin bath	Six treatments (200 ppm/1 hour)	10,000 m ³ total treated volume	High	Weather dependent/very laborious	12 tons of formalin
Medicated feed	Four 10 d treatments (10 mg drug /kg fish/day)	100 tons treated fish biomass	Unknown-low	Fish anorexia & palatability of diet/lack of registered compounds	40 kg drug delivered; <10 kg released in parent & active metabolized forms
Functional feed	Daily (0.3 % of diet, 60 days)	100 tons treated fish biomass	Low in small lab scale, higher in field	Fish anorexia	6 kg Harmless

*hypothetical calculation due to uneaten feed, leaching, undigested & metabolized drug- based on information from other fish antimicrobials

11. Conclusions & recommendations

Amongst the results obtained in this evaluation we would like to stress the evidence that only a very low number of substances or commercial products are fully available in Mediterranean Aquaculture. There is a high urgency for effective new chemicals as an alternative to the few available substances as some of them (formalin) can be banned in the future. As it is described in this document, PerformFISH has also made his contribution approaching the knowledge of two relevant antiparasitic molecules (Mebendazole and Toltrazuril) through a pharmacokinetic study. However, this effort should be complemented in the future with further studies on efficacy and safety at laboratory but also at field scale. This could also be tested in WP6.

Finally, we would like to highlight the relevance of the integrated control strategies for parasitic diseases. There is still a lot of room for improvement, refinement and reduction of the amount of product to be used and using the synergic effect of combined management/therapeutic strategies can help to achieve the main target of the control of the parasitic diseases in the Mediterranean farming.

List of Tables

Table 1. Important bacterial diseases of European sea bass and gilthead sea bream .8	
Table 2. Registered antibacterials for animal farming/aquaculture in EU/Mediterranean countries.....14	14
Table 3. Properties of important antibacterial groupsfor animal farming/aquaculture in EU/Mediterranean countries.....18	18

Table 4. Selected pharmacokinetics of antibacterials in European sea bass and gilthead sea bream.....	37
Table 5. MICs values of several antibiotics recorded on bacterial pathogens from European sea bass and gilthead sea bream and other marine fish species available in international literature and also obtained from PerformFISH	42
Table 6. Main ectoparasites found in farmed gilthead sea bream (GSB) and European sea bass (ESB). Impact is related to the prevalence, frequency of the parasitosis, its pathological effect and economic costs in aquaculture. VH= very high (highlighted in green), H= high (highlighted in orange), M= moderate, L = low, O = Occasional U= Unclear	76
Table 7. Main endoparasites found in farmed gilthead sea bream (GSB) and European sea bass (ESB). Impact is related to the frequency of the parasitosis, its pathological effect and economic costs in aquaculture. VH= very high (highlighted in green), H= high (highlighted in orange), M= moderate, L = low, O = Occasional. U = Unclear	77
Table 8. List of antiparasitic substances against parasites of European sea bass and gilthead sea bream under EMA consideration for use in fish and commercial products (medicines) specifically licenced for salmonids and/or European sea bass and gilthead seabream	81
Table 9. Chemical substances experimentally used against parasites of European sea bass, gilthead sea bream and other sparids	82
Table 10. Advantages and disadvantages of different bath applications	88
Table 11. Suggested doses and exposure times of formalin according to rearing systems.....	91
Table 12. Alternative/natural compounds used against parasites of European sea bass & gilthead sea bream and other sparids.....	97
Table 13. Environmental pollution schemes from different treatment approaches against <i>S. chrysophii</i> in gilthead sea bream; hypothetical therapy model of 20,000 m ³ total net volume /100,000 kg fish, during 60 d in summer	107

List of Figures

Figure 1: Simulation of the evolution of the daily mortality (scale in the left side, number of dead fish collected perday) 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 <i>P. damsela</i> subsp. <i>piscicida</i> or <i>V. anguillarum</i>	25
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).	27
Figure 3: Early administration strategy: high antibiotic coverage is maintained during all the outbreak.....	28
Figure 4: Late administration strategy: lower and more variable antibiotic coverage during the outbreak. This is the most frequent situation found in the field.....	29
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.....	29

Figure 6: Pharmacokinetic/Pharmacodynamic parameters affecting antibiotic potency57

Figure 7: Environmental fate of orally administered antibacterials (OTC) in euryhaline fish farming sites; adapted and modified from Rigos and Troisi (2005)72

Figure 8: PerformFISH contribution to fighting parasites80

ANNEX I Pharmacokinetics of Oral Lincomycin in European sea bass (*Dicentrarchus labrax*)

1. Introduction

Lincomycin (LCM) is a lincosamide natural antibacterial drug obtained from *Streptomyces linconensis*. It interferes with the protein synthesis binding to the 50s ribosomal subunit at the same place that phenicols and macrolide drugs bind. It is active mostly against gram-positive bacteria and has limited used against gram-negative bacteria (anaerobic)(Papich, 2016). Lincosamides are primarily used in pet animals to treat gram-positive bacterial infections and can be either bacteriostatic or bactericidal, depending on the antibiotic concentration and the susceptibility of the organism. It has been also used in swine, cattle and sheep (Papich, 2016). No data about the pharmacokinetics of this drug in fish has been found however in the accessible literature.

LCM is not included in the lists of antimicrobials critically important and with highest priority according WHO (2017). These drugs may be eventually banned for use in food-producing animals. The aforementioned fact explained why LCM was explored in Performfish.

2. Materials and methods

2.1. Fish

The pharmacokinetic trial was carried out at the facilities of HCMR in Athens. There, fifty healthy European sea bass averaging 93 g were separated in 3 cylindrical fiber glass tanks (800 L), receiving open flow sea water (38 psu) at a temperature of 23°C with aeration. Oxygen content was kept close to saturation by bubbling air through air stones and the photoperiod was kept at 12 h dark–12 h light. Fish were acclimatized for 2 weeks before the administration of medicated feed.

2.2. Medicated feed & distribution

During acclimatization fish received a conventional extruded diet (Table 1) which contained 42% protein and 18% fat. The diet was prepared locally. Hand feeding was performed once a day at 12.00 hours.

Table 1. Composition of medicated diet

Ingredients	Moisture	Protein	Fat	Fiber	Starch	Ash	Energy
Fishmeal 70 LT FF Skagen	6.8	69.2	10.2	0.0	0.0	12.6	20.4
Wheat meal	8.0	10.2	1.6	2.3	60.6	1.5	15.8
Wheat Gluten	5.5	79.5	6.1	0.0	7.4	0.8	22.3

Corn gluten	10.9	60.0	3.8	1.5	13.6	1.0	21.3
Soybean meal 48	9.2	46.3	2.2	3.4	4.7	6.1	17.4
Soy protein concentrate (Soycomil)	7.9	62.7	0.2	3.5	0.0	6.5	18.6
Fishoil - COPPENS	2.0	0.0	98.0	0.0	0.0	0.0	39.0
MCP	2.6	0.0	0.0	0.0	0.0	30.0	0.0
L-Lysine	1.0	95.0	0.0	0.0	0.0	0.0	0.0
DL-Methionine	1.0	58.0	0.0	0.0	0.0	0.0	0.0
LINCOMICIN							

Following acclimatization, an extruded medicated diet was delivered to the experimental fish at the same as above feeding conditions. The drug as incorporated to the pellets during oil coating. Medicated feed with LCM was given for 5 consecutive days at a concentration of 100mg/kg fish (2% BW daily). Medicated diet was readily consumed by the fish with no signs of palatability issues.

2.3. Blood sampling and sample preparation

Sampling was set on days 1,3 and 5 during medication. The time points per sampling day were set at 2, 4, 8 and 23h. Five fish per sampling point were used. Approximately 1 ml of blood was taken from the caudal vein of anesthetized fish (clove oil) with Microlance 23G needles and immediately stored (24 h) at 4°C until plasma preparation. Plasma was isolated from the heparin-treated blood samples by centrifugation at 4000 rpm for 10 min and stored at 20°C. Plasma samples were shipped to UoB in dry ice for chromatographical analysis of DXC.

2.4. Analytical method

The analytical method consisted of a liquid-liquid extraction and a subsequent separation of the analyte by the means of High Performance Liquid Chromatography with QQQ-MS. The quantifying method consisted of interpolation along a regression line constructed from the results of a calibration curve (LCM concentration vs LCM Area). The quantifying procedure was performed automatically by the HPLC software. Each day of analysis, a calibration curve and a quality control samples set were analyzed with the samples.

Reagents

- Lincomycin Hydrochloride (LCM-HCl) 95.6% purity: European Pharmacopoeia, ref. L0650000.
- Methanol (Fisher Scientific, ref. M/4056/17)
- Formic Acid for LC/MS (Fisher Scientific, ref. A117-50)
- Water Milli Q

Equipment

- Analytical Scales: KERN AEJ 120-4M / Sartorius BP100S
- Refrigerator 4 °C / Freezer -20 °C: Fagor / Rommer 460 HC
- Lab extractor hood: CaptairFiltair 936
- Vortex: Reax Heidolph
- Multivortex Mixer: VX-2500 VWR International
- Magnetic stirrer: Stuart SB161
- Centrifuge: Heraeus FRESCO 200
- Micropipettes: Gilson Pipetman F5, F10, and F25 /
Rainin 100 µL and 1 mL
- Water Purification System: Millipore Milli-RX45
- HPLC:
 - Separation Module: Agilent 1290 series
 - Thermostated Autosampler: Agilent 1290 series
 - Column Oven: Agilent 1290 series
 - MS-QQQ: Agilent 6420
 - Ionization source: ESI
 - Personal Computer: HP
 - Software: Agilent Mass-Hunter

Other Material

- Polypropylene Eppendorf 1.5 mL Tubes (Sudelab, ref. 175508)
- HPLC Polypropylene Vials 2 mL with insert, and caps (Akady, ref. 8136 and ref. 3839)
- Pipette Tips 200 µL and 1 mL (Sudelab, ref. 200016 and ref. 162222X)
- Volumetric Flasks 100 mL (Afora)
- Chromatographic Column C₁₈Zorbax Eclipse Plus 1.8 µm 5 x 0.21 cm (Agilent)

Solutions

The LCM standard solutions were prepared as follows:

LCM primary solution (0.1 mg/mL): LCM standard (0.01 g 100% purity) was placed in a 100 mL glass volumetric flask and 50 mL of Milli Q water were added. The flask was then placed in an ultrasonic bath for 20 sec, and the volume completed up to 100 mL with Milli Q water. The primary solution was aliquoted and conserved at -20°C, approximately.

LCM secondary solutions (0.01 and 0.001 mg/mL): Solution 0.01 mg/mL: A volume of 100 microliter (100 µL) of LCM primary solution was diluted with Milli Q water up to a

D3.3-Therapeutics for MMFF

final volume of 1 mL. Solution 0.001 mg/mL: A volume of 100 µL of LCM 0.01 mg/mL solution was diluted with Milli Q water up to a final volume of 1 mL.

Other solutions

Formic Acid 0.1%: 1 ml of Formic acid was dissolved in 1 L of Milli Q water.

Methanol with 0.1% of formic acid: 1 ml of Formic acid was dissolved in 1 L of Methanol.

Blank Samples

Blank samples were obtained from non-treated animals of European seabass. They were kept frozen at -20°C (± 5°C) until analysis, and the presence of interferences was checked before use.

Sample Extraction Procedure

A volume of 50 µL of plasma was placed in a 1.5 mL Eppendorf tube and the corresponding volume of standard solution was added, when applicable. After adding 200 µL of methanol, the samples were multivortexed for 5 min and then centrifuged at 14,800 r.p.m. for 5 min at 4°C. The upper phase was transferred to a clean polypropylene HPLC vial with insert for chromatographic analysis.

Calibration Curves preparation

The calibration curves were constructed with blank plasma spiked with increasing LCM concentrations, as follows:

SAMPLE IDENTIFICATION	LCM CONC. (µg/mL)	VOLUME OF LCM SOLUTION IN 100 µL PLASMA
A	0.1	5 µL 0.001 mg/mL
B	0.2	10 µL 0.001 mg/mL
C	0.5	25 µL 0.001 mg/mL
D	1.0	5 µL 0.01 mg/mL
E	2.0	10 µL 0.01 mg/mL
F	5.0	25 µL 0.01 mg/mL
G	10.0	5 µL 0.1 mg/mL
H	20.0	10 µL 0.1 mg/mL

Chromatographic conditions

- HPLC System: Agilent 1206
- Software: AgilentMassHunter
- Mobile Phase: Milli Q water (Formic acid 0.1%):Methanol (0.1% Formic acid) in a gradient of solvents
- Stationary Phase: Column C₁₈ 5 µm (20 x 0.46 cm)
- Flow rate: 1.5 mL/min
- Column temperature: 50 °C
- Samples temperature: 10°C

- Injection Volume: 1 μ L
- Chromatogram time: 8 min

QQQ-MS Detection conditions

- Ionization source: ESI
- Ionization polarity: Positive
- Precursor ion: 407.2 uma
- Quantifyer transition: 126.1 (Dwell 100, Fr 150, CE 29, CAV 4)
- Qualifyer transition: 70.1 (Dwell 100, Fr 150, CE 89, CAV 4)

3. Results

3.1. Validation results

The intraday and interday accuracy results were between the range of -30 and 15% and the precision values were lower than 20% along the concentrations of the calibration curves. The lower limit of quantification (LLOQ) and the lower limit of detection (LLOD) were established at 0.1 μ g/ml and 0.02 μ g/ml respectively. The calibration curve showed to be linear between a 0.1 to 20 μ g/ml range. The LCM showed to be stable at storing conditions (-20 $^{\circ}$) at least for 30 days without any degradation.

3.2. PK Study results

Table 2 shows the plasma concentration results of LCM in European sea bass. Each result is expressed as a mean of 5 samples from 5 different animals. As it can be seen the LCM is rapidly absorbed and significant concentrations remain in plasma up to 23h post treatment. High concentrations were observed with peak concentration situated near the 4-8 h after treatment.

Table 2. Plasma concentration results of LCM in European sea bass

Time (h)	Plasma concentration (μ g/ml)					
	Day 1		Day 2		Day 3	
	Mean	SD	Mean	SD	Mean	SD
2	4.0	2.1	5.4	2.8	4.5	1.7
4	6.0	2.6	8.6	4.9	8.3	0.9
8	12.5	6.4	10.4	6.3	6.9	1.5
23	1.9	1.4	2.3	1.8	2.2	0.4

Figure 1 depicts the mean pharmacokinetic profile of LCM in the European sea bass. We do not observe any accumulating factor during the 5 days of treatment. In addition, concentrations higher than 1 μ g/ml were observed in all analyzed samples exceeding this level during all the treatment.

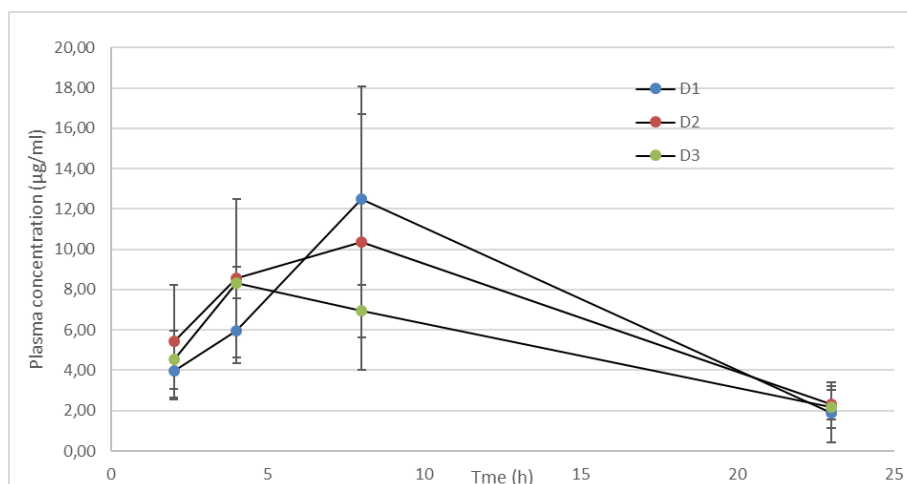


Figure 1. Plasma-concentration profile of LCM in European sea bass

4. Discussion

LCM in this study displayed a promising distribution profile in European sea bass plasma following a 5-day oral administration of 100mg/kg fish. Interestingly, the highest concentration reached values as high as 13 µg/ml reflecting an extremely high absorption in the circulation of European sea bass when compared with other registered (oxytetracycline, enrofloxacin & thiamphenicol) (Intorre, Cecchini, Bertini, Cognetti Varriale, Soldani, Mengozzi, 2000; Intorre, Castells, CristoFol, Bertini, Soldani, Arboix, 2002; Rigos, Nengas, Athanassopoulou, Alexis, 2004b) and newly evaluated antibacterials in PerformFISH (PerformFISH Technical docs). Promising PKs of LCM have been demonstrated also in livestock production animals (Meemansha, Dumka, 2018; Sreeshitha Gouri, Venkatachalam, Dumka, 2014). The LCM plasma levels remained relatively high during the 24h cycle confirming one medicated meal per treatment day. This is a desirable pattern since the $T_c > MIC$ ratio has been suggested as the most appropriate for lincosamides.

ANNEX II Pharmacokinetics of Oral Doxycycline in European sea bass (*Dicentrarchus labrax*)

1. Introduction

Doxycycline (DXC) is a semi-synthetic tetracycline antibiotic, derived from oxytetracycline. It has wide use in pet animals where may be administered orally or intravenously. Tetracycline antibiotics are broad-spectrum and bacteriostatic. Their mechanism of action is through the inhibition of protein synthesis, and the alteration of cytoplasmic membrane permeability within the susceptible organism. Interestingly, doxycycline is more lipid-soluble than other tetracycline antibiotics an indication that it can penetrate more easily biological membranes and may reach higher levels in some of the difficult to penetrate tissues. Based on the above, DXC can be a promising alternative to oxytetracycline which is widely used in aquaculture with contradicting results. It has been used empirically in fish farming (pangasius) in some southeast Asian countries but very few scientific information is available about its use in aquaculture conditions. Finally, DXC is not included in the lists of antimicrobials critically important and with highest priority according WHO (2017). These drugs may be eventually banned for use in food-producing animals. The aforementioned facts explained why DXC was explored in Performfish.

2. Materials and methods

2.1. Fish

The pharmacokinetic trial was carried out at the facilities of HCMR in Athens. There, fifty healthy European sea bass averaging 122 g were separated in 3 cylindrical fiber glass tanks (800 L), receiving open flow sea water (38 psu) at a temperature of 22°C with aeration. Oxygen content was kept close to saturation by bubbling air through air stones and the photoperiod was kept at 12 h dark–12 h light. Fish were acclimatized for 2 weeks before the administration of medicated feed.

2.2. Medicated feed & distribution

During acclimatization fish received a conventional extruded diet (Table 1) which contained 42% protein and 18% fat. The diet was prepared locally. Hand feeding was performed once a day at 12.00 hours.

Table 1. composition of medicated diet

Ingredients	Moisture	Protein	Fat	Fiber	Starch	Ash	Energy
	%	%	%	%	%	%	MJ/kg
Fishmeal 70 LT FF Skagen	6.8	69.2	10.2	0.0	0.0	12.6	20.4
Wheat meal	8.0	10.2	1.6	2.3	60.6	1.5	15.8
Wheat Gluten	5.5	79.5	6.1	0.0	7.4	0.8	22.3
Corn gluten	10.9	60.0	3.8	1.5	13.6	1.0	21.3

Soybean meal 48	9.2	46.3	2.2	3.4	4.7	6.1	17.4
Soy protein concentrate (Soycomil)	7.9	62.7	0.2	3.5	0.0	6.5	18.6
Fishoil - COPPENS	2.0	0.0	98.0	0.0	0.0	0.0	39.0
MCP	2.6	0.0	0.0	0.0	0.0	30.0	0.0
L-Lysine	1.0	95.0	0.0	0.0	0.0	0.0	0.0
DL-Methionine	1.0	58.0	0.0	0.0	0.0	0.0	0.0
DOXYCYCLINE							

Following acclimatization, an extruded medicated diet was delivered to the experimental fish at the same as above feeding conditions. The drug as incorporated to the pellets during oil coating. Medicated feed with DXC 15% (Tetoros 150) was given for 5 consecutive days at a concentration of 100 mg/kg fish (2% BW daily). Medicated diet was readily consumed by the fish with no signs of palatability issues.

2.3. Blood sampling and sample preparation

Sampling was set on days 1,3 and 5 during medication. The time points per sampling day were set at 2, 4, 8 and 23h. Five fish per sampling point were used. Approximately 1 mL of blood was taken from the caudal vein of anesthetized fish (clove oil) with Microlance 23G needles and immediately stored (24 h) at 4°C until plasma preparation. Plasma was isolated from the heparin-treated blood samples by centrifugation at 4000 rpm for 10 min and stored at 20°C. Plasma samples were shipped to UoB in dry ice for chromatographical analysis of DXC.

2.4. Analytical method

The analytical method consisted of a liquid-liquid extraction and a subsequent separation of the analyte by means of High Performance Liquid Chromatography with UV-Vis detection. The quantifying method consisted of interpolation along a regression line constructed from the results of a calibration curve (DXC concentration vs DXC Area). The quantifying procedure was performed automatically by the HPLC software. Each day of analysis, a calibration curve and a quality control samples set were analyzed with the samples.

Materials

Doxycycline hydrochloride (98.3% purity) from Sigma-Aldrich was used as reference standard.

Reagents

- Acetonitrile (VWR Chemicals, ref. 83639.320)
- Oxalic acid (Scharlau, ref. AC17200500)
- Ethylenediaminetetraacetic acid (EDTA) (Scharlau, ref. AC0963)
- Perchloric acid 60% (Fisher Scientific, ref. P/1290/PB05)
- Distilled water

Equipment

- Analytical Scales: KERN AEJ 120-4M / Sartorius BP100S

D3.3-Therapeutics for MMFF

- Refrigerator 4 °C / Freezer -20 °C: Fagor / Rommer 460 HC
- Ultrasonic bath: Branson 2510
- Lab extractor hood: CaptairFiltair 936
- Vortex: Reax Heidolph
- Multivortex Mixer: VX-2500 VWR International
- Magnetic stirrer: Stuart SB161
- Vacuum Pump: Büchi Vac V500
- Centrifuge: Heraeus FRESCO 200
- Micropipettes: Gilson Pipetman F5, F10, and F25 / Rainin 100 µL and 1 mL
- Water Purification System: Millipore Milli-RX45
- HPLC HP 1100 Series:
 - Quaternary Pump: G1311A
 - UV Detector: G1315A
 - Column Oven: G1316A
 - Autosampler and Thermostat: G1329A and G1330A
 - Degasser: G1322A
 - Personal Computer: LG
 - Software: OpenLABEZChrom A.02.02(1.3.4)-version

Other Materials

- Polypropylene Eppendorf 1.5 mL Tubes (Sudelab, ref. 175508)
- HPLC Polypropylene Vials 2 mL with insert, and caps (Akady, ref. 8136 and ref. 3839)
- Pipette Tips 200 µL and 1 mL (Sudelab, ref. 200016 and ref. 162222X)
- Volumetric Flasks 100 mL (Afora)
- Sterile Membrane Filters 0.45 µm (Pall Corporation, ref. GN-6 Grid)
- Mobile Phase Filter System (Fisher Scientific, ref. 5810/3)
- Chromatographic Column C₈Spherisorb 5 µm 20 x 0.46 cm (Waters, ref. PSS839534)

Solutions

The DXC Working Standard Solutions were prepared as follows.

DXC primary solution (0.1 mg/mL):DXC standard (0.0110 g 98.3% purity) was placed in a 100 mL glass volumetric flask and 50 mL of oxalic acid 2.53 g/L solution were added. The flask was then placed in an ultrasonic bath for 20 sec, and the volume completed up to 100 mL with oxalic acid 2.53 g/L solution. The primary solution was aliquoted and conserved at -20 °C, approximately.

DXC secondary solutions (0.01 and 0.001 mg/mL): Solution 0.01 mg/mL: A volume of 100 microliter (100 µL) of DXC primary solution was diluted with distilled water up to a final volume of 1 mL.Solution 0.001 mg/mL: A volume of 100 µL of DXC 0.01 mg/mL solution was diluted with distilled water up to a final volume of 1 mL.

Other solutions

Oxalic acid 2.53 g/L solution

Oxalic acid (2.53 g) was dissolved with filtered distilled water up to a final volume of 1 L.

EDTA 5 g/L solution

Ethylenediaminetetraacetic acid (EDTA, 0.5 g) was dissolved with distilled water up to a final volume of 100 mL.

Blank Samples

Blank samples were obtained from non-treated animals of European seabass. They were kept frozen at -20 °C (\pm 5 °C) until analysis, and the presence of interferences was checked before use.

Sample Extraction Procedure

A volume of 100 μ L of plasma was placed in a 1.5 mL Eppendorf tube and the corresponding volume of standard solution was added, when applicable. After adding 50 μ L of EDTA solution, the samples were let at room temperature for 2 min, and then approximately 10 μ L of perchloric acid was added. The samples were multivortexed for 2 min and then centrifuged at 14,000 r.p.m. for 10 min at 4 °C. The upper phase was transferred to a polypropylene HPLC vial with insert for chromatographic analysis.

Calibration Curves preparation

The calibration curves were constructed with blank plasma spiked with increasing DXC concentrations, as follows:

SAMPLE IDENTIFICATION	DXC CONC. (μ g/mL)	VOLUME OF DXC SOLUTION IN 100 μ L PLASMA
A	0.10	10 μ L 0.001 mg/mL
B	0.50	5 μ L 0.01 mg/mL
C	1.00	10 μ L 0.01 mg/mL
D	2.50	25 μ L 0.01 mg/mL
E	5.00	5 μ L 0.1 mg/mL
F	10.00	10 μ L 0.1 mg/mL

Chromatographic conditions

- HPLC System: Agilent HP 1100 Series
- Software: OpenLAB EZChrom A.02.02(1.3.4)
- Mobile Phase: Oxalic acid 2.53 g/L :Acetonitrile 50:50
- Stationary Phase: Column C₈ 5 μ m (20 x 0.46 cm)
- Flow rate: 1.5 mL/min
- UV-Vis Detection: Wavelength of 346 nm
- Column temperature: 50 °C
- Samples temperature: 4 °C
- Injection Volume: 25 μ L
- Chromatogram time: 4 min

3. Results

3.1. Validation results

The intraday and interday accuracy results were between the range of -30 and 15% and the precision values were lower than 20% along the concentrations of the calibration curves. The lower limit of quantification (LLOQ) and the lower limit of

detection (LLOD) were established at 0.1 µg/ml and 0.0351 µg/ml respectively. The calibration curve showed to be linear between a 0.1 to 10 µg/ml range. The DXC showed to be stable at storing conditions (-20°) at least for 30 days without any degradation.

3.2. PK Study results

Table 2 show the plasma concentration results of DXC in European sea bass. Each result is expressed as a mean of 5 samples from 5 different animals. As it can be seen the DXC is rapidly absorbed and a certain accumulation factor can be detected along the two days of treatment.

Table 2. Plasma concentration results of DXC in European sea bass.

Time (h)	Concentration	
	µg/ml	SD
2	0.126	0.031
4	0.374	0.200
8	0.521	0.078
23	0.455	0.110
26	0.498	0.110
28	0.431	0.118
36	0.565	0.119
47	0.714	0.360

Figure 1 depicts the mean pharmacokinetic profile of DXC in European sea bass. As it can be seen a high level of variability can be observed in each time point. This variability is due to the fact that each time point is a result of the mean of 5 animals. Also we have to take in account that such variabilities are habitual when the treatment is given to the animals mixed in feed. In this case the consumed dose is not the same for all the animals and, also, they do not eat at the same time. So the dose schedule, itself, show high variability.

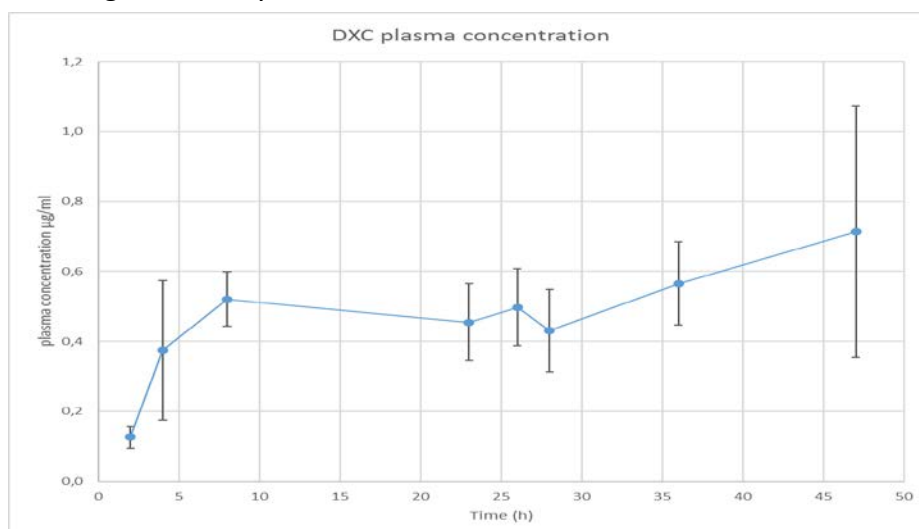


Figure 1: Plasma concentration profile of DXC in European sea bass after administration of DXC in feed

4. Discussion

DXC absorption was measured to be relatively low in this trial which is in agreement with other tetracycline trials in euryhaline species (Rigos et al. 2003,2004). Moreover, its absorption profile appeared to be slow since no dropping effect was evident during a 24h period. This pattern possible suggests a sequential dosing of DXC in European sea bass (perhaps 3 dosings in 5 day period).

Concerning PK/PD integration, tetracyclines are bacteriostatic drugs with co-dependent action (mostly concentration-dependent) thus, the AUC_{0-24}/MIC index would be the most appropriate . For DXC, the AUC/MIC_{90} was calculated to be more than 20 for the first days of treatment based on relatively low MIC values of important bacterial pathogens (produced also in PFF). These promising in vitro values of DOX are reflected in lab trials where the compounds appeared very effective when orally delivered against repeated incidences of heavy *V. harveyi* infections in European sea bass juveniles (Rigos et al., lab observations). Additional large-scale trials of DCS against important bacterial pathogens of European sea bass remain to confirm then above conclusions.

ANNEX III Pharmacokinetics of Oral Spectinomycin in European sea bass (*Dicentrarchus labrax*)

1. Introduction

Spectinomycin (SPT) is an aminocyclitol closely related to the aminoglycoside antibiotic group. SPT is a broad-spectrum drug produced by *Streptomyces spectabilis* and is active against gram-negative bacteria. It binds to the 30s ribosomal subunit of the bacteria inhibiting the protein synthesis. The compound is not registered for use in Mediterranean farmed finfish but seems to be lightly used in the past in combination with lincomycin in Norway (Norwegian Veterinary Institute 2016). SPT is currently entered into Annex I of Council Regulation (EEC) No. 2377/90 and has given an MRL of 200-5000 µg/kg depending on the tissue of food-producing animals.

Finally, SPT is not included in the lists of antimicrobials critically important and with highest priority according WHO (2017). These drugs may be eventually banned for use in food-producing animals. Moreover, the compound has shown promising PKs in other farmed animals (Abu-Basha et al. 2007). The aforementioned facts explained why SPT was explored in Performfish.

2. Materials and methods

2.1. Fish

The pharmacokinetic trial was carried out at the facilities of HCMR in Athens. There, fifty healthy European sea bass averaging 152 g were separated in 3 cylindrical fiber glass tanks (800 L), receiving open flow sea water (38 psu) at a temperature of 26°C with aeration. Oxygen content was kept close to saturation by bubbling air through air stones and the photoperiod was kept at 12 h dark–12 h light. Fish were acclimatized for 2 weeks before the administration of medicated feed.

2.2. Medicated feed & distribution

During acclimatization fish received a conventional extruded diet (Table 1) which contained 42% protein and 18% fat. The diet was prepared locally. Hand feeding was performed once a day at 12.00 hours.

Table 1. Composition of medicated diet

Ingredients	Moisture	Protein	Fat	Fiber	Starch	Ash	Energy
	%	%	%	%	%	%	MJ/kg
Fishmeal 70 LT FF Skagen	6.8	69.2	10.2	0.0	0.0	12.6	20.4
Wheat meal	8.0	10.2	1.6	2.3	60.6	1.5	15.8
Wheat Gluten	5.5	79.5	6.1	0.0	7.4	0.8	22.3
Corn gluten	10.9	60.0	3.8	1.5	13.6	1.0	21.3
Soybean meal 48	9.2	46.3	2.2	3.4	4.7	6.1	17.4

Soy protein concentrate (Soycomil)	7.9	62.7	0.2	3.5	0.0	6.5	18.6
Fishoil- COPPENS	2.0	0.0	98.0	0.0	0.0	0.0	39.0
MCP	2.6	0.0	0.0	0.0	0.0	30.0	0.0
L-Lysine	1.0	95.0	0.0	0.0	0.0	0.0	0.0
DL-Methionine	1.0	58.0	0.0	0.0	0.0	0.0	0.0
SPECTINOMYCIN							

Following acclimatization, an extruded medicated diet was delivered to the experimental fish at the same as above feeding conditions. The drug as incorporated to the pellets during oil coating. Medicated feed with SPT (ESPECTINOCEN 50%) was given for 5 consecutive days at a concentration of 50mg/kg fish (2% BW daily). Medicated diet was readily consumed by the fish with no signs of palatability issues.

2.3. Blood sampling and sample preparation

Sampling was set on days 1,3 and 5 during medication. The time points per sampling day were set at 2, 4, 8 and 23h. Five fish per sampling point were used. Approximately 1 ml of blood was taken from the caudal vein of anesthetized fish (clove oil) with Microlance 23G needles and immediately stored (24h) at 4oC until plasma preparation. Plasma was isolated from the heparin-treated blood samples by centrifugation at 4000 rpm for 10 min and stored at 20°C. Plasma samples were shipped to UoB in dry ice for chromatographical analysis of SPT.

2.4. Analytical method

The analytical method consisted of a liquid-liquid extraction and a subsequent separation of the analyte by means of High Performance Liquid Chromatography with QQQ-MS. The quantifying method consisted of interpolation along a regression line constructed from the results of a calibration curve (SPT concentration vs SPT Area). The quantifying procedure was performed automatically by the HPLC software. Each day of analysis, a calibration curve and a quality control samples set were analyzed with the samples.

Reagents

- Spectinomycin Hydrochloride pentahydrate: Fluka, ref. 85555.
- Methanol (Fisher Scientific, ref. M/4056/17)
- Formic Acid for LC/MS (Fisher Scientific, ref. A117-50)
- Water Milli Q

Equipment

- Analytical Scales: KERN AEJ 120-4M / Sartorius BP100S
- Refrigerator 4 °C / Freezer -20 °C: Fagor / Rommer 460 HC
- Lab extractor hood: CaptairFiltair 936

D3.3-Therapeutics for MMFF

- | | |
|-------------------------------|--|
| • Vortex: | Reax Heidolph |
| • Multivortex Mixer: | VX-2500 VWR International |
| • Magnetic stirrer: | Stuart SB161 |
| • Centrifuge: | Heraeus FRESCO 200 |
| • Micropipettes: | Gilson Pipetman F5, F10,
and F25 / Rainin 100 µL and 1 mL |
| • Water Purification System: | Millipore Milli-RX45 |
| • HPLC: | |
| • Separation Module: | Agilent 1290 series |
| • Thermostatized Autosampler: | Agilent 1290 series |
| • Column Oven: | Agilent 1290 series |
| • MS-QQQ: | Agilent 6420 |
| • Ionization source: | ESI |
| • Personal Computer: | HP |
| • Software: | Agilent Mass-Hunter |

Other Materials

- Polypropylene Eppendorf 1.5 mL Tubes (Sudelab, ref. 175508)
- HPLC Polypropylene Vials 2 mL with insert, and caps (Akady, ref. 8136 and ref. 3839)
- Pipette Tips 200 µL and 1 mL (Sudelab, ref. 200016 and ref. 162222X)
- Volumetric Flasks 100 mL (Afora)
- Chromatographic Column Infinity Lab Poroshell HILIC-Z 2.7 µm 10 x 0.21 cm (Agilent)

Solutions

SPT primary solution (0.1 mg/mL)

SPT standard (0.0149 g) was placed in a 100 mL glass volumetric flask and 50 mL of Milli Q water were added. The flask was then placed in an ultrasonic bath for 20 sec, and the volume completed up to 100 mL with Milli Q water. The primary solution was aliquoted and conserved at -20 °C, approximately.

SPT secondary solutions for plasma (0.1, 0.01, 0.001 mg/mL): Solutions were obtained by serial 1/10 dilutions with Milli Q water.

SPT secondary solutions for Gastrointestinal contents. Solutions 0.1, 0.05, 0.025, 0.01, 0.005, 0.0025 and 0.001 mg/ml were prepared from SPT primary solution diluting with Milli Q water.

Other solutions

Formic Acid 0.1%: 1 ml of Formic acid was dissolved in 1 L of Milli Q water.

Methanol with 0.1% of formic acid: 1 ml of Formic acid was dissolved in 1 L of Methanol.

Blank Samples

Blank samples were obtained from non-treated animals of European seabass). They were kept frozen at -20 °C (± 5 °C) until analysis, and the presence of interferences was checked before use.

Sample Extraction Procedure

Plasma. A volume of 50 μL of plasma was placed in a 1.5 mL Eppendorf tube and the corresponding volume of standard solution was added, when applicable. After adding 150 μL of methanol, the samples were multivortexed for 5 min and then centrifuged at 14,800 r.p.m. for 5 min at 4 $^{\circ}\text{C}$. The upper phase was transferred to a clean polypropylene HPLC vial with insert for chromatographic analysis.

Gastrointestinal contents. A volume of 50 μL of gastrointestinal content was placed in a 1.5 mL Eppendorf tube and 1 ml of MilliQ water or standard solution. The samples were multivortexed for 5 min and then centrifuged at 14,800 r.p.m. for 5 min at 4 $^{\circ}\text{C}$. The upper phase was transferred to a clean polypropylene HPLC vial with insert for chromatographic analysis.

Calibration Curves preparation

Plasma. The calibration curves were constructed with blank plasma spiked with increasing SPT concentrations, as follows:

SAMPLE IDENTIFICATION	SPT CONC. ($\mu\text{g}/\text{mL}$)	VOLUME OF SPT SOLUTION IN 100 μL PLASMA
A	0.10	5 μL 0.001 mg/mL
B	0.2	10 μL 0.001 mg/mL
C	0.5	25 μL 0.001 mg/mL
D	1	5 μL 0.01 mg/mL
E	2	10 μL 0.01 mg/mL
F	5	25 μL 0.01 mg/mL

Gastrointestinal content. The calibration curves were constructed with blank samples spiked with 1 ml of SPT standard solution, as follows:

SAMPLE IDENTIFICATION	SPT CONC. ($\mu\text{g}/\text{g}$)	SPT SOLUTION IN 50 μL sample
A	20	0.001 mg/mL
B	50	0.0025 mg/mL
C	100	0.005 mg/mL
D	200	0.01 mg/mL
E	500	0.025 mg/mL
F	1000	0.05 mg/mL
G	2000	0.1 mg/mL

Chromatographic conditions

- HPLC System: Agilent 1290
- Software: AgilentMassHunter
- Mobile Phase: Milli Q water (Formic acid 0.1%):Methanol (0.1% Formic acid) in a gradient of solvents
- Stationary Phase: Lab Poroshell HILIC-Z 2.7 μm 10 x 0.21 cm
- Flow rate: 0.2 mL/min
- Column temperature: 30 $^{\circ}\text{C}$

D3.3-Therapeutics for MMFF

- Samples temperature: 10°C
- Injection Volume: 1 µL
- Chromatogram time: 10 min

QQQ-MS Detection conditions

- Ionization source: ESI
- Ionization polarity: Positive
- Precursor ion: 351.2 uma
- Quantifyer transition: 33.2 (Dwell 300, Fr 170, CE 15, CAV 4)
- Qualifyer transition: 207.1 (Dwell 300, Fr 170, CE 18, CAV 4)

3. Results

3.1. Validation results

The intraday and interday accuracy results in both matrices were between the range of -30 and 15% and the precision values were lower than 20% along the concentrations of the calibration curves. The lower limit of quantification (LLOQ) and the lower limit of detection (LLOD) were established at 0.1 µg/ml and 0.02 µg/ml respectively. The calibration curve showed to be linear between a 0.1 to 5 µg/ml range in plasma and between 20 µg/ml and 2000 µg/ml in gastrointestinal content. The SPT showed to be stable at storing conditions (-20°C) at least for 30 days without any degradation.

3.2. PK Study results

Plasma

Table 2 show the plasma concentration results of SPT in European sea bass. Each result is expressed as a mean of 5 samples from 5 different animals. As it can be seen the SPT is rapidly absorbed and significant concentrations remain in plasma up to 23 h post treatment.

Table 2. Plasma concentration results of SPT in European sea bass

Time (h)	Plasma concentration (µg/ml)					
	Day 1		Day 2		Day 3	
	Mean	SD	Mean	SD	Mean	SD
2	0.85	0.17	0.80	0.37	0.54	0.38
4	0.85	0.12	0.92	0.44	1.16	0.51
8	1.01	0.43	1.35	0.39	1.26	0.51
23	0.12	0.07	0.08	0.06	0.04	0.05

Figure 1 depicts the mean pharmacokinetic profile of SPT in European sea bass. We do not observe any accumulating factor during the 5 days of treatment.

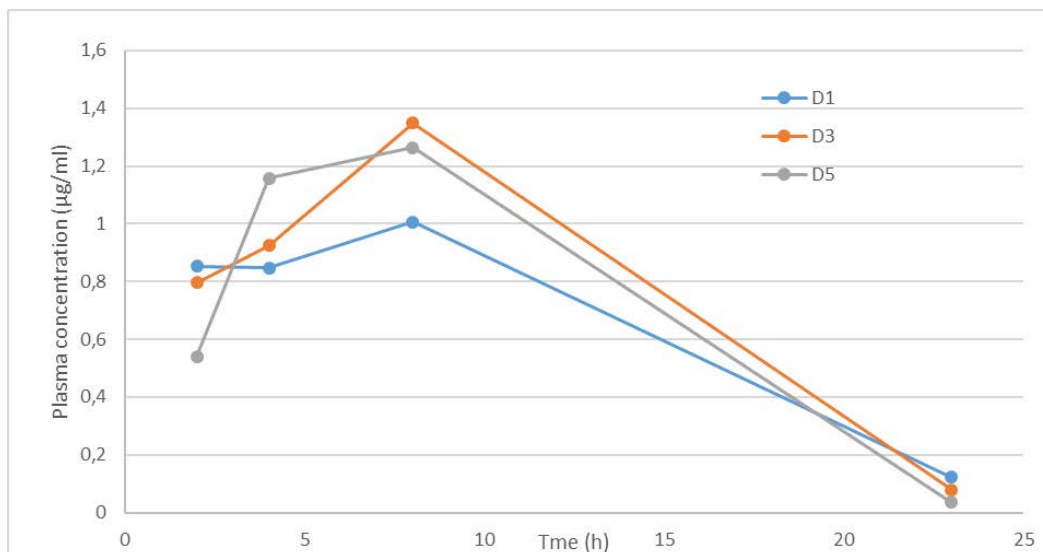


Figure 1: Plasma-concentration profile of SPT in European sea bass

Gastrointestinal content.

The concentrations of SPT in the gastrointestinal content were determined in treated animals in day 3 of treatment (23h post-administration) and during the 5th day of treatment. Figure 2 shows the SPT concentrations observed in the samples. As it can be seen extremely high concentrations were found.

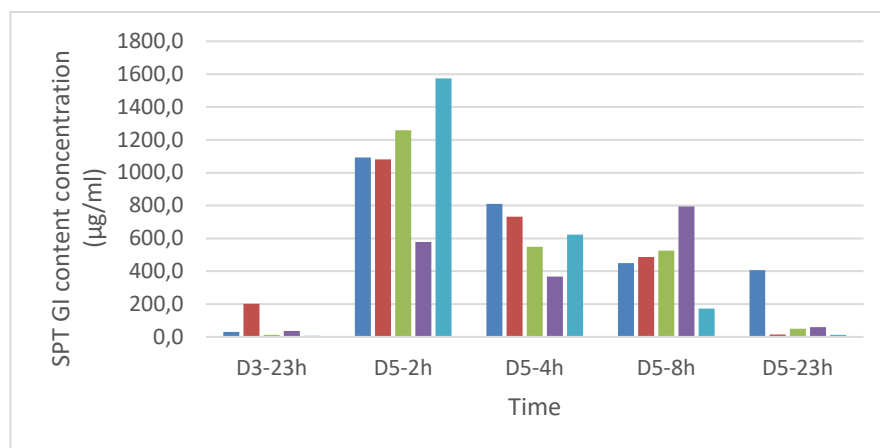


Figure 2. Gastrointestinal content concentrations of SPT in European sea bass.

4. Discussion

SPE in this study displayed an adequate distribution profile in European sea bass plasma following 5 day oral administration of 50mg/kg fish. The highest concentration reached values of 1.3mg/ml which is close to the concentrations obtained with other antibacterials including oxytetracycline (Rigos et al. 2004) but lower compared to what measured in lincomycin in the same species (PerformFISH). The Cmax/MIC ratio has been suggested as the most appropriate for aminocyclitols thus the MIC values against

D3.3-Therapeutics for MMFF

important bacterial pathogens of European sea bass will be necessary for the appropriate integration of PK/PD. The extremely high SPT concentrations measured in gastrointestinal content compared to the low concentrations in plasma can indicate low values of bioavailability of orally-administered SPT. The variability between individuals observed in the SPT concentration is a direct expression of the variability in the feed intake. Relatively low bioavailability has been demonstrated also in broilers (Abu-Basha et al. 2007).

ANNEX IV Pharmacokinetics of Oral Mebendazole in gilthead sea bream (*Sparus aurata*)

1. Introduction

Mebendazole (MBZ) is a benzimidazole anthelmintic widely used in human and veterinary medicine (and pet animals). In veterinary medicine, its recommended dosage when administered orally ranges from 8 to 15 mg/kgBW (EMA 2001). Mebendazole is used in human medicine for the treatment of intestinal nematodes and hydatidosis. Currently, mebendazole is included in Annex III of Council Regulation (EEC) No. 2377/90 and has given an MRL of 60 – 400 µg/kg depending on the tissue of producing animal (EMA 2001). Although there have been some in vitro and in vivo trials of MBZ against monogeneans and microsporidians (Buchmann, Bjerregaard, 1990; Kim et al. 1998; Schmahl et al. 1998), its absorption has never been investigated in farmed fish. Due to its wide and effective use generally in medicine it was decided to be included in Performfish.

2. Materials and methods

2.1. Fish

The pharmacokinetic trial was carried out at the facilities of HCMR in Athens. There, fifty healthy gilthead sea bream averaging 232 g were separated in 3 cylindrical fiber glass tanks (800 L), receiving open flow sea water (38 psu) at a temperature of 25°C with aeration. Oxygen content was kept close to saturation by bubbling air through air stones and the photoperiod was kept at 12 h dark–12 h light. Fish were acclimatized for 2 weeks before the administration of medicated feed.

2.2. Medicated feed & distribution

During acclimatization fish received a conventional extruded diet (Table 1) which contained 44% protein and 18% fat. The diet was prepared locally.

Table 1. Composition of medicated diet

Ingredients	Moisture	Protein	Fat	Fiber	Starch	Ash	Energy
	%	%	%	%	%	%	MJ/kg
Fishmeal 70 LT FF Skagen	6.8	69.2	10.2	0.0	0.0	12.6	20.4
Wheat meal	8.0	10.2	1.6	2.3	60.6	1.5	15.8
Wheat Gluten	5.5	79.5	6.1	0.0	7.4	0.8	22.3
Corn gluten	10.9	60.0	3.8	1.5	13.6	1.0	21.3
Soybean meal 48	9.2	46.3	2.2	3.4	4.7	6.1	17.4

Soyproteinconcentrate (Soycomil)	7.9	62.7	0.2	3.5	0.0	6.5	18.6
Fishoil - COPPENS	2.0	0.0	98.0	0.0	0.0	0.0	39.0
MCP	2.6	0.0	0.0	0.0	0.0	30.0	0.0
L-Lysine	1.0	95.0	0.0	0.0	0.0	0.0	0.0
DL-Methionine	1.0	58.0	0.0	0.0	0.0	0.0	0.0
MEBENDAZOLE							

Following acclimatization, an extruded medicated diet was delivered to the experimental fish at the same as above feeding conditions. The drug as incorporated to the pellets during oil coating. A single dosing of medicated feed with MBZ was given at a concentration of 200 mg/kg fish (1% BW). Medicated diet was readily consumed by the fish with no signs of palatability issues.

2.3. Blood sampling and sample preparation

Sampling was set at 2, 4, 8, 12, 24, 36, 48 and 60h post medication. Five fish per sampling point were used. Approximately 1 ml of blood was taken from the caudal vein of anesthetized fish (clove oil) with Microlance 23G needles and immediately stored (24h) at 4°C until plasma preparation. Plasma was isolated from the heparin-treated blood samples by centrifugation at 4000 rpm for 10 min and stored at 20°C. Plasma samples were shipped to UoB in dry ice for chromatographical analysis of MBZ.

2.4. Analytical method

The analytical method consisted of a liquid-liquid extraction and a subsequent separation of the analyte by means of High Performance Liquid Chromatography with UV-Vis detection. The quantifying method consisted of interpolation along a regression line constructed from the results of a calibration curve (MBZ concentration vs MBZ Area). The quantifying procedure was performed automatically by the HPLC software. Each day of analysis, a calibration curve and a quality control samples set were analyzed with the samples.

Materials

The MBZreference standard (100% purity) was purchased from Sigma-Aldrich.

Reagents

- Acetonitrile (VWR Chemicals, ref. 83639.320)
- Methanol (Fisher Scientific, ref. M/4056/17)
- Chlorhidric Acid (Scharlau, ref. AC07361000)
- Sodium chloride (JT Baker, ref. 0277)
- Phosohoric acid 85% (Sigma, ref. 30417)

- Distilled water

Equipment

- Analytical Scales: KERN AEJ 120-4M / Sartorius BP100S
- Refrigerator 4 °C / Freezer -20 °C: Fagor / Rommer 460 HC
- Ultrasonic bath: Branson 2510
- Lab extractor hood: CaptairFiltair 936
- Vortex: Reax Heidolph
- Multivortex Mixer: VX-2500 VWR International
- Magnetic stirrer: Stuart SB161
- Vacuum Pump: Büchi Vac V500
- Centrifuge: Heraeus FRESCO 200
- Micropipettes: Gilson Pipetman F5, F10, and F25
/ Rainin 100 µL and 1 mL
- Water Purification System: Millipore Milli-RX45
- HPLC Waters Alliance:
 - Separation Module: Waters 2695
 - UV Detector: Waters2996
 - Column Oven: Waters Column Heater
 - Personal Computer: LG Alliance
 - Software: Empower 2 Software Build 2154

Other Materials

- Polypropylene Eppendorf 1.5 mL Tubes (Sudelab, ref. 175508)
- HPLC Polypropylene Vials 2 mL with insert, and caps (Akady, ref. 8136 and ref. 3839)
- Pipette Tips 200 µL and 1 mL (Sudelab, ref. 200016 and ref. 162222X)
- Volumetric Flasks 100 mL (Afora)
- Sterile Membrane Filters 0.45 µm (Pall Corporation, ref. GN-6 Grid)
- Mobile Phase Filter System (Fisher Scientific, ref. 5810/3)
- Chromatographic Column C₁₈Ultrapase 5 µm 20 x 0.46 cm (Akady)

Solutions

D3.3-Therapeutics for MMFF

The MBZ primary standard solution (0.1 mg/mL) was prepared as follows: MBZ standard (0.01 g 100% purity) was placed in a 100 mL glass volumetric flask and 50 mL of acetonitrile were added. Two drops of HCL 37% were added to dissolve MBZ. The flask was then placed in an ultrasonic bath for 20 sec, and the volume completed up to 100 mL with acetonitrile. The primary solution was aliquoted and conserved at -20 °C, approximately. The MBZ secondary solutions (0.01 and 0.001 mg/mL) were prepared as follows: Solution 0.01 mg/mL: A volume of 100 microliter (100 µL) of MBZ primary solution was diluted with acetonitrile up to a final volume of 1 mL. Solution 0.001 mg/mL: A volume of 100 µL of MBZ 0.01 mg/mL solution was diluted with acetonitrile up to a final volume of 1 mL.

Other solutions

HCl 0.1M: 0.83 ml of HCl 37% was dissolved with distilled water up to a final volume of 100 mL. H₃PO₄ 0.1%: For the mobile phase preparation 1 ml of H₃PO₄ was dissolved in 1 L of filtrated distilled water.

Resuspension solution: Mix 200 µL of Methanol with 800µL of H₃PO₄ 0.1% solution.

Blank Samples

Blank samples were obtained from non-treated animals of gilthead sea bream. They were kept frozen at -20 °C (± 5 °C) until analysis, and the presence of interferences was checked before use.

Sample Extraction Procedure

A volume of 100 µL of plasma was placed in a 1.5 mL Eppendorf tube and the corresponding volume of standard solution was added, when applicable. After adding 200 µL of HCl 0.1M solution, the samples were let at room temperature for 2 min, and then 400 µL of Ice cold acetonitrile was added for protein precipitation. The samples were multivortexed for 1 min. 50 mg of solid NaCl were added to the samples, shaken in a multivortex during 5 min, and then centrifuged at 14,000 r.p.m. for 5 min at 4 °C. The upper organic phase was transferred to a clean Eppendorf tube and dried under N₂ stream at 40°C. The samples were then resuspended with 100 µL of resuspension solution and placed in polypropylene HPLC vial with insert for chromatographic analysis.

Calibration Curves preparation

The calibration curves were constructed with blank plasma spiked with increasing MBZ concentrations, as follows:

SAMPLE IDENTIFICATION	MBZ (µg/mL)	CONC.	VOLUME OF MBZ SOLUTION IN 100 µL PLASMA
A	0.10		10 µL 0.001 mg/mL
B	0.25		25 µL 0.001 mg/mL
C	1.00		10 µL 0.01 mg/mL
D	5.00		5 µL 0.0 mg/mL
E	10.00		10 µL 0.1 mg/mL

Chromatographic conditions

- HPLC System: Waters Alliance
- Software: Empower2 Software Build 2154
- Mobile Phase: H₃PO₄ 0.1% :Methanol in a gradient of solvents
- Stationary Phase: Column C₁₈ 5 µm (20 x 0.46 cm)
- Flow rate: 1.5 mL/min
- UV-Vis Detection: Wavelength of 240 nm
- Column temperature: 50 °C
- Samples temperature: Room temperature
- Injection Volume: 25 µL
- Chromatogram time: 6 min

3. Results & Discussion

3.1. Validation results

The intraday and interday accuracy results were between the range of -30 and 15% and the precision values were lower than 20% along the concentrations of the calibration curves. The lower limit of quantification (LLOQ) and the lower limit of detection (LLOD) were established at 0.1µg/ml and 0.02µg/ml respectively. The calibration curve showed to be linear between a 0.1 to 10 µg/ml range. The MBZ showed to be stable at storing conditions (-20°) at least for 30 days without any degradation.

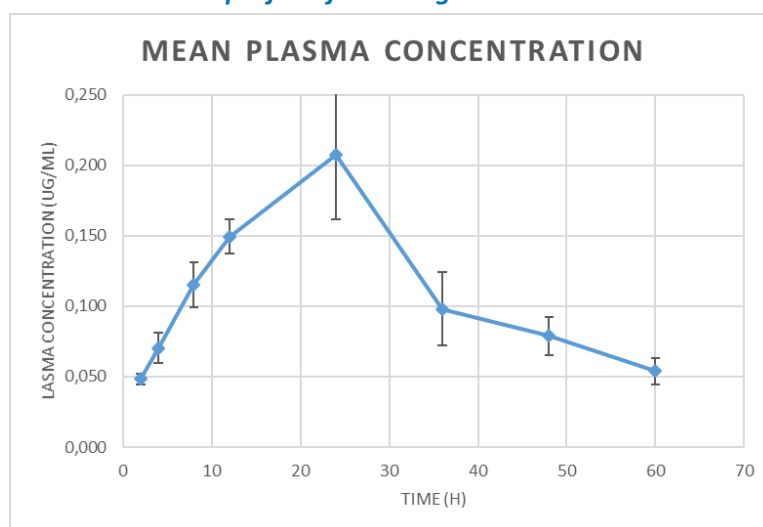
Table 2. Plasma concentration results of MBZ in gilthead sea bream

Time (h)	mean plasma concentration µg/mL	SD
2	0.049	0.004
4	0.070	0.011
8	0.115	0.016
12	0.149	0.012
24	0.207	0.045
36	0.098	0.026
48	0.079	0.013
60	0.054	0.009

Figure 2 depicts the mean pharmacokinetic profile of MBZ in gilthead sea bream. As it can be seen a reduced variability was observed in plasma concentrations at the beginning and end of sampling. The obtained higher rates of variability were after 24 and 36h of treatment. This reduced variability in the time points where concentrations

were low it is a positive result because all the animals treated show a considerable availability of drug taking in account that the drug was given with the diet.

Figure 2: Plasma-concentration profile of MBZ in gilthead sea bream



3.2. PK Study results

Table 2 shows the plasma concentration results of MBZ in gilthead sea bream. Each result is expressed as a mean of 5 samples from 5 different animals. As it can be seen the MBZ is rapidly absorbed and its concentrations remain in plasma up to 60 h post treatment. These levels are however lower compared to those measured when other antimicrobials are orally given to gilthead sea bream. Actually, MBZ has been blamed for reduced absorption due to its low solubility (de la Torre-Iglesias et al. 2014). The fact that MBZ plasma levels remain for a considerable period post-treatment suggest that a single dosing could be sufficient in parasitized gilthead sea bream, but this remains to be confirmed by clinical trials.

ANNEX V Oral Tortrazuril in gilthead sea bream (*Sparus aurata*)

1. Introduction

Toltrazuril (TTZ) is a triazine-based antiprotozoal drug that has specific activity against apicomplexan coccidial infections. Although its mechanism of action is unclear, TTZ may inhibit metabolic enzymes or decrease pyrimidine synthesis of the target parasites. It is widely used in livestock production including chickens, turkeys, pigs and cattle for the treatment of coccidiosis. TTZ is included in Annex I of Council Regulation (EEC) No 2377/90 with an MRL set at 100-600 µg/kg, depending on the animal tissue. In fish it has evaluated in vitro and in vivo against fresh water monogenean and ciliates of fish with mixed results (Schmahl&Mehlhorn, 1988; Schmahl et al., 1989; Jaafar et al., 1999). Mehlhorn et al. (1988) demonstrated TTZ effectiveness against endoparasites as well. In euryhaline fish such as sharpsnout sea bream, it was orally administered with limited success against a myxosporean (*Myxobolus* sp.). To be noted that its absorption has not been investigated in farmed fish. Since it has displayed some encouraging findings against ecto- and endoparasites of fish it was decided to be included in Performfish trials.

2. Materials and methods

2.1. Fish

The pharmacokinetic trial was carried out at the facilities of HCMR in Athens. There, fifty healthy gilthead sea bream averaging 355 g were separated in 3 cylindrical fiber glass tanks (800 L), receiving open flow sea water (38 psu) at a temperature of 22°C with aeration. Oxygen content was kept close to saturation by bubbling air through air stones and the photoperiod was kept at 12 h dark–12 h light. Fish were acclimatized for 2 weeks before the administration of medicated feed.

2.2. Medicated feed & distribution

During acclimatization fish received a conventional extruded diet prepared locally (Table 1) which contained 44% protein and 18% fat.

Table 1. Composition of medicated diet

Ingredients	Moisture	Protein	Fat	Fiber	Starch	Ash	Energy
	%	%	%	%	%	%	MJ/kg
Fishmeal 70 LT FF Skagen	6.8	69.2	10.2	0.0	0.0	12.6	20.4
Wheat meal	8.0	10.2	1.6	2.3	60.6	1.5	15.8
Wheat Gluten	5.5	79.5	6.1	0.0	7.4	0.8	22.3

Corngluten	10.9	60.0	3.8	1.5	13.6	1.0	21.3
Soybean meal 48	9.2	46.3	2.2	3.4	4.7	6.1	17.4
Soy protein concentrate (Soycomil)	7.9	62.7	0.2	3.5	0.0	6.5	18.6
Fishoil - COPPENS	2.0	0.0	98.0	0.0	0.0	0.0	39.0
MCP	2.6	0.0	0.0	0.0	0.0	30.0	0.0
L-Lysine	1.0	95.0	0.0	0.0	0.0	0.0	0.0
DL-Methionine	1.0	58.0	0.0	0.0	0.0	0.0	0.0
TOLTRAZURIL							

Following acclimatization, an extruded medicated diet was delivered to the experimental fish at the same as above feeding conditions. The drug as incorporated to the pellets during oil coating. A single dosing of medicated feed with TTZ (Baycox) was given at a concentration of 10 mg/kg fish (1% BW). Medicated diet was readily consumed by the fish with no signs of palatability issues.

2.3. Blood sampling and sample preparation

Sampling was set at 2, 4, 8, 12, 24, 48, 96, 192, 288 and 432h post medication. Five fish per sampling point were used. Approximately 1 ml of blood was taken from the caudal vein of anesthetized fish (clove oil) with Microlance 23G needles and immediately stored (24h) at 4°C until plasma preparation. Plasma was isolated from the heparin-treated blood samples by centrifugation at 4000 rpm for 10 min and stored at 20°C. Plasma samples were shipped to UoB in dry ice for chromatographical analysis of TTZ.

2.4. Analytical method

The analytical method consisted of a liquid-liquid extraction and a subsequent separation of the analyte by means of High Performance Liquid Chromatography with QQQ-MS. The quantifying method consisted of interpolation along a regression line constructed from the results of calibration curves (TTZ concentration, TTZ-SO and TTZ-SO₂ vs its Areas). The quantifying procedure was performed automatically by the HPLC software. Each day of analysis, a calibration curve and a quality control samples set were analyzed with the samples.

Materials

Reagents

- Toltrazuril Sigma, ref. 34000
- Toltrazuril sulfoxide, ref. 33815
- Toltrazuril sulphone, ref. 33816
- Methanol (Fisher Scientific, ref. M/4056/17)
- Acetonitrile (Fisher Scientific, ref. 10489553)

D3.3-Therapeutics for MMFF

- Formic Acid for LC/MS (Fisher Scientific, ref. A117-50)
- Water Milli Q

Equipment

- Analytical Scales: KERN AEJ 120-4M / Sartorius BP100S
- Refrigerator 4 °C / Freezer -20 °C: Fagor / Rommer 460 HC
- Lab extractor hood: CaptairFiltair 936
- Vortex: Reax Heidolph
- Multivortex Mixer: VX-2500 VWR International
- Magnetic stirrer: Stuart SB161
- Centrifuge: Heraeus FRESCO 200
- Micropipettes: Gilson Pipetman F5, F10, and F25 / Rainin 100 µL and 1 mL
- Water Purification System: Millipore Milli-RX45
- HPLC:
 - Separation Module: Agilent 1290 series
 - Thermostated Autosampler: Agilent 1290 series
 - Column Oven: Agilent 1290 series
 - MS-QQQ: Agilent 6420
 - Ionization source: ESI
 - Personal Computer: HP
 - Software: Agilent Mass-Hunter

Other Materials

- Polypropylene Eppendorf 1.5 mL Tubes (Sudelab, ref. 175508)
- HPLC Polypropylene Vials 2 mL with insert, and caps (Akady, ref. 8136 and ref. 3839)
- Pipette Tips 200 µL and 1 mL (Sudelab, ref. 200016 and ref. 162222X)
- Volumetric Flasks 100 mL (Afora)
- Chromatographic Column Zorbax SB-C18 1.8 µm 50 x 0.46 cm (Agilent)

Solutions

Working Standard Solutions

TTZ, TTZ-SO and TTZ-SO₂ primary solutions (0.4 mg/mL): TTZ, TTZ-SO or TTZ-SO₂ standards (0.010 g) were placed in a 25 mL glass volumetric flask and 15 mL of methanol were added. The flask were then placed in an ultrasonic bath for 20 sec, and the volume completed up to 25 mL with methanol. The primary solutions were aliquoted and conserved at -20 °C, approximately.

Secondary solutions (0.1, 0.01, 0.001 and 0.0001 mg/mL): 0.1 mg/mL solution was prepared mixing 100 µL of TTZ, TTZ-SO and TTZ-SO₂ primary solutions in a 1 ml glass vial and adding 100 µL of methanol. This combined solution was serially diluted 1/10 with methanol to obtain the other solutions.

Other solutions

Formic Acid 0.1%: 1 ml of Formic acid was dissolved in 1 L of Milli Q water.

Acetonitrile with 0.1% of formic acid: 1 ml of Formic acid was dissolved in 1 L of acetonitrile.

Blank Samples

Blank samples were obtained from non-treated gilthead sea bream. They were kept frozen at $-20\text{ }^{\circ}\text{C}$ ($\pm 5^{\circ}\text{C}$) until analysis, and the presence of interferences was checked before use.

Sample Extraction Procedure

A volume of $50\text{ }\mu\text{L}$ of plasma was placed in a 1.5 mL Eppendorf tube and the corresponding volume of standard solution was added, when applicable. After adding $100\text{ }\mu\text{L}$ of methanol, the samples were multivortexed for 5 min and then centrifuged at $14,800\text{ r.p.m.}$ for 5 min at $4\text{ }^{\circ}\text{C}$. The upper phase was transferred to a clean polypropylene HPLC vial with insert for chromatographic analysis.

Calibration Curves preparation

The calibration curves were constructed with blank plasma spiked with increasing TTZ, TTZ-SO and TTZ-SO₂ concentrations, as follows:

SAMPLE IDENTIFICATION	TTZ CONC. ($\mu\text{g}/\text{mL}$)	VOLUME OF STANDARD SOLUTION IN $100\text{ }\mu\text{L}$ PLASMA
A	0.05	$25\text{ }\mu\text{L}$ $0.0001\text{ mg}/\text{mL}$
B	0.1	$5\text{ }\mu\text{L}$ $0.001\text{ mg}/\text{mL}$
C	0.2	$10\text{ }\mu\text{L}$ $0.001\text{ mg}/\text{mL}$
D	0.5	$25\text{ }\mu\text{L}$ $0.001\text{ mg}/\text{mL}$
E	1	$5\text{ }\mu\text{L}$ $0.01\text{ mg}/\text{mL}$
F	2	$10\text{ }\mu\text{L}$ $0.01\text{ mg}/\text{mL}$
G	5	$25\text{ }\mu\text{L}$ $0.01\text{ mg}/\text{mL}$

Chromatographic conditions

- HPLC System: Agilent 1290
- Software: AgilentMassHunter
- Mobile Phase: Milli Q water (Formic acid 0.1%):Acetonitrile (0.1% Formic acid) in a gradient of solvents
- Stationary Phase: Zorbax SB-C18 $1.8\text{ }\mu\text{m}$ $50\text{ x }0.46\text{ cm}$
- Flow rate: $0.5\text{ mL}/\text{min}$
- Column temperature: $40\text{ }^{\circ}\text{C}$
- Samples temperature: 10°C
- Injection Volume: $5\text{ }\mu\text{L}$
- Chromatogram time: 4 min

QQQ-MS Detection conditions

- Ionization source: ESI
- Ionization polarity: negative
- Transition conditions:

Drug	Parent ion UMA	TransitionsUMA	DWELL	Frag.	CE	CAV
TTZ	424.1	424.1	200	110	13	4
		42.2	200	110	13	4
TTZ-SO	440.1	440.1	200	130	17	4
		42.1	200	130	17	4
TTZ-SO ₂	440.1	440.1	200	130	17	4
		42.1	200	130	17	4

3. Results & Discussion

3.1. Validation results

The intraday and interday accuracy results in both matrices were between the range of -30 and 15% and the precision values were lower than 20% along the concentrations of the calibration curves. The lower limit of quantification (LLOQ) and the lower limit of detection (LLOD) were established at 0.1µg/ml and 0.02 µg/ml respectively. The calibration curve showed to be linear between a 0.1 to 5 µg/ml range in plasma and between 20 µg/ml and 2000 µg/ml in gastrointestinal content. The TTZ showed to be stable at storing conditions (-20°) at least for 30 days without any degradation.

3.2. PK Study results

Table 2 show the plasma concentration results of TTZ and its metabolites in gilthead sea bream plasma. Each result is expressed as a mean of 5 samples from 5 different animals. As it can be seen the TTZ is rapidly absorbed and significant concentrations remain in plasma up to 20 days post treatment after a single dose. Interestingly, its metabolites in plasma detected at low levels and seem to remain for a larger time in circulation compared to the parent compound. These findings suggest for a single dosing of TTZ in gilthead sea bream and perhaps in other euryhaline or marine farmed spp., however, this hypothesis remain to be elucidated in future work. Whether these prolonged TTZ plasma levels are sufficient against ecto- or endoparasites of gilthead sea bream has to be confirmed by lab-scale and/or field clinical trials.

Table 2. Plasma concentration results of TTZ and its metabolites in gilthead sea bream

Plasma concentrations($\mu\text{g/ml}$)						
Time	TTZ-SO		TTZ-SO ₂		TTZ	
	Mean	SD	Mean	SD	Mean	SD
2 h	0.12	0.06	0.00	0.00	18.03	3.49
4 h	0.19	0.07	0.00	0.00	24.25	8.53
8 h	0.45	0.08	0.00	0.00	32.82	8.86
12 h	0.61	0.20	0.01	0.01	38.01	17.88
1 day	1.59	1.01	0.18	0.18	40.96	28.40
2 days	2.74	1.69	0.91	0.91	31.20	12.98
5 days	1.96	1.37	1.20	1.20	18.15	10.87
9 days	1.33	0.50	3.08	3.08	7.41	2.11
13 days	0.46	0.21	1.70	1.70	3.32	1.61
19 days	0.09	0.07	1.28	1.28	0.41	0.38
25 days	0.01	0.01	0.65	0.65	0.05	0.04

Figure 1 depicts the mean pharmacokinetic profile of TTZ and its metabolites in the sea bream. As it can be seen the elimination profile of TTZ and its metabolites is very slow with a suspected elimination half-life of 60 h for TTZ and TTZ-SO, and about 180h for the TTZ-SO₂. This results indicates that a single administration give significant concentrations in order to suspect good efficacy of the treatment.

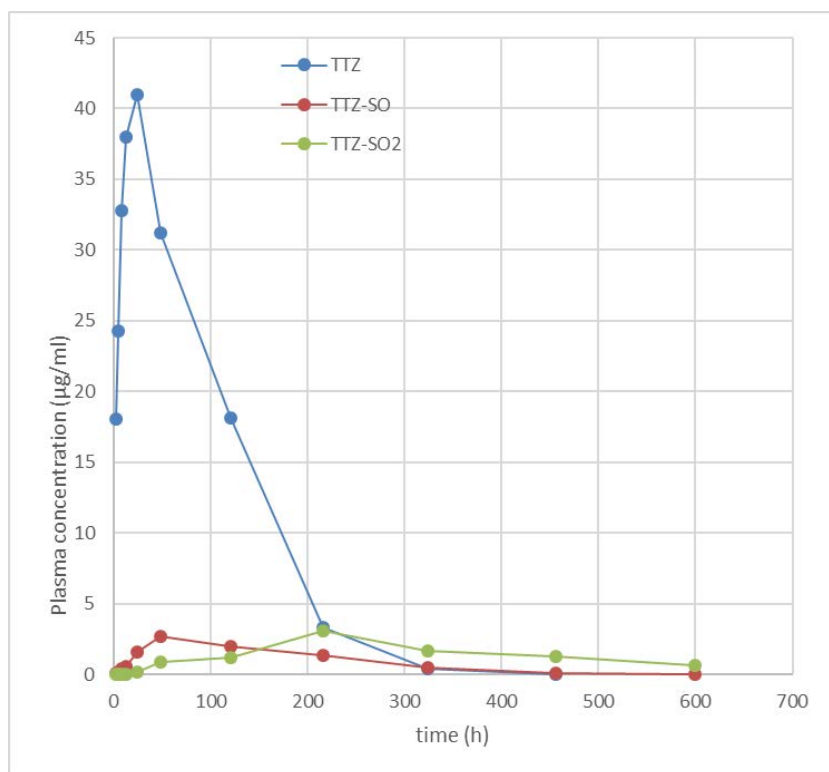


Figure 1: Plasma-concentration profile of TTZ and its metabolites in gilthead sea bream

REFERENCES

- Aamri, F.E., Caballero M.J., Real, F., Acosta, F., Déniz, S., Román, L., Padilla, D., 2014. *Streptococcus iniae* in Gilthead Seabream (*Sparus aurata*, L.) and Red Porgy (*Pagrus pagrus*, L.): Ultrastructural Analysis. *Veterinary Pathology*. 52, 209-212.
- Abdel-Aziz, M., Eissa, A.E., Hanna, M., Okada, M.A., 2013. Identifying some pathogenic *Vibrio*/*Photobacterium* species during mass mortalities of cultured Gilthead seabream (*Sparus aurata*) and European seabass (*Dicentrarchus labrax*) from some Egyptian coastal provinces. *International Journal of Veterinary Science and Medicine*. 1, 87-95.
- Abu-Basha, E. A., Gehring, R., Albwa'neh, S. J.2007. Pharmacokinetics and bio-availability of spectinomycin after i.v., i.m., s.c. and oral administration in broiler chickens. *J. vet. Pharmacol. Therap.* 30, 139–14.
- Alderman, D., Hastings, T.S. (2003). Antibiotic use in aquaculture: development of antibiotic resistance-potential for consumer health risks. *International Journal of Food Science & Technology*. 33. 139 - 155.
- Álvarez-Pellitero, P. 2004. Report about fish parasitic diseases. pp 103-130. *In*: P. Álvarez-Pellitero, J.L. Barja, B. Basurco, F. Berthe and A.E.Toranzo AE [eds.] *Mediterranean aquaculture diagnostic laboratories: results of the survey on Mediterranean aquaculture diagnostic laboratories conducted within the framework of the CIHEAM/FAO network on "Technology of Aquaculture in the Mediterranean"*. Options Méditerranéennes, B/49 Etudes et Recherches, Options Méditerranéennes. CIHEAM/ FAO Publications, France.
- ALVAREZ-PELLITERO, P., SITJÀ-BOBADILLA, A., FRANCO-SIERRA, A., 1993. Protozoan parasites of wild and cultured sea bass, *Dicentrarchus labrax* (L.), from the Mediterranean area. *Aquaculture Research*. 24, 101-108.
- ALVAREZ-PELLITERO, P., ADILLA, A.S.-B., FRANCO-SIERRA, A., PALENZUELA, O., 1995. Protozoan parasites of gilthead sea bream, *Sparus aurata* L., from different culture systems in Spain. *Journal of Fish Diseases*. 18, 105-115.
- Antonelli, L., Quilichini, Y., Marchand, B., 2010. Biological study of *Furnestinia echeneis* Euzet and Audouin 1959 (Monogenea: Monopisthocotylea: Diplectanidae), parasite of cultured Gilthead sea bream *Sparus aurata* (Linnaeus 1758) (Pisces: Teleostei) from Corsica. *Aquaculture*. 307, 179-186.
- Antonelli, L., Quilichini, Y., Marchand, B., 2012. *Lernanthropus kroyeri* (Van Beneden and Hesse 1851) parasitic Copepoda (Siphonostomatoidae, Lernanthropidae) of European cultured sea bass *Dicentrarchus labrax* (Linnaeus 1758) from Corsica: ecological and morphological study. *Parasitology Research*. 110, 1959-1968.
- Athanasopoulou, F., 1998. A case report of *Pleistophora* SP. infection in cultured sea bream (*sparus aurata* L.) in Greece. *Bulletin of European Association of Fish Pathologists* 18, 19-21.
- Athanasopoulou, F., Bouboulis, D., Martinsen, B., 2001. In vitro treatments of deltamethrin against the isopod parasite *Ceratothoa oestroides*, a pathogen of seabass *Dicentrarchus labrax* L. *Bulletin of the European Association of Fish Pathologists*. 21, 26-29.
- Athanasopoulou, F., Ragias, B., Tavla, J., Christofiliogiannis, P., Liberis, N., 2008. Preliminary trials on the efficacy and toxicity of ivermectin against *Lernanthropus kroyeri* Van Beneden, 1851 in cultured sea bass *Dicentrarchus labrax* L. *Aquaculture Research* 32, 77-79.
- Athanasopoulou, F., Karagouni, E., Dotsika, E., Ragias, B., Tavla, J., Christofiloyanis, P., Vatsos, I., 2004b. Efficacy and toxicity of orally administered anti-coccidial drugs for innovative treatments of *Myxobolus* sp. infection in Puntazzo puntazzo. *Diseases of Aquatic Organisms*. 62, 217-226.
- Athanasopoulou, F., Karagouni, E., Dotsika, E., Ragias, V., Tavla, J., Christofiloyanis, P., 2004a. Efficacy and toxicity of orally administered anticoccidial drugs for innovative treatments of *Polysporoplasma sparisi* (Sitja-Bobadilla and Alvarez-Pellitero 1985) infection in *Sparus aurata* L. *Journal of Applied Ichthyology*. 20, 345-354.

- Athanassopoulou F., Pappas I.S., Bitchava K. An overview of the treatments for parasitic disease in Mediterranean aquaculture. In : Rogers C. (ed.), Basurco B. (ed.). The use of veterinary drugs and vaccines in Mediterranean aquaculture. Zaragoza : CIHEAM, 2009. p. 65-83 (Options Méditerranéennes : Série A. Séminaires Méditerranéens; n. 86)
- Bouboulis, D., Athanasopoulou, F., Tyrpenou, A., 2004. Experimental treatments with diflubenzuron and deltamethrin of sea bass, *Dicentrarchus labrax* L., infected with the isopod, *Ceratothoa oestroides*. *Journal of Applied Ichthyology*. 20, 314-317.
- Avendaño-Herrera, R., Toranzo, A.E., Beatriz, M., 2006. Tenacibaculosis infection in marine fish caused by *Tenacibaculum maritimum*: a review. *Diseases of Aquatic Organisms*. 71, 255-266
- Avsever, M.L., Çavuşoğlu, C., Eskiizmirli, S., Türe, M., Korun, J., Çamkerten, İ., 2016. First isolation of *Mycobacterium marinum* from sea bass (*Dicentrarchus labrax*) and gilthead sea bream (*Sparus auratus*) cultured in Turkey. *Bulletin of the European Association of Fish Pathology*. 36, 193.
- Balebona, M.C., Zorrilla, I., Moriñigo, M.A., Borrego, J.J., 1998. Survey of bacterial pathologies affecting farmed gilt-head sea bream (*Sparus aurata* L.) in southwestern Spain from 1990 to 1996. *Aquaculture*. 166, 19-35.
- Bernardet, J.-F., Kerouault, B., Michel, C., 1994. Comparative Study on *Flexibacter maritimus* Strains Isolated from Farmed Sea Bass (*Dicentrarchus labrax*) in France.
- Bernardet, J.F., 1998. Cytophaga, Flavobacterium, Flexibacter and Chryseobacterium infections in cultured marine fish. *Fish Pathology*. 33, 229–238.
- Blaser, J., Stone, B.B., Grown, M.C., Zinner, S.H., 1987. Comparative study with enoxacin and netilmicin in a pharmacodynamic model to determine importance of ratio of antibiotic peak concentration to MIC for bactericidal activity and emergence of resistance. *Antimicrobial Agents and Chemotherapy*. 31, 1054–1060.
- Bonev, B., Hooper, J., Parisot, J., 2008. Principles of assessing bacterial susceptibility to antibiotics using the agar diffusion method. *Journal of Antimicrobial Chemotherapy*. 61, 1295-1301.
- Bouboulis, D., Athanasopoulou, F., Tyrpenou, A., 2004. Experimental treatments with diflubenzuron and deltamethrin of sea bass, *Dicentrarchus labrax* L., infected with the isopod, *Ceratothoa oestroides*. *Journal of Applied Ichthyology*. 20, 314-317.
- Bragoni, G., Romestand, B., Trilles, J.P. 1984. Parasitoses à Cymothoadien chez le loup (*Dicentrarchus labrax* Linnaeus, 1758) en élevage. I. Ecologie parasitaire dans le cas de l'étang de Diana (Haute Corse). *Crustaceana* 47 :44-51.
- Branson, E., 2001. Clinical relevance of Minimum Inhibitory Concentrations (MICs). *Aquaculture*. 196, 289–298.
- Bruun, M.S., Madsen, L., Dalsgaard, I., 2003. Efficiency of oxytetracycline treatment in rainbow trout experimentally infected with *Flavobacterium psychrophilum* strains having different in vitro antibiotic susceptibilities. *Aquaculture*. 215, 11-20.
- BSAC, 1991. A guide to sensitivity testing. Report of the working party on antibiotic sensitivity testing of the British Society for Antimicrobial Chemotherapy. *Journal of Antimicrobial Chemotherapy*. 23 (Suppl. D), 1–47.
- Burgess, D.S., Frei, C.R., Lewis, I.J.S., Fiebelkorn, K.R., Jorgensen, J.H., 2007. The contribution of pharmacokinetic–pharmacodynamic modelling with Monte Carlo simulation to the development of susceptibility breakpoints for *Neisseria meningitidis*. *Clinical Microbiology and Infection*. 13, 33-39.
- Caffara, M., Quaglio, F., Marcer, F., Florio, D., Fioravanti, M., 2010. Intestinal microsporidiosis in European seabass (*Dicentrarchus labrax* L.) farmed in Italy. *Bulletin of the European Association of Fish Pathologists*. 30, 237-240.
- Candan, A., Kucker, A., Karatas, F., 1996. Pasteurellosis in cultured Sea bass (*Dicentrarchus labrax*) in Turkey. *Bulletin of the European Association of Fish Pathologists*. 16, 150-153.
- Castells, Intorre, Bertini, Cristòfol, Soldani, Arboix, 2000. Oral single-dose pharmacokinetics of thiamphenicol in the sea-bass (*Dicentrarchus labrax*). *Journal of Veterinary Pharmacology and Therapeutics*. 23, 53-54.
- Chelossi, E., Vezzulli, L., Milano, A., Branzoni, M., Fabiano, M., Riccardi, G., Banat, I.M., 2003. Antibiotic resistance of benthic bacteria in fish-farm and control sediments of the Western Mediterranean. *Aquaculture*. 219, 83-97.
- Cecchini, S., Cognetti-Varriale, A. 2003. Dehydration is more effective for the control of embryonic development and larval hatching of *Diplectanum aequans* (Monogenea, Diplectanidae) than formalin and trichlorphon. *Aquaculture International* 11: 261.

D3.3-Therapeutics for MMFF

- Čolak, S., Barić, R., Kolega, M., Mejdandžić, D., Mustačić, B., Petani, B., Župan, I., Šarić, T., 2018. Effect of the pesticide deltamethrin as a treatment of *Ceratomyxa oestroides* infestations of farmed sea bass *Dicentrarchus labrax*. *Aquaculture*. 500.
- Colorni, A. (1985). Aspects of biology of *Cryptocaryon irritans* and hyposalinity as a control measure in cultured sea bream *Sparus aurata*. *Diseases of Aquatic Organisms - Diseases of Aquatic Organisms* 1, 19-22.
- Colorni, A., Burgess, P., 1997. *Cryptocaryon irritans* Brown 1951, the cause of 'white spot disease' in marine fish: an update. *Aquarium Sciences and Conservation*. 1, 217-238.
- Colorni, A., Diamant, A., 2005. Hyperparasitism of trichodinid ciliates on monogenean gill flukes of two marine fish. *Diseases of Aquatic Organisms*. 65, 177-180.
- Colorni A, Padrós F. Diseases and health management. In: Pavlidis MA, Mylonas CC, editors. *Sparidae: biology and aquaculture of gilthead sea bream and other species*. Oxford: Wiley; 2011.
- Coyne, R., Samuelsen, O., Bergh, P., Andersen, K., Pursell, L., Dalsgaard, I., Smith, P., 2004b. On the validity of setting breakpoint minimum inhibition concentrations at one quarter of the plasma concentration achieved following oral administration of oxytetracycline. *Aquaculture*. 239, 23-35.
- Coyne, R., Bergh, O., Samuelsen, O., Andersen, K., Lunestad, B.T., Nilsen, H., Dalsgaard, I., Smith, P., 2004a. Attempt to validate breakpoint MIC values estimated from pharmacokinetic data obtained during oxolinic acid therapy of winter ulcer disease in Atlantic salmon (*Salmo salar*). *Aquaculture*. 238, 51-66.
- Craig, W., 2002. Pharmacodynamics of antimicrobials: general concepts and applications. in: Nightingale, C., Murakawa, T., Ambrose, P. (Eds.), *Antimicrobial Pharmacodynamics in Theory and Clinical Practice*. Marcel Dekker, Oxford, pp. 1-22.
- Craig, W., 2007. Pharmacodynamics of antimicrobials: general concepts and applications. in: Nightingale, C., Ambrose, P., Drusano, G., Murakawa, T. (Eds.), *Antimicrobial Pharmacodynamics in theory and clinical practice*. Informa Health Care, New York, pp. 1-20.
- Craig, W., Gudmundsson, S., 1996. Post-antibiotic effect. in: Lorian, V. (Ed.), *Antibiotics in laboratory medicine*. Williams and Wilkins, Baltimore, pp. 296-329.
- della Rocca, G., Di Salvo, A., Malvisi, J., Sello, M., 2004a. The disposition of enrofloxacin in seabream (*Sparus aurata* L.) after single intravenous injection or from medicated feed administration. *Aquaculture*. 232, 53-62.
- della Rocca, G., Zaghini, A., Zanoni, R., Sanguinetti, V., Zanchetta, S., Di Salvo, A., Malvisi, J., 2004b. Seabream (*Sparus aurata* L.): disposition of amoxicillin after single intravenous or oral administration and multiple dose depletion studies. *Aquaculture*. 232, 1-10.
- Diamant, A., Ucko, M., Paperna, I., Colorni, A., Lipshitz, A., 2005. *Kudoa iwatai* (Myxosporidia: Multivalvulida) in wild and cultured fish in the Red Sea: Redescription and molecular phylogeny. *The Journal of Parasitology*. 91, 1175-1189.
- Di Salvo, A., della Rocca, G., Terzetti, E., Malvisi, J., 2013. Florfenicol depletion in edible tissue of rainbow trout, *Oncorhynchus mykiss* (Walbaum), and sea bream, *Sparus aurata* L. *Journal of Fish Diseases*. 36, 685-693.
- Doukas, Athanassopoulou, Karagouni, Dotsika, 2008. *Aeromonas hydrophila* infection in cultured sea bass, *Dicentrarchus labrax* L., and *Puntazzo puntazzo* Cuvier from the Aegean Sea.
- Dragesco, A., Dragesco, J., Coste, F., Gasc, C., Romestand, B., Raymond, J.-C., Bouix, G., 1995. *Philasterides dicentrarchi*, n. sp., (ciliophora, scuticociliatida), a histophagous opportunistic parasite of *Dicentrarchus labrax* (Linnaeus, 1758), a reared marine fish. *European Journal of Protistology*. 31, 327-340.
- Drusano, G.L., 2004. Antimicrobial pharmacodynamics: critical interactions of 'bug and drug'. *Nature Reviews Microbiology* 2, 289-300.
- Dudley, M.N., 1991. Pharmacodynamics and pharmacokinetics of antibiotics with special reference to the fluoroquinolones. *The American Journal of Medicine*. 91, S45-S50.
- Dyková, I., Figueras, A., Peric, Z. 2000. *Neoparamoeba* Page, 1987: light and electron microscopic observations on six strains of different origin. *Dis. Aquat. Org.* 43:217-223.
- Eliopoulos, G.M., Eliopoulos, G.M., Roberts, M.C., 2003. Tetracycline Therapy: Update. *Clinical Infectious Diseases*. 36, 462-467.
- Elkamel, A., Hawke, J., G. Henk, W., Thune, R., 2003. *Photobacterium damsela* subsp. *piscicida* Is Capable of Replicating in Hybrid Striped Bass Macrophages. *Journal of Aquatic Animal Health*. 15, 175-183.

D3.3-Therapeutics for MMFF

- Estensoro, I., Jung-Schroers, V., Alvarez-Pellitero, P., Steinhagen, D., Sitjà-Bobadilla, A., 2012. Effects of *Enteromyxum leei* (Myxozoa) infection on gilthead sea bream (*Sparus aurata*) (Teleostei) intestinal mucus: Glycoprotein profile and bacterial adhesion. *Parasitology Research*. 112.
- EMA, 1995a. Oxytetracycline, Tetracycline, Chlortetracycline. Summary report (3). Committee for Veterinary Medicinal Products / The European Agency for the Evaluation of Medicinal Products. EMA/MRL/023/95.
- EMA, 1995b. Sulphonamides Summary report (2). Committee for Veterinary Medicinal Products / The European Agency for the Evaluation of Medicinal Products. EMA/MRL/026/95.
- EMA, 1997a. Doxycycline. Summary report (2). Committee for Veterinary Medicinal Products / The European Agency for the Evaluation of Medicinal Products. EMA/MRL/270/97-FINAL.
- EMA, 1997b. Trimethoprim, Summary report (2). Committee for Veterinary Medicinal Products / The European Agency for the Evaluation of Medicinal Products. EMA/MRL/255/97-FINAL.
- EMA, 1998. Sarafloxacin (Salmonidae) Summary report (2). Committee for Veterinary Medicinal Products / The European Agency for the Evaluation of Medicinal Products. EMA/MRL/394/98-FINAL.
- EMA, 2000. Lincomycin, Summary report (2). Committee for Veterinary Medicinal Products / The European Agency for the Evaluation of Medicinal Products. EMA/MRL/749/00-FINAL-corr.
- EMA, 2002. Flumequine (extension to all food producing species) Summary report (4). Committee for Veterinary Medicinal Products / The European Agency for the Evaluation of Medicinal Products. EMA/MRL/823/02-FINAL.
- EMA, 2002b. Enrofloxacin (extension to all food producing species). Summary report (5). Committee for Veterinary Medicinal Products / The European Agency for the Evaluation of Medicinal Products. EMA/MRL/820/02-FINAL.
- EMA, 2002c. Danofloxacin (extension to all food producing species) Summary report (6). Committee for Veterinary Medicinal Products / The European Agency for the Evaluation of Medicinal Products. EMA/MRL/812/02-FINAL.
- EMA, 2002d. Florfenicol (extension to all food producing species) Summary report (6). Committee for Veterinary Medicinal Products / The European Agency for the Evaluation of Medicinal Products. EMA/MRL/822/02-FINAL.
- EMA, 2002e. Spectinomycin (extension to all food producing species) Summary report (5). Committee for Veterinary Medicinal Products / The European Agency for the Evaluation of Medicinal Products. EMA/MRL/826/02-FINAL.
- EMA, 2005a. Oxolinic acid (extension to all food producing species) Summary report (5). Committee for Veterinary Medicinal Products / The European Agency for the Evaluation of Medicinal Products. EMA/MRL/41090/2005-FINAL.
- EMA, 2006. Thiamphenicol (extension to pigs and extrapolation all food producing species) Summary report (6). Committee for Veterinary Medicinal Products / The European Agency for the Evaluation of Medicinal Products. EMA/CVMP/162614/06-FINAL.
- EMA, 2008. Penicillines, Summary report. Committee for Veterinary Medicinal Products / The European Agency for the Evaluation of Medicinal Products. EMA/MRL/Revision 1.
- Essam, H.M., Abdellrazeq, G.S., Tayel, S.I., Torky, H.A., Fadel, A.H., 2016. Pathogenesis of *Photobacterium damsela* subspecies infections in sea bass and sea bream. *Microbial Pathogenesis*. 99, 41-50.
- Fleurbaey, R., Sauvegrain, C., Marques, A., Le Breton, A., Guereaud, C., Cherel, Y., Wyers, M., 2008. Histopathological changes caused by *Enteromyxum leei* infection in farmed sea bream *Sparus aurata*. *Diseases of Aquatic Organisms*. 79, 219-228.
- Gjurčević, E., kužir, s., Baždarić, B., Matanović, K., Debelić, I., Marino, F., Drašner, K., Rosenthal, B.M., 2017. New data on *Eimeria dicentrarchi* (Apicomplexa: Eimeriidae), a common parasite of farmed European sea bass (*Dicentrarchus labrax*) from the mid-eastern Adriatic. *Veterinarski Archiv*. 87, 77-86.
- Giavenni, R. 1985. Infestation by *Diplectanum aequans* (Wagener 1857) Diesing 1858 in cultured sea bass (*Dicentrarchus labrax* L.) *Rivista Italiana di Piscicoltura e Ittiopatologia*.
- Golomazou, E., Athanasopoulou, F., Karagouni, E., Vagianou, S., Tsantilas, I., Karamanis, D., 2006. Efficacy and Toxicity of Orally Administered Anti-Coccidial Drug Treatment on *Enteromyxum leei* Infections in Sharpnose Seabream (*Diplodus puntazzo* C.). *The Israeli Journal of Aquaculture, Bamiddeh*. 58, 157-169.

D3.3-Therapeutics for MMFF

- González-Lanza, C., Alvarez-Pellitero, P., Sitjà-Bobadilla, A., 1991. Diplectanidae (Monogenea) infestations of sea bass, *Dicentrarchus labrax* (L.), from the Spanish Mediterranean area. *Parasitology Research*. 77, 307-314.
- Haldar, S., Maharajan, A., Chatterjee, S., Hunter, S.A., Chowdhury, N., Hinenoya, A., Asakura, M., Yamasaki, S., 2010. Identification of *Vibrio harveyi* as a causative bacterium for a tail rot disease of sea bream *Sparus aurata* from research hatchery in Malta. *Microbiological Research*. 165, 639-648.
- Hispano, C. 2016 *Avaluació d'una infestació de Gnathia maxillaris* (Montagu, 1804) (Crustacea:Isopoda:Gnathiidae), a partir del seguiment i eradicació d'un brot a un sistema d'aquaris de grans dimensions. PhD Thesis. Universitat de Barcelona.
- Holzer, A.S., Montero, F.E., Repullés, A., Nolan, M.J., Sitjà-Bobadilla, A., Alvarez-Pellitero, P., Zarza, C., Raga, J.A., 2008. *Cardicola aurata* sp. n. (Digenea: Sanguinicolidae) from Mediterranean *Sparus aurata* L. (Teleostei: Sparidae) and its unexpected phylogenetic relationship with *Paradeontacylix McIntosh*, 1934. *Parasitology International*. 57, 472-482.
- Horton, T., Okamura, B., 2001. Cymothoid isopod parasites in aquaculture: A review and case study of a Turkish sea bass (*Dicentrarchus labrax*) and sea bream (*Sparus auratus*) farm. *Diseases of Aquatic Organisms*. 46, 181-188.
- Horton, T., Okamura, B., 2003. Post-haemorrhagic anaemia in sea bass, *Dicentrarchus labrax* (L.), caused by blood feeding of *Ceratothoa oestroides* (Isopoda: Cymothoidae). *Journal of Fish Diseases*. 26, 401-406.
- Intorre, L., Cecchini, S., Bertini, S., Cognetti Varriale, A.M., Soldani, G., Mengozzi, G., 2000. Pharmacokinetics of enrofloxacin in the seabass (*Dicentrarchus labrax*). *Aquaculture*. 182, 49-59.
- Intorre, L., Castells, G., CristoFol, C., Bertini, S., Soldani, G., Arboix, M., 2002. Residue depletion of thiamphenicol in the sea-bass. *Journal of Veterinary Pharmacology and Therapeutics*. 25, 59-63.
- Jaafar, R.M., Buchmann, K., 2011. Toltrazuril (Baycox vet.) in feed can reduce *Ichthyophthirius multifiliis* invasion of rainbow trout (Salmonidae). *Acta Ichthyologica Et Piscatoria* 41(1):63-66.
- Kahlmeter, G., Brown, D.F.J., Goldstein, F.W., MacGowan, A.P., Mouton, J.W., Osterlund, A., Rodloff, A., Steinbakk, M., Urbaskova, P., Vatopoulos, A., 2003. European harmonization of MIC breakpoints for antimicrobial susceptibility testing of bacteria. *Journal of Antimicrobial Chemotherapy*. 52, 145-148.
- Katharios, P., Papandroulakis, N., Divanach, P., 2006. Treatment of *Microcotyle* sp. (Monogenea) on the gills of cage-cultured red porgy, *Pagrus pagrus* following baths with formalin and mebendazole. *Aquaculture*. 251, 167-171.
- Kim, K.H. Park, S_I, Jee B-Y, 1998, Efficacy of oral administration of praziquantel and mebendazole against *Microcotyle sebastis* (Monogenea) infestation of cultured rockfish (*Sebastes schlegeli*). *Fish Pathology*, 33(5), 467-471,
- Kolygas, M.N., Gourzioti, E., Vatsos, I.N., Athanassopoulou, F., 2012. Identification of *Tenacibaculum maritimum* strains from marine farmed fish in Greece. *Vet Record*. 170, 623.
- Korun, J., Timur, G., 2008. MARINE VIBRIOS ASSOCIATED WITH DISEASED SEA BASS (*Dicentrarchus labrax*) IN TURKEY. *Journal of FisheriesSciences.com*. DOI: 10.3153/jfscom.200800.
- Leal, J. F., Neves, M. G., Santos, E. B. and Esteves, V. I. (2018), Use of formalin in intensive aquaculture: properties, application and effects on fish and water quality. *Rev Aquacult*, 10: 281-295. doi:10.1111/raq.12160
- Lees, P., Concordet, D., Alliabadi, F., Toutain, P.-L., 2006. Drug selection and optimization of dosage schedules to minimise antimicrobial resistance. in: Aarestrup, F. (Ed.). ASM Press, Washington, pp. 49-72.
- Malvisi, J., Rocca, G.d., Anfossi, P., Giorgetti, G., 1996. Tissue distribution and residue depletion of oxytetracycline in sea bream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*) after oral administration. *Aquaculture*. 147, 159-168.
- Malvisi, J., Rocca, G.d., Anfossi, P., Giorgetti, G., 1997. Tissue distribution and depletion of flumequine after in-feed administration in sea bream (*Sparus aurata*). *Aquaculture*. 157, 197-204.
- Malvisi, J., Della Rocca, G., Anfossi, P., Tomasi, L., Di Salvo, A., Zanchetta, S., Magni, A., Sello, M., Giorgetti, G., 2002. Tissue distribution and residue depletion of thiamphenicol after multiple oral dosing in seabass (*Dicentrarchus labrax* L.) and seabream (*Sparus aurata* L.). *Journal of Applied Ichthyology*. 18, 35-39.

D3.3-Therapeutics for MMFF

- Manera, M., Dezfuli, B., 2003. *Lernanthropus kroyeri* infections in farmed sea bass *Dicentrarchus labrax*: Pathological features. *Diseases of Aquatic Organisms*. 57, 177-180.
- Mathieu-Daude F., Faye N., Coste F., Monier J. F., Marques A., Bouix G. 1992. Occurrence of a microsporidiosis in marine cultured gilthead sea bream from the Languedoc Coast: a problem of specificity in the genus *Glugea* (Protozoa, Microspora). *Bulletin of the European Association of Fish Pathologists* 12, (SI-IO).
- Mladineo, I. 2002. Prevalence of *Ceratomyxa oestroides* (Risso, 1826), a cymothoid isopode parasite, in cultured sea bass *Dicentrarchus labrax* L., on two farms in middle Adriatic Sea. *Acta Adriat.* 43:97-102.
- Montgomery-Brock, Diana & T. Sato, Vernon & Brock, James & Tamaru, Clyde. 2007. The Application of Hydrogen Peroxide as a Treatment for the Ectoparasite *Amyloodinium ocellatum* (Brown 1931) on the Pacific Threadfin *Polydactylus sexfilis*. *Journal of the World Aquaculture Society*. 32. 250 - 254.
- Morsy, K., Rahman Bashtar, A., Abdel-Ghaffar, F., Al-Quraishy, S., 2013. Morphological and phylogenetic description of a new xenoma-inducing microsporidian, *Microsporidium aurata* nov. sp., parasite of the gilthead seabream *Sparus aurata* from the Red Sea. *Parasitology Research*. 112.
- Mazzolini, E., Vismara, D., Geschia, G., Malvisi, J., Fabris, A., Passera, A., Danielis, L., Giorgetti, G.I., 1997. In vitro activity of several antibiotics against clinical isolated of marine fish pathogens, *Int. Workshop: "Aquaculture Application of Controlled Grud and Vaccine Delivery"*, 21-23 May, 1997, Udine, Italy.
- McKellar, Q.A., Sanchez, B.S.F., Jones, D.G., 2004. Pharmacokinetic/pharmacodynamic relationships of antimicrobial drugs used in veterinary medicine. *Journal of Veterinary Pharmacology and Therapeutics*. 27, 503-514.
- Meemansha, S., Dumka, V., 2018. Pharmacokinetics of lincomycin following intravenous administration in febrile goats. *Indian J. Anim. Res.* 52, 605-609.
- Mehlhorn H, G Schmahl and A Haberkorn 1988. Toltrazuril effective against a broad spectrum of protozoan parasites. *Parasitology Research* 75(1):64-6.
- Miller, R.A., Reimschuessel, R., 2006. Epidemiologic cutoff values for antimicrobial agents against *Aeromonas salmonicida* isolates determined by frequency distributions of minimal inhibitory concentration and diameter of zone of inhibition data. *American Journal of Veterinary Research*. 67, 1837-1843.
- Miranda, C.D., Godoy, F.A., Lee, M.R., 2018. Current Status of the Use of Antibiotics and the Antimicrobial Resistance in the Chilean Salmon Farms. *Front Microbiol.* 2018 Jun 18;9:1284.
- Nilsen, A., Nielsen, K.V, Biering, E., Bergheim, A (2017). Effective protection against sea lice during the production of Atlantic salmon in floating enclosures. *Aquaculture*, 466, 41-50.
- Odenholt, I., 2001. Pharmacodynamic effects of subinhibitory antibiotic concentrations. *International Journal of Antimicrobial Agents*. 17, 1-8.
- Ogut, H., Uzun, E., 2014. Incidence and prevalence of *Diplectanum aequans* and its influence on the fitness of juvenile sea bass (*Dicentrarchus labrax*) in the Black Sea. *Aquaculture Research*. 45, 742-748.
- Öktener, A., Alaş, A., Solak, K., 2009. Occurrence of *Diplectanum aequans* (Wagener, 1857) on the cultured sea bass, *Dicentrarchus labrax* (Linnaeus, 1758) from the Black Sea of Turkey. *Bulletin of the European Association of Fish Pathologists*. 29, 102-103.
- Overton, K. , Samsing, F. , Oppedal, F. , Stien, L. H. and Dempster, T. 2018., Lowering treatment temperature reduces salmon mortality: a new way to treat with hydrogen peroxide in aquaculture. *Pest. Manag. Sci.* 74: 535-540
- Öztürk, R.Ç., Altınok, I., 2014. Bacterial and Viral Fish Diseases in Turkey. *Turkish Journal of Fisheries and Aquatic Sciences*. 14:, 275-297.
- Palenzuela, O., Alvarez-Pellitero, P., Sitjà-Bobadilla, A., 1999. Glomerular disease associated with *Polysporoplasma sparis* (Myxozoa) infections in cultured gilthead sea bream, *Sparus aurata* L. (Pisces: Teleostei). *Parasitology*. 118 (Pt 3), 245-256.
- Palenzuela, O., Sitjà-Bobadilla, A., Alvarez-Pellitero, P. 1997. *Ceratomyxa sparusaurati* (Protozoa: Myxosporidia) infections in cultured gilthead sea bream *Sparus aurata* (Pisces: Teleostei) from Spain: aspects of the host-parasite relationship. *Parasitol. Res.* 83:539-548, 1997.
- Palenzuela, O., Redondo, M., Cali, A., Takvorian, P., Alonso-Naveiro, M., Alvarez-Pellitero, P., Sitjà-Bobadilla, A., 2014. A new intranuclear microsporidium, *Enterosporea nucleophila* n. sp.,

D3.3-Therapeutics for MMFF

- causing an emaciative syndrome in a piscine host (*Sparus aurata*), prompts the redescription of the family Enterocytozoonidae. *International Journal for Parasitology*, 44:189-203.
- Palenzuela, O., del Pozo, R., Piazzon, M.C., Isern-Subich, M.M., Ceulemans, S., Coutteau, P., Sitjà-Bobadilla, A., 2017. Functional feed additives can reduce the impact of an *Enteromyxum leei* infection on performance and disease severity: evidence from an experimental challenge with gilthead sea bream. *International Aquafeed*:14–1.
- Papapanagiotou, E.P., Batzias, G.C., Iossifidou, E.G., Psomas, I.E., 2002. Sulfadimethoxine and Ormetoprim residue study in cultured gilthead sea bream (*Sparus aurata*, L.) *Revue de Medecine Veterinaire*. 152, 669-674.
- Papapanagiotou, E., P Trilles, J., 2001. Cymothoid parasite *Ceratothoa parallela* inflicts great losses on cultured gilthead sea bream *Sparus aurata* in Greece. *Diseases of Aquatic Organisms*. 45, 237-239.
- Papapanagiotou, E., P Trilles, J., Photis, G., 1999. First record of *Emetha audouini*, a cymothoid isopod parasite, from cultured sea bass *Dicentrarchus labrax* in Greece. *Diseases of Aquatic Organisms*. 38, 235-237.
- Paperna, I. 1980. Study of *Caligus minimus* (Otto, 1821), (Caligidae Copepoda) infections of the sea bass *Dicentrarchus labrax* (L.) in Bardawil lagoon. *Ann. Parasitol.* 55:687-706.
- Papich, M.G., 2016. Lincomycin Hydrochloride, Lincomycin Hydrochloride Monohydrate. in: Papich, M.G. (Ed.), *Saunders Handbook of Veterinary Drugs (Fourth Edition)*. W.B. Saunders, St. Louis, pp. 450-451.
- Parra, L., García, L., Sendra, S., Lloret, J., 2018. The Use of Sensors for Monitoring the Feeding Process and Adjusting the Feed Supply Velocity in Fish Farms 2018, *Journal of Sensors*, Volume 2018, Article ID 1060987, 14 pages, <https://doi.org/10.1155/2018/1060987>.
- Pepin, J.-F., Emery, E., 1993. Marine cytophaga-like bacteria (CLB) isolated from diseased reared sea bass (*Dicentrarchus labrax* L.) from French mediterranean coast. *Bulletin of the European Association of Fish Pathologists*. 13, 165-167.
- Pereira, J. C., Abrantes, I., Martins, I., Barata, J., Frias, P., Pereira I., 2011. Ecological and morphological features of *Amyloodinium ocellatum* occurrences in cultivated gilthead seabream *Sparus aurata* L.: A case study. *Aquaculture*, 310: 289-297.
- Pérez-Sánchez, J., Bermejo-Nogales, A., Benedito-Palos, L., Estensoro, I., Petropoulos, Y., Browdy, C.L., Sitjà-Bobadilla, A. 2015. Effects of dietary NE[®]150 on growth performance, antioxidant status and expression of immune and intestinal integrity related genes in gilthead sea bream (*Sparus aurata* L.). *Fish & Shellfish Immunology* 44:117-128.
- Piazzon, M.C., Galindo-Villegas, J., Pereiro, P., Estensoro, I., Calduch-Giner, J.A, Gómez-Casado, E., Novoa, B., Mulero, V., Sitjà-Bobadilla, A., Pérez-Sánchez, J. 2016. Differential modulation of IgT and IgM upon parasitic, bacterial, viral and dietary challenges in a perciform fish. *Frontiers in Immunology*, 7: 637.
- Piazzon, M.C., Calduch-Giner, J.A., Fouz, B., Estensoro, I. Simó-Mirabet, P., Puyalto, M., Karalazos, V., Palenzuela, O., Sitjà-Bobadilla, A*, Pérez-Sánchez, J. (2017). Under control: how a dietary additive can restore the gut microbiome and proteomic profile, and improve disease resilience in a marine teleostean fish fed vegetable diets. *BMC Microbiome*, 5 (164):18-23.
- Picard-Sánchez, A., Estensoro, I., Del Pozo, R., Piazzon, M.C., Palenzuela, O. Sitjà-Bobadilla, A. 2019. Acquired protective immune response in a fish-myxozoan model encompasses specific antibodies and inflammation resolution. *Fish & Shellfish Immunology*, 90: 349-362.
- Poher, I., Blanc, G., Lousouarn, S., 2003. Pharmacokinetics of oxolinic acid in sea-bass, *Dicentrarchus labrax* (L., 1758), after a single rapid intravascular injection. *Journal of Veterinary Pharmacology and Therapeutics*. 20, 267-275.
- Poley, J. D., Braden, L. M., Messmer, A. M., Igboeli, O. O., Whyte, S. K., Macdonald, A., ... Fast, M. D. (2018). High level efficacy of lufenuron against sea lice (*Lepeophtheirus salmonis*) linked to rapid impact on moulting processes. *International journal for parasitology. Drugs and drug resistance*, 8(2), 174–188.
- Pujalte, M.J., Sitjà-Bobadilla, A., Macián, M.C., Belloch, C., Álvarez-Pellitero, P., Pérez-Sánchez, J., Uruburu, F., Garay, E., 2003. Virulence and Molecular Typing of *Vibrio harveyi* Strains Isolated from Cultured Dentex, Gilthead Sea Bream and European Sea Bass. *Systematic and Applied Microbiology*. 26, 284-292.

D3.3-Therapeutics for MMFF

- Ragias, V., Tontis, D., Athanassopoulou, F. 2004 Incidence of an intense *Caligus minimus* Otto 1821, *C.pageti* Russel, 1925, *C.mugilis* Brian, 1935 and *C.apodus* Brian, 1924 infection in lagoon cultured sea bass (*Dicentrarchus labrax* L.) in Greece. *Aquaculture* 242:727-733.
- Rangel, L.F., Rocha, S., Borkhanuddin, M.H., Cech, G., Castro, R., Casal, G., Azevedo, C., Severino, R., Székely, C., Santos, M.J. 2014. *Ortholinea aurata* n. sp. (Myxozoa, Ortholineidae) infecting the urinary bladder of the gilthead seabream *Sparus aurata* (Teleostei, Sparidae), in a Portuguese fish farm. *Parasitol Res.* 113:3427-37.
- Riera, E. 2018. Use of cleaner fish as biological control for parasites in aquaculture and the evaluation of new species for Mediterranean Aquaculture. Bachelor's thesis. Degree in veterinary medicine. Facultat de Veterinaria. Universitat Autònoma de Barcelona
- Rigos, G., Troisi, G.M., 2005. Antibacterial Agents in Mediterranean Finfish Farming: A Synopsis of Drug Pharmacokinetics in Important Euryhaline Fish Species and Possible Environmental Implications. *Reviews in Fish Biology and Fisheries.* 15, 53-73.
- Rigos, G., Alexis, M., Nengas, I., 1999. Leaching, palatability and digestibility of oxytetracycline and oxolinic acid included in diets fed to seabass *Dicentrarchus labrax* L. *Aquaculture Research.* 30, 841-847.
- Rigos, G., Nengas, I., Alexis, M., 2006. Oxytetracycline (OTC) uptake following bath treatment in gilthead sea bream (*Sparus aurata*). *Aquaculture.* 261, 1151-1155.
- Rigos, G., Alexis, M., Andriopoulou, A., Nengas, I., 2002a. Pharmacokinetics and tissue distribution of oxytetracycline in sea bass, *Dicentrarchus labrax*, at two water temperatures. *Aquaculture.* 210, 59-67.
- Rigos, G., Fountoulaki, E., Cotou, E., Dotsika, E., Dourala, N., Karacostas, I., 2013. Tissue distribution and field evaluation of caprylic acid against natural infections of *Sparicotyle chrysophrii* in cage-reared gilthead sea bream *Sparus aurata*. *Aquaculture.* s 408–409.
- Rigos, G., Alexis, M., Andriopoulou, A., Nengas, I., 2002c. Temperature-dependent pharmacokinetics and tissue distribution of oxolinic acid in sea bass, *Dicentrarchus labrax* L., after a single intravascular injection. *Aquaculture Research.* 33, 1175-1181.
- Rigos, G., Tyrpenou, A., Nengas, I., Alexis, M., 2002d. A pharmacokinetic study of flumequine in sea bass, *Dicentrarchus labrax* (L.), after a single intravascular injection. *Journal of Fish Diseases.* 25, 101-105.
- Rigos, G., Nengas, I., Athanassopoulou, E., Alexis, M., 2004b. Bioavailability of oxytetracycline in sea bass, *Dicentrarchus labrax* (L.). *Journal of Fish Diseases.* 27, 119-122.
- Rigos, G., Nengas, I., Alexis, M., Tyrpenou, A.E., Troisi, G.M., 2003b. Tissue distribution and residue depletion of oxolinic acid in gilthead sea bream (*Sparus aurata*) and sharpnose sea bream (*Diplodus puntazzo*) following multiple in-feed dosing. *Aquaculture.* 224, 245-256.
- Rigos, G., Nengas, I., Tyrpenou, A.E., Alexis, M., Troisi, G.M., 2003c. Pharmacokinetics and bioavailability of oxytetracycline in gilthead sea bream (*Sparus aurata*) after a single dose. *Aquaculture.* 221, 75-83.
- Rigos, G., Mladineo, I., Nikoloudaki, C., Vrbatovic, A., Kogiannou, D., 2016. Application of compound mixture of caprylic acid, iron and mannan oligosaccharide against *Sparicotyle chrysophrii* (Monogenea: Polyopisthocotylea) in gilthead sea bream, *Sparus aurata*. *Folia Parasitologica.* 63.
- Rigos, G., Alexis, M., Tyrpenou, A.E., Nengas, I., Piper, I., Troisi, G., 2002b. Pharmacokinetics of oxolinic acid in gilthead sea bream, *Sparus aurata* L. *Journal of Fish Diseases.* 25, 401-408.
- Rigos, G., Tyrpenou, A., Nengas, I., Alexis, A.E.M., Athanassopoulou, F., Troisi, G.M., 2004a. Poor bioavailability of oxytetracycline in sharpnose sea bream *Diplodus puntazzo*. *Aquaculture.* 235, 489-497.
- Rigos, G., Fountoulaki, E., Cotou, E., Dotsika, E., Dourala, N., Karacostas, I., 2013. Tissue distribution and field evaluation of caprylic acid against natural infections of *Sparicotyle chrysophrii* in cage-reared gilthead sea bream *Sparus aurata*. *Aquaculture.* s 408–409.
- Rigos, G., Athanassios, E.T., Ioannis, N., Maria, Y., Maria, K., Maria, A., Gera, M.T., 2003a. Pharmacokinetics of flumequine and in vitro activity against bacterial pathogens of gilthead sea bream *Sparus aurata*. *Diseases of Aquatic Organisms.* 54, 35-41.
- Rigos, G., Zonaras, V., Nikoloudaki, X., Cotou, E., Henry, M., Varo, I., Alexis, M., 2013. Distribution and depletion of sulfadiazine after a multiple per os dosing in gilthead sea bream (*Sparus aurata*) fed two different diets. *Mediterranean Marine Science.* 14, 377-383.

D3.3-Therapeutics for MMFF

- Romero Gonzales, R., Fernandez Fernandez, R., Vidal, J.L.M., Muros, M.J.S., Frenich, A.G., 2010. Depletion of Veterinary Drugs Used in Aquaculture after Administration in Feed to Gilthead Seabream (*Sparus aurata*). *Journal of Food Protection*. 73, 1664-1670.
- Rosa, J., Leston, S., Castro, M., Freitas, A., Barbosa, J., Pardal, M.Â., Rema, P., Dias, J., Ramos, F., 2018. Evaluation of antimicrobials residues in farmed gilthead seabream (*Sparus aurata*) after admSchmahl G, Mehlhorn H. 1988. Treatment of fish parasites. 4. Effects of sym. triazinone (toltrazuril) on *Monogenea Parasitol Res.* 75(2):132-43.
- Roubal, F.R., 1994. Attachment of eggs by *Lamellodiscus acanthopagri* (*Monogenea: Diplectanidae*) to the gills of *Acanthopagrus australis* (*Pisces: Sparidae*), with evidence for auto-infection and postsettlement migration. *Can J Zool* 72:87–95.
- Sanders, J.J., Watral, V., Kent, M.L. 2012. Microsporidiosis in zebrafish research facilities. *ILAR J.* 2012 ; 53: 106–113. doi:10.1093/ilar.53.2.106.
- Santos, M.J., Cavaleiro, F., Campos, P., Sousa, A., Teixeira, F., Martins, M., 2010. Impact of amoeba and scuticociliatidia infections on the aquaculture European sea bass (*Dicentrarchus labrax* L.) in Portugal. *Veterinary Parasitology*. 171, 15-21.
- Sarusic, G., 1999. Preliminary report of infestation by isopod *Ceratothoa oestroides* (Risso,1826), in marine cultured fish. *Bulletin of the European Association of Fish Pathologists*. 19, 110-112.
- Seoud, S.S., Zaki, V.H., Ahmed, G.E., Abd El-Khalek, N.K. 2017. Studies on *Amyloodinium* infestation in European seabass (*Dicentrarchus labrax*) fishes with special reference for treatment. *International Journal of Fisheries and Aquatic Studies* 5: 276-287.
- Schmahl G, Mehlhorn H, Taraschewski H. 1989. Treatment of fish parasites: 5. The effects of sym. triazinone (Toltrazuril) on fish parasitic ciliophora (*Ichthyophthirius multifiliis* FOUQUET, 1876, *Apiosoma amoebae* GRENFELL, 1884, *Trichodina* sp. EHRENBERG, 1831). *Eur J Protistol.* 1989 Feb 24;24(2):152-61. inistration through medicated feed. *Food Control*. 86, 110-116.
- Schmidt, S., Barbour, A., Sahre, M., Rand, K.H., Derendorf, H., 2008. PK/PD: new insights for antibacterial and antiviral applications. *Current Opinion in Pharmacology*. 8, 549-556.
- Serdoz, F., Voinovich, D., Perissutti, B., Grabnar, I., Hasa, D., Ballestrazzi, R., 2010. An innovative Oxytetracycline self-emulsifying formulation for fish diets: preparation, characterisation and oral bioavailability in rainbow trout (*Oncorhynchus mykiss*) and in European sea bass (*Dicentrarchus labrax*). *Journal of Drug Delivery Science and Technology*. 20, 431-437.
- Shinn, A.P., Pratoomyot, J., Bron, J.E., Paladini, G., Brooker, E.E., Brooker, A.J. 2015. Economic impacts of aquatic parasites on global finfish production. *Global Aquaculture Advocate* 18, 58-61.
- Shojaee AliAbadi, F., Lees, P., 2000. Antibiotic treatment for animals: effect on bacterial population and dose regime optimisation. *International Journal of Antimicrobial Agents*. 14, 307–313.
- Sitjà-Bobadilla, A., Alvarez-Pellitero, 1992. Effect of Fumagillin treatment on sea bass (*Dicentrarchus labrax* L.) parasitized by *Sphaerospora testicularis* (*Myxosporea Bivalvulida*). *Dis. Aquat.Org.*,14: 171-178.
- Sitjà-Bobadilla, A. & Alvarez-Pellitero, P. 1993. Population dynamics of *Sphaerospora dicentrarchi* Sitjà-Bobadilla et Alvarez-Pellitero, 1992 and *S. testicularis* Sitjà-Bobadilla et Alvarez-Pellitero, 1990 (*Myxosporea: Bivalvulida*) infections in wild and cultured sea bass (*Dicentrarchus labrax* L.): *Parasitology* 106: 39-45.
- Sitjà-Bobadilla, A. & Alvarez-Pellitero, P. 2001. *Leptotheca sparidarum* n. sp. (*Myxosporea: Bivalvulida*), a parasite from cultured common dentex (*Dentex dentex* L.) and gilthead sea bream (*Sparus aurata* L.) (*Teleostei. Sparidae*). *J. Eukaryot. Microbiol.*, 48: 627-639.
- Sitjà-Bobadilla, A., Palenzuela, O. & Alvarez-Pellitero, P. 1996. Light microscopic description of, *Eimeria sparis* sp. nov. and *Goussia sparis* sp. nov. (*Protozoa: Apicomplexa*) from gilthead sea bream (*Sparus aurata* L.) (*Pisces: Teleostei*). *Parasitol. Res.*, 82: 323-332.
- Sitjà-Bobadilla, A., de Felipe, M.C., Alvarez-Pellitero, P., 2006. In vivo and in vitro treatments against *Sparicotyle chrysophrii* (*Monogenea: Microcotylidae*) parasitizing the gills of gilthead sea bream (*Sparus aurata* L.). *Aquaculture*. 261, 856-864.
- Sitjà-Bobadilla, A., Redondo, M.J., Alvarez-Pellitero, P., 2010. Occurrence of *Sparicotyle chrysophrii* (*Monogenea: Polyopisthocotylea*) in gilthead sea bream (*Sparus aurata* L.) from different mariculture systems in Spain. *Aquaculture Research*. 41, 939-944.
- Sitjà-Bobadilla, A., Palenzuela, O. 2012. *Enteromyxum* species. In: "Fish Parasites: Pathobiology and Protection". Chapter 9: 163-176, CABI Publishing. Eds: P.T.K. Woo & K. Buchmann.
- Sitjà-Bobadilla, A., Zarza, C. Fouz, B. 2014. Pathology. In: "The Biology of sea bass". Ed. F.J. Sánchez-Vázquez & J.A. Muñoz-Cueto. CRC Press, Boca Ratón. pp. 287-341.

D3.3-Therapeutics for MMFF

- Smith, P., 2008. A cost-benefit analysis of the application of pharmacokinetic/pharmacodynamic-based approaches to setting disc diffusion breakpoints in aquaculture: A case study of oxolinic acid and *Aeromonas salmonicida*. *Aquaculture*. 284, 2-18.
- Smith, P., Hiney, M., Samuelsen, O.B., 1994. Bacterial resistance to anti-microbial agents used in fish farming; a critical evaluation on method and meaning. *Annual Reviews of Fish Diseases*. 4, 273-313.
- Smyrli, M., Prapas, A., Rigos, G., Kokkari, C., Pavlidis, M., Katharios, P., 2017. *Aeromonas veronii* Infection Associated with High Morbidity and Mortality in Farmed European Seabass *Dicentrarchus labrax* in the Aegean Sea, Greece.
- Sreeshitha Gouri, S., Venkatachalam, D.V., Dumka, V., 2014. Pharmacokinetics of lincomycin following single intravenous administration in buffalo calves. *Tropical Animal Health and Production*. 46.
- Stamm, J.M., 1989. In vitro resistance by fish pathogens to aquacultural antibacterials, including the quinolones difloxacin (A-56619) and sarafloxacin (A-56620). *Journal of Aquatic Animal Health*. 1, 135-141.
- Terceti, M.S., Ogut, H., Osorio, C.R., 2016. *Photobacterium damsela* subsp. *damsela*, an emerging fish pathogen in the Black Sea: evidence of a multiclonal origin. *Applied and Environmental Microbiology*. 82, 3736-3745.
- Toksen, E., 2007. Treatment trials of gill parasite *Diplectanum aequans* (Monogenea: Diplectanidae) of cultured gilthead sea bass (*Dicentrarchus labrax*) in Aegean sea. *Ekoloji*. 16, 66-71.
- Toksen, E., Cagirgan, H., Tanrikul, T., Saygi, H., 2006. The effect of emamectin benzoate in the control of *Lernanthropus kroyeri* (van Beneden, 1851) (Lernanthropidae) infestations in cultured sea bass, *Dicentrarchus labrax* (Linnaeus, 1758). *Turkish Journal of Veterinary and Animal Sciences*. 30, 405-409.
- Tokşen, E., Değirmenci, U., Cankurt, M. 2010. The effect of Trichlorfon on the control of *Lernanthropus kroyeri* (van Beneden, 1851) (Lernanthropidae) infestations in Cultured Sea Bass, *Dicentrarchus labrax* (Linnaeus, 1758). *Bulletin of the European Association of Fish Pathologists* 30(6) 205-210
- Toksen, E, Nemli, E, Degirmenci, Ugur, Karacalar, U. 2013. The effects of azamethiphos and trichlorfon on the control of *Diplectanum aequans* (Monogenea: Diplectanidae) infestations in cultured broodstock sea bass, *Dicentrarchus labrax*. *Bulletin of the European Association of Fish Pathologists* 33(5) 144-149
- Toksen, E., Cagirgan, H., Tanrikul, T., Saygi, H., 2006. The effect of emamectin benzoate in the control of *Lernanthropus kroyeri* (van Beneden, 1851) (Lernanthropidae) infestations in cultured sea bass, *Dicentrarchus labrax* (Linnaeus, 1758). *Turkish Journal of Veterinary and Animal Sciences*. 30, 405-409.
- Tokşen, E, Nemli, E. Cankurt, M. 2009. The effect of Teflubenzuron on the control of *Lernanthropus kroyeri* (van Beneden, 1851) (Lernanthropidae) infestations in cultured sea bass, *Dicentrarchus labrax* (Linnaeus, 1758) *Bull. Eur. Ass. Fish Pathol.*, 29(6) 205-209.
- Toranzo, A.E., Barreiro, S.n., Casal, J.F., Figueras, A., Magarinos, B., Barja, J.L., 1991. Pasteurellosis in cultured gilthead seabream (*Sparus aurata*): first report in Spain. *Aquaculture*. 99, 1-15.
- Toutain, P.L., Bousquet-Melou, A., Martinez, M., 2007. AUC/MIC: A PK/PD index for antibiotics with a time dimension or simply a dimensionless scoring factor? *Journal of Antimicrobial Chemotherapy*. 60, 1185-1188.
- Tyrpenou, A.E., Kotzamanis, Y.P., Alexis, M.N., 2003. Flumequine depletion from muscle plus skin tissue of gilthead seabream (*Sparus aurata* L.) fed flumequine medicated feed in seawater at 18 and 24 °C. *Aquaculture*. 220, 633-642.
- Tyrpenou, A.E., Iossifidou, E.G., Psomas, I.E., Fotis, G.D., 2002. DETERMINATION OF SARAFLOXACIN RESIDUES IN GILTHEAD SEABREAM (*SPARUS AURATA* L.) TISSUES BY HPLC-SFD-PDA. *Journal of Liquid Chromatography & Related Technologies*. 25, 747-758.
- Ucko, M., Colorni, A., 2005. *Mycobacterium marinum* Infections in Fish and Humans in Israel.
- Ucko, M., Colorni, A., Kvitt, H., Diamant, A., Zlotkin, A., Knibb, W.R., 2002. Strain Variation in *Mycobacterium marinum* Fish Isolates. *Applied and Environmental Microbiology*. 68, 5281-5287.
- Uzun, E., Ogut, H., 2015. The isolation frequency of bacterial pathogens from sea bass (*Dicentrarchus labrax*) in the Southeastern Black Sea. *Aquaculture*. 437, 30-37.
- Vagianou, S., Athanassopoulou, F., Ragias, V., Di Cave, D., Leontides, L., Golomazou, E. 2006a. Prevalence and pathology of ectoparasites of Mediterranean sea bream and sea bass reared

- under different environmental and aquaculture conditions. *Israeli Journal of Aquaculture-Bamidgheh* 58:78-88.
- Vagianou, S., Bitchava, K. and Athanassopoulou, F. 2006b. Sea lice (*Ceratothoa oestroides*), (Risso, 1826), infestation in Mediterranean aquaculture: new information. *J. Hell. Vet. Med. Soc.* 57: 223-229.
- Vagianou, S., Bitchava K., Athanassopoulou, F. 2017. Sea lice (*Ceratothoa oestroides*), (Rissa, 1826), infestation in Mediterranean aquaculture: new information. *Journal of the Hellenic Veterinary Medical Society*, 57, 223-229.
- Vallejos-Vidal, E., Reyes-López, F., Teles, M., MacKenzie, S. (2016) The response of fish to immunostimulant diets. *Fish & Shellfish Immunology* 56: 34-69,
- Vardali, S.C., Kotzamanis, Y.P., Tyrpenou, A.E., Samanidou, V.F., 2017. Danofloxacin depletion from muscle plus skin tissue of European sea bass (*Dicentrarchus labrax*) fed danofloxacin mesylate medicated feed in seawater at 16°C and 27°C. *Aquaculture*. 479, 538-543.
- Vendramin, N., Zrncic, S., Padrós, F., Oraic, D., Le Breton, A., Zarza, C., Olesen, N., 2016. Fish health in Mediterranean Aquaculture, past mistakes and future challenges. *Bulletin of the European Association of Fish Pathologists*. 36, 38-45.
- WHO, 2017. Guidelines on use of medically important antimicrobials in food-producing animals, World Health Organization 2017, ISBN 978-92-4-155013-0
- Xu, D., Rogers, W.A., 1994. Leaching Loss from Oxytetracycline Medicated Feeds. *Journal of Applied Aquaculture*. 4, 29-38.
- Xu, N. , Gong, B. , Dong, J. , Yang, Y., Ai, X. 2016, Single Intravascular and Oral Dose Pharmacokinetics of Mebendazole in Blunt Snout Bream, *Megalobrama amblycephala*. *J World Aquacult Soc*, 47: 685-690.
- Yardimci, R., Timur, G., 2015. Isolation and Identification of *Tenacibaculum maritimum*, the Causative Agent of Tenacibaculosis in Farmed Sea Bass (*Dicentrarchus labrax*) on the Aegean Sea Coast of Turkey.
- Yavuzcan, H, Ergonul, M. 2010. Is prophylactic formalin exposure a stress source for gilthead sea bream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*)?. *Ankara Üniversitesi Veteriner Fakültesi Dergisi* 57, 113-118 .
- Yiagnisis, M., Kolygas, M., Athanassopoulou, F., 2016. EFFECTS OF OREGANO ESSENTIAL OIL ON ENTEROMYXUM LEEI (MYXOZOA) INFECTION ON GILTHEAD SEA BREAM (*SPARUS AURATA L.*) *Aquaculture Europe EAS*, Edinburgh, Scotland.
- Yokoyama, H. and Shirakashi, S. 2007. Evaluation of hyposalinity treatment on infection with *Enteromyxum leei* (Myxozoa) using anemonefish *Amphiprion* spp. as experimental host. *Bulletin of the European Association of Fish Pathologists* 2,74-78.
- Zlotkin, A., Hershko, H., Eldar, A., 1998. Possible Transmission of *Streptococcus iniae* from Wild Fish to Cultured Marine Fish. *Applied and Environmental Microbiology*. 64, 4065-4067.
- Zonaras, V., Tyrpenou, A., Alexis, M., Koupparis, M., 2016. Determination of sulfadiazine, trimethoprim, and N4-acetyl-sulfadiazine in fish muscle plus skin by Liquid Chromatography–Mass Spectrometry. Withdrawal-time calculation after in-feed administration in gilthead sea bream (*Sparus aurata L.*) fed two different diets. *Journal of Veterinary Pharmacology and Therapeutics*. 39, 504-513.
- Zorrilla, I., Moriñigo, M.A., Castro, D., Balebona, M.C., Borrego, J.J., 2003. Intraspecific characterization of *Vibrio alginolyticus* isolates recovered from cultured fish in Spain. *Journal of Applied Microbiology*. 95, 1106-1116.