Universitat Autònoma de Barcelona

Fouling management in cage aquaculture. New strategies on nets, fish health and economic impact

Jordi Comas Morte

PHD.THESIS
PROGRAMA DE DOCTORAT EN AQÜICULTURA, U.A.B
PROGRAMA DE DOCTORATS INDUSTRIALS, GENERALITAT DE CATALUNYA
2018

Fouling management in cage aquaculture

New strategies, effects on nets, fish health and economic impact

JORDI COMAS MORTE

Dpt. Cell Biology, Physiology and Immunology (Biociències)
Universitat Autònoma de Barcelona

DIRECTORS

LLUÍS TORT BARDOLET
DAVID PARRA GÓMEZ

Als meus pares,

Als meus fills, la Itzel, l'Otger, l'Oleguer i la petita Lucía…la Sileta

A la Marieta

Contents

Preface 06

Abstract 07

Resum 09

Aknowledgements 15

List of tables 17

List of figures 17

Abbreviations and acronyms 19

General Introduction 21

The progress of Aquaculture 21
Fouling in cage aquaculture 24
Effects on farm structures and fish 25
Fouling control strategies in aquaculture 28

Aims of the thesis 33

Materials and methods 35

Study area 35
Experimental conditions 35
Fish husbandry and feeding 38
Water quality monitoring and copper determination in water 38
Cage and net operations 39
Sampling schedule 40
Skin mucus, blood collection, and tissue sampling 41

On-site cleaning strategy efficiency and effects on nets 43

Introduction 43
Materials and methods 44
Results 47
Discussion 50
Conclusions 52

Growth and health features 53

Introduction 53
Materials and methods 55
Results 58
Discussion 64
Conclusions 69

Fish welfare, primary and secondary stress responses 71

Fish welfare
Introduction 71
Materials and methods 74

The primary stress response
Introduction 77
Materials and methods 79
Results 80
Discussion 82
Conclusions 84

The secondary stress response

The metabolic response
Blood and skin mucus biochemical parameters as stress and health status indicators 86
Materials and methods 87
Results 88
Discussion 92
Conclusions 95

The immune response
Introduction 86
Materials and methods 101
Results 103
Discussion 108
Conclusions 110

The antioxidant response
Introduction 112
Materials and methods 114
Results 114
Discussion 119
Conclusions 120
Chapter conclusions 121

Economic impact of the on-site cleaning strategy. A basic approach 123

Sea bream production cost structure in Spain 123
Net and treatment effect 124
Operational effect 126
Conclusions 127

General discussion and conclusions 131

References 140

Annexes 153

Preface

The academy and the industry, still today, not always work and walk together. Basic science and research provide the knowledge and the tools to its more applicable areas which must give scientific and consistent answers to global progress worldwide in a knowledge transfer process that not always occurs.

The origin of this thesis is an agreement between the academy and the industry, the entire project has been partially funded by an Industrial PhD program from the Catalan Government (Programa de Doctorats Industrials 2014) specifically oriented and designed to promote this kind of initiatives. In the abbreviations list of this work appear together words like EBITDA or PAMPs giving an idea of the broad range of aspects, from mechanical to immuno-physiological and even economics that have been here studied, giving a smell of multidisciplinary work to this thesis. The general purpose of this study has been to get a results outcome easy transferable to the industry's uncovered needs.

When designing the work, it was mandatory to think in an experimental setting under real working conditions as the pursued results had to reflect what actually happens at commercial scale. Although, from the academic point of view this might represent some difficulties as often what occurs outside the laboratory is difficult to control.

From the very beginning the project was courageously engaged from all the partners and the final result has fulfilled the expectations of all of them. The obtained results will provide consistency and scientific support to the industry in their decision making as well as indicate future directions in fouling control. Both the PhD student and the University department have gained a worthy experience conducting research in an industrial real working environment and have generated network for further collaboration.

Abstract

There is an open debate in the aquaculture industry regarding fouling management strategies. The primary aim of this study was to contrast a newly implemented technology, the on-site cleaning, to the existing and widely used copper based antifouling paints to more efficiently control an issue that affects fish farming worldwide, the fouling production. The experimental work was planned and conducted in real working conditions simulating part of a sea bream growth cycle. Despite knowing that working in field conditions might represent some difficulties, it was thought that the obtained results would much better explain what happens in the real life rather than those from in vitro experiments or in laboratory conditions. As the study was set within an industrial PhD framework a wide scope investigation was considered, and therefore, a wide range of parameters including the effects on nets, fish health and performance were analyzed prior to very basically approaching the situation from the economic impact point of view.

Nets are the strongbox that protect the most important asset of pisciculture companies, the biomass. For this reason, in this work, the efficiency of paint and coating products was analyzed, and special attention was paid to the effect of cleaning on the essential property of the nets, their resistance. Afterwards, those aspects resulting from the different rearing conditions generated by one or the other fouling management strategy and that were thought to have an effect on health, and performance, of the fish were studied. Growth was analyzed, as one of the main goals of the aquaculture industry, but also the presence of parasites, specifically the monogenean Sparicotyle chrysophrii (Monogenea: Polyopisthocotylea) causing high damage to the sea bream industry throughout the Mediterranean, and the skin microbiota, since the proliferation of these organisms is often associated with the hygienic status of the nets. Additionally, a histological study in gills was performed to determine the magnitude of the possible effects of different maneuvers on this tissue, which is essential for the survival of fish due to its multifunctionality. The content of copper in gills, liver and muscle was also analyzed, not only to see if it could pose a danger to animals, but also because of the concern that heavy metal accumulation generates among fish consumers. We also wanted to see whether the two strategies tested had different effects on the animals in terms of welfare impairment and stress response. Therefore, the main indicator of the primary stress response, cortisol, as well as those of the subsequent secondary responses (metabolic, immunological and antioxidant responses) were analyzed in plasma and

skin mucus. We also wondered if the two strategies caused differences at molecular level, so we analyzed the modulation of the gene expression of those genes involved in the stress response. This analysis was carried out in liver, gills and spleen, as organs mainly involved in homeostasis and the immune response of animals, or in the case of gills because they are specially exposed to the effects of rearing conditions.

Finally, a very basic approach to the economic impact that the new methodology may have in the production cost compared to the existing one was done, since one of the main motivations of the industry is precisely to reduce the operational costs involved in fouling management.

The outcome set of results suggested that in terms of welfare impairment and stress response, the on-site cleaning strategy did not suppose any advantage or disadvantage compared to the old strategy of net replacing, as no major differences were detected between them. However, negative consequences as netting damage, parasite infestation, fish gill damage or poor growth were detected. This is due to the current use of the on- site cleaning technology. Apart from the biological analyses, the economic approach revealed that the high cleaning frequency required to keep nets in fairly good conditions originates elevated costs that counteracts the initial advantages pursued by the industry, such as the decrease of the amount of nets used and the allocated resources (operation time and manpower). The use of the on-site cleaning technology might be very useful in specific aquaculture environments like the massive offshore cages currently endeavored. But the bottleneck that the high cleaning frequency represents, and its consequences must be tackled and solved to make this alternative a reliable solution if it is to be applied extensively in the industry. As a result of this investigation, an alternative midway among the two tested scenarios, reduced copper coating involving less cleaning operations, emerges as a promising option which, under our point of view deserves further attention and research.

Resum

Avui en dia, la problemàtica causada per la formació i gestió del fouling en granges marines genera un debat obert a nivell global en la indústria aqüícola. Així doncs, l'objectiu d'aquest treball ha estat comparar una tecnologia recentment implementada en la gestió de xarxes, la neteja in situ, amb la ja existent i majoritàriament utilitzada estratègia de l'ús de pintures antifouling (amb l'òxid de coure, Cu2O, com a principi actiu) per controlar i gestionar de manera més eficient un problema que afecta la indústria aqüícola de forma global, la producció de fouling. En aquesta tesi, el treball experimental es va planificar i realitzar en condicions reals de producció, simulant una part del cicle d'engreix de l'orada, Sparus aurata. Tot i saber que treballar en condicions de camp podria representar algunes dificultats, es va pensar que els resultats obtinguts explicarien molt millor què passa en la vida real que no pas els obtinguts en experiments in vitro o en condicions controlades de laboratori. Atès que aquesta és una tesi dins el marc d'un doctorat industrial, es va posar com a objectiu realitzar una aproximació generalista i, conseqüentment es van analitzar una àmplia gamma de paràmetres per identificar els efectes de les estratègies seleccionades sobre les xarxes, la salut i rendiment productiu dels peixos, per finalment realitzar una aproximació molt bàsica des del punt de vista de l'impacte econòmic.

Les xarxes són la caixa forta que protegeix l'actiu més important de les empreses productores de peix, la biomassa. Per aquest motiu, en aquest treball, es va analitzar l'eficiència dels productes de recobriment de les xarxes, i es va parar especial atenció en l'efecte de la neteja sobre la propietat bàsica de les xarxes, la seva resistència. Seguidament es van estudiar aquells aspectes derivats de les condicions de cultiu generades per l'una o l'altra estratègia de gestió del fouling i que es creia que podien tenir un efecte sobre la salut, i per tant rendiment productiu, dels peixos. Així doncs, es va analitzar el creixement, com a principal objectiu de les instal·lacions d'engreix, però també la presència de paràsits, concretament el monogeni Sparicotyle chrysophrii (Monogenea: Polyopisthocotylea) causant d'elevats danys en la indústria de l'orada a tot el Mediterrani, i la microbiota cutània, ja que sovint s'associa la proliferació d'aquests organismes amb l'estat higiènic de les xarxes. Addicionalment es va realitzar un estudi histològic a nivell branquial per determinar la magnitud dels possibles efectes de les diferents maniobres sobre aquest teixit, bàsic per la supervivència dels peixos degut a la seva multi-funcionalitat. També es va analitzar el contingut de coure en bràncquies, fetge i múscul, no només per veure si podia suposar un perill pels animals, sinó també per la preocupació que

sovint genera l'acumulació de metalls pesats entre els consumidors de peix. A més, es va voler veure quins eren els efectes, i si hi havia diferències, entre les dues estratègies testades a nivell de benestar dels animals i de resposta d'estrès. Per això es van analitzar en plasma i moc de la pell el principal indicador de la resposta primària envers una situació d'estrès, el cortisol així com els de les subseqüents respostes secundària (resposta metabòlica, immunològica i antioxidant). També es va voler comprovar si les dues estratègies contrastades provocaven diferències a nivell molecular, per això es va analitzar la modulació de l'expressió d'aquells gens involucrats en la resposta a l'estrès. Aquesta anàlisi es va realitzar en fetge, brànquia i melsa com a òrgans majoritàriament involucrats en l'homeòstasi i resposta immune dels animals, o en el cas de les brànquies com a òrgan especialment exposat als efectes de les condicions de cultiu.

Finalment es va realitza una aproximació bàsica des del punt de vista de l'impacte econòmic que pot suposar l'aplicació de la nova metodologia comparant-la amb la ja existent. I és que una de les motivació principals de la indústria és precisament abaratir els costos operacionals derivats de la gestió del fouling.

L'anàlisi del conjunt de resultats obtinguts suggereix que, en termes de benestar i estrès, l'estratègia de neteja de xarxes in situ no suposa cap avantatge ni desavantatge clar en comparació amb l'estratègia de substitució periòdica de xarxes tractades amb pintures antifouling ja que no s'aprecien diferències clares i significatives entre les dues metodologies. No obstant això, dels resultats també es desprèn que degut a l'actual mode d'aplicació i l'estat de desenvolupament de la tecnologia, l'estratègia de neteja in situ comporta una sèrie de conseqüències que afecten negativament el rendiment de les instal·lacions. La neteja in situ, compromet la integritat física de les xarxes afeblint la seva càrrega de ruptura, afavoreix la proliferació de paràsits i bacteris en els peixos i produeix severs danys branquials. D'altra banda l'ús de coure sembla que no suposa cap perill ni pels animals ni pels consumidors, ja que els nivells detectats després de 7 mesos són molt baixos, i en el cas de la musculatura, que és la part que es consumeix, els nivells estan per sota del llindar de detecció de la tècnica utilitzada.

Deixant de banda els aspectes biològics, l'enfoc econòmic revela que l'elevada freqüència de neteja necessària per mantenir les xarxes en condicions acceptables, implica un elevat cost que contraresta els avantatges inicials que suposa la neteja in situ com ara la disminució de la quantitat de xarxes i recursos utilitzats (temps d'operació i mà d'obra) que precisament són els

objectius que persegueix la indústria y que la motiva a buscar alternatives a les pràctiques ja existents. L'ús de la tecnologia de neteja in situ pot ser molt útil en entorns específics, com les grans gàbies offshore que actualment estan entrant en funcionament en la indústria del salmó. Però el cost i les conseqüències de l'elevada freqüència de neteja necessària avui en dia no estan ben resolts, i s'han d'abordar i solucionar perquè aquesta alternativa sigui una solució fiable i aplicable de manera general a la indústria.

Com a resultat d'aquesta investigació, apareix una opció que mereix atenció i recerca addicional la de xarxes tractades amb un component de Cu menor que l'habitual, que comporta menys neteges. Aquesta opció ofereix una interessant alternativa a mig camí entre les dues estratègies contrastades en la present tesi.

… "la vida és molt tenaç"…

Andreu Oliva de la Esperanza S.J.

Rector de la UCA,
Universidad Centroamericana, *José Simeón Cañas*,
San Salvador

Acknowledgements

As everything in this life, the work here presented is not the result obtained from the work, effort and commitment of a single person. Especially in this case, it owes a great debt to many people.

First and foremost my thanks go to my mentors, Professor. Lluís Tort and Albert Comas (General Manager, Delta Aqua Redes-Mørenot Spain). They have been the promoters and leaders in this quest, and without their help, tolerance and flexibility this project could not have become a reality. Albert and Lluís are those kind of people who do not think difficulties are enough reason to stop pursuing an idea, and this mentality allows the progress in the world. So, I would like to thank them together with Dr. David Parra their advice, help, encouragement and guidance during the elaboration and writing of this thesis.

I would also like to express my warm thanks to Bente Lund Jacobsen (CEO) and Nina Hildre (Export Manager Aquaculture), Mørenot Aquaculture AS, for their patience and comprehension throughout all this time.

Many thanks to Dr. Dolors Furones as Director of IRTA Sant Carles de La Ràpita for making me feel like home during the time I was conducting the field work of my experiment, and very especially to Magda Monllaó, Josep Lluís Celades and Marta Sastre who very enthusiastically contributed and helped in water quality monitoring and fish sampling. I would also thank to Dr. Alicia Estevez for helping me in gill parasites counting.

I am very grateful to my lab mates Drs. Mariana Teles, Felipe Reyes, Eva Vallejos and Ali Khansary who very kindly provided me help in PCR analysis and received me as one more in the lab from the very first moment. My special recognition to Dr. Joan Carles Balasch for his advice and help in publishing this thesis.

Thanks to Núria Barba, Saioa Medizuri and Mar del Castillo from INC, U.A.B. for their kindness in training me in the use of the microscope and getting gill micro-sections.

I wish to express my gratitude to the team Delta Aqua Redes-Mørenot Spain, Teresa, Josué and Andreu for their support and very especially to Mercedes Pardo and Àngel Pascual from for their commitment with me and their support in designing, drawing and helping in the construction of the experimental nets and cages. Thank you very much to all of you guys!!

A warm hug to Juanma Mancheño (Delta Aqua Redes-Mørenot Spain), and Toni Sahuquillo. There are bright, and they were always there when I needed them.

A big hug to all my friends for their support in many ways throughout the past years that permitted me to look always ahead.

And, a very special acknowledgement to Prof. Rosa Flos who first inoculated the Aquaculture virus into me more than twenty years ago and to Dr. Carlos-Antonio Martínez Palacios whose words encouraging me to pursue a PhD have always resonated into my mind since 2001.

Last but not least, I could not have gone this far without all my family…

Very especially, I want to deeply acknowledge the endless support I have received from my parents. In this, as in many other occasions, both their support and unconditional love have allowed me to succeed in this personal challenge. And Maria,…Maria is walking with me. I have many many things to acknowledge her, but the essential is that she is unconditionally walking with me.

This work has been partially supported by the AGAUR Industrial Doctorates, Generalitat de Catalunya, resolution ECO/1426/2014 exp. number 2014 DI 061 and Mørenot Aquaculture AS.

Tables

Chapter 2

Table 1. Water parameters monitored in the pond during the experiment.

Chapter 4

Table 2. Gill score for gill scoring protocol.
Table 3. Presence of *Sparicotyle chrysophrii* at the end of the trial.

Chapter 5

Table 4. Actions susceptible to produce stress in farming conditions and works assessing their effects.
Table 5. Sequences and of primers used for quantitative real-time PCR analysis.

Chapter 6

Table 6. Costs structures in sea bream farming in Spain.
Table 7. Relatives weights and costs of net and treatment factors in S1 and S2.
Table 8. Relatives weights and costs of the operational factors in S1 and S2.
Table 9. Required on site cleaning operations for a growth cycle of sea bream in Spain.
Table 10. Overall effect of on-site cleaning on the cost compared to the use of AF treated nets.
Table 11. Effect of on-site cleaning on production cost and business margin.
Table 12. Effect of a 2% lack of performance on cost and gross profit margin.

Figures

Chapter 1

Figure 1. United Nations world's population growth projection.
Figure 2. Salmar's Ocean Farming 1 moored in Frøya, Norway in September 2017.
Figure 3. Nets waiting for service in a net service station in Shetland, UK.
Figure 4. Heading of a row of cages in a mooring analysis.
Figure 5. Example of a typical 2x5 cages grid in Norway. Source: Aqua knowledge, A.S., 2016.
Figure 6. Heavily fouled net in a sea bream cage in the Mediterranean.
Figure 7. Simulation of the effect of current forces on a conic net. Source: Aquastructures, 2016.
Figure 8. Fouled with *M. edulis*, and hydroids grid rope. Courtesy of T. Meyer.
Figure 9. Summary of antifouling strategies. Source CRAB Final Activity Report, 2006.

Chapter 2

Figure 10. Registered pond temperatures during the experiment
Figure 11. Technical 3D view drawing of an experimental cage. Mørenot, 2015
Figure 12. View of the experimental lay out.
Figure 13. Experimental groups.
Figure 14. Net bottom center lifting during net replacing procedure. Figure 15. On-site cleaning operation in a cage during the experiment. **Figure 16**. Experimental lay out.

Chapter 3

Figure 17. Net panel lifting maneuver for panel picture capture.
Figure 18. Example of image thresholding process with imageJ software prior to PNA analysis. Figure 19. Effect of netting treatment in PNA and PNA/PNO changes during the experiment. Figure 20. View of panels from C1 (top), C2 (center) and C3 (bottom) along the experiment.
Figure 21. Results of BS tests performed during the experiment.
Figure 22. Portion of net panel of C2 after on-site cleaning at the end of the experiment. View from the inside out.

Chapter 4

Figure 23. Growth evolution during the experiment.
Figure 24. SGR at the end of the experiment compared to expected values.
Figure 25. Initial and final values of hepatosomatic (HSI) and spleen-somatic (SSI) indexes.

Figure 26. Copper content in liver and gills at the beginning and at the end of the experiment.
Figure 27. Skin microbiota richness at the beginning and at the end of the experiment expressed as log H (Shannon's index H).
Figure 28. Example of a scored "6" gill arch of C1 after net changing at the end of the experiment.
Figure 29. Gill score at the end of the experiment.
Figure 30. View of a seriously damage gill in C3. **Figure 31.** Gill damaged produced in gills in C2 and C3.

Chapter 5

Figure 32. Cortisol release over the experiment.
Figure 33. *hsp70* gene expression fold change in liver and spleen.
Figure.34. Relative expression of *hsp70* gene in liver and spleen.
Figure 35. Plasma and skin mucus glucose and lactate levels during the experiment.
Figure 36. Plasma and mucus proteins and triglycerides.
Figure 37. Creatinine and ALT in plasma and skin mucus during the experiment.
Figure 38: Citric acid cycle or tricarboxylic acid cycle (TCA). Figure 39. General mechanisms of the fish immune response. Figure 40. Model representing the stress spectrum.
Figure 41. Lysozyme activity throughout the experiment.
Figure 42. Complement activity throughout the experiment.
Figure 43. IgM in plasma throughout the experiment.
Figure 44. Fold change expression of genes encoding complement protein C3 and interleukins IL6 and IL1ß between the beginning and the end of the experiment (right before cage operation).
Figure 45. Fold change expression of *igm* gene in gill and liver between the beginning and the end of the experiment (right before cage operation).
Figure 46. Relative expression efficiency of *c3 il6* and *il1ß* during cage operation at the end of the experiment.
Figure 47. Relative expression efficiency of *igm* gene during cage operation at the end of the experiment.
Figure 48. The sources and cellular responses to reactive oxygen species (ROS).
Figure 49. Fold change expression of genes encoding SOD, CAT, GPX and CYP1A1 in gill between the beginning and the end of the experiment (right before cage operation).
Figure 50. Fold change expression of genes encoding SOD, CAT, GPX, CYP1A1 and MT in liver between the beginning and the end of the experiment (right before cage operation).
Figure 51. Fold change expression of genes encoding SOD, CAT, GPX, CYP1A1 and MT in spleen between the beginning and the end of the experiment (right before cage operation).
Figure 52. Relative expression efficiency of *c3 il6* and *il1ß* during cage operation at the end of the experiment.
Figure 53. Relative expression efficiency of *c3,* il6 and *il1ß* during cage operation at the end of the experiment.
Figure 54. Relative expression efficiency of *c3*, *il6* and *il1ß* during cage operation at the end of the experiment.

Chapter 7

Figure 55. Temperature profile of the experimental pond compared to seawater temperature in the experimental area.

Abbreviations

A: Abundance
ACH50: Alternative complement pathway activity
AF: Antifouling
ALT/ALAT: Alanine aminotransferase
ANOVA: Analysis of variance
BCR: B cell receptors
BS: Breaking strength
C1, C2, and C3: Experimental conditions/ groups 1, 2, and 3
C3: Complement c3 protein
CAC: Citric acid cycle
CAT : Catalase
Cd: Cadmium
cDNA: Complementary DNA
CoA: CoenzymeA
COGS: Cost of goods sold
CRAB: Collective research on aquaculture biofouling
Cu: Copper
CYP1A1: Gene encoding a member of the cytochrome P450 superfamily of enzymes
DME: Drug metabolizing enzymes
DNA: Deoxyribonucleic acid
DOC: Dissolved organic carbon
EBITDA: Earnings before interest, taxes, depreciation and amortization
EDTA : Ethylenediaminetetraacetic acid
EGTA: Ethylene glycol tetraacetic acid
ELISA: Enzyme-linked immunosorbent assay
EXW: Ex-works
FAO: Food and agriculture organization of the United Nations
FCR: Feed conversion rate
GHRP: Growth hormone-releasing peptide
GLM: General linear model
GIALT: Gill associated lymphoid tissue
GPO: Glycerol phosphate oxidase
GPX : Glutathione peroxidase
HNO3: Nitric acid
Hg: Mercury
HSI: Hepatosomatic index
HSP: Heat shock protein
H2SO4: Sulphuric acid
IFCC: International Federation of Clinical Chemistry
IGM : Immunoglobuline M
IGs: Immunoglobulines
IL1ß : Interleukine 1ß
IL6 : Interleukin 6
ILT: Interbranchial lymphoid tissue
LOD: Lactate oxidase
MALT: Mucosal associated lymphoid tissue
MHC: Major histocompatibility complex
MI: Mean intensity
MS222: Ethyl 3-aminobenzoate methanesulfonate
MT: Metallothionein
NaCl: Sodium chloride
NK: Natural killer cells
P: Prevalence
PAMPs: Pathogen associated molecular patterns
Pb: Lead
PBS: Phosphate-buffered saline
PNA: Percentage net aperture
PNO: Percentage not occlusion
PRRs: Pattern-recognition receptors
ROS: Reactive oxygen species
ROV: Remotely operated vehicle
rRNA: Ribosomal ribonucleic acid
RT-qPCR: Quantitative reverse transcription polymerase chain reaction
SGR: Specific Growth Rate
SOD: Super oxid dismutase
SSI: Spleen somatic index
TCA: Tricarboxylic acid cycle
TCR: T cell receptors
TMB: Tetramethylbenzidine
UV light: Ultra violet light
Zn: Zinc

General Introduction

The progress of Aquaculture

Fish protein and fatty acids are healthy and one essential component for human nutrition. Seafood was very early incorporated in the human diet as supports the fact that fish bones and shells have been found in ancient middens of hunters and gatherers communities (Nash, 2011). Thus, we could stablish the origin of the aquaculture activity in the old Sumerian practice 2500BC (Stickney, 2011) of keeping captured wild caught fish for a period of time to ensure access to fresh animal protein. Since then, along the history, aquaculture has undergone a process of improvement and development that has evolved into a modern industry that nowadays provides half of the seafood that is consumed in the world (FAO, 2016; Stickney, 2011).

The industrial revolution in the 19th Century promoted fisheries and opened vast fishing areas when commercial fishing vessels were powered first by steam and later by combustion engines instead of wind and manpower. However, as the world population grew and with it the seafood demand, overfishing during the 20th century depleted fish stocks, especially in European waters. Since then, aquaculture experienced substantial advances, for instance first attempts to arise salmon in cages were done in Washington State and Norway after the 2nd World War (Nash, 2011). In 2016, Norway's production of salmon was above 1.2 million tonnes (Statistics, 2016).

Today, world's population keeps growing and according to the latest United Nations projections it will reach 9.8 billion in 2050 and 11.2 billion in 2100 (United Nations, Department of Economic and Social Affairs, 2017), **(Figure 1)**. This will generate and increasing demand of aquatic food sources which aquaculture will try to cope with as capture fisheries smoothly decline year after year (FAO, 2016).

Fish farming is a primary sector industry where operation costs are high and profit margins narrow, that is especially true in the case of cage aquaculture where fish production is carried out often in a tough and difficult environment. Cage aquaculture represents already more than 65% of the finfish production in Europe and Americas with approximately 4.3 million tons produced in 2014 and is increasingly being introduced all over the world to places where conditions allow it (FAO, 2016).

In cage aquaculture, fouling formation, which is the attachment and growth of living organisms on surfaces immersed in the marine environment, has always been a major concern as it has many implications on both the inte-

grity of the farm structures and health status of the fish (Douglas-Helders et al., 2003; Edwards et al., 2015).

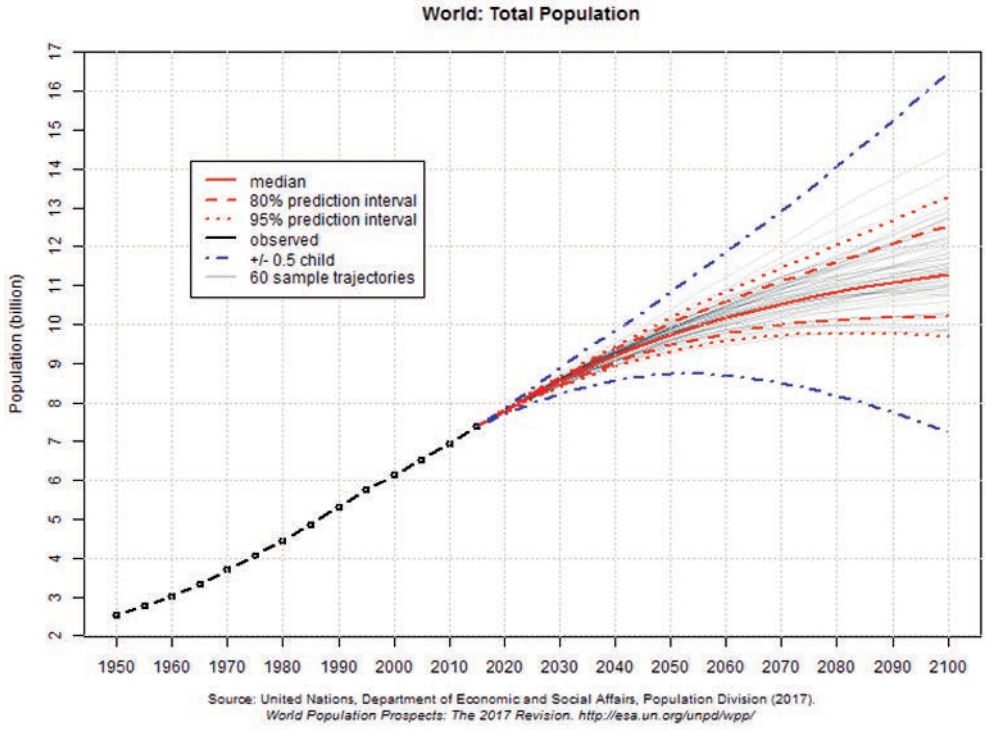


Figure 1. United Nations world's population growth projection.

Like twenty years ago, fouling is still today a difficult and expensive issue to manage (Hodson et al., 1997) and its cost has been estimated to be 5%-10% of the total production cost (Lane and Willemsen, 2004). Specially complicated is the management of fouling in aquaculture nets, in particular in a moment in which places for aquaculture begin to be scarce and the industry need to find offshore solutions which imply bigger cages and nets **(Figure 2)**.

Over time many different strategies have been approached to fight fouling in aquaculture nets being the most effectives those involving the use of antifouling paints (Douglas- Helders et al., 2003). Despite some concerns about the use of copper due to its toxicity, during the past decades the use of antifouling paints containing cuprous oxide have proved to be highly effective and widely used by the industry worldwide. Although there is no indication that copper based products will be prohibited in the near future

(Willemsen and Science, 2016), the industry is looking for more sustainable and cheaper solutions to reduce the use of copper and the operational costs. Apart from the release of copper into the environment when it leaches out the paint, this strategy involves the use of a large quantity of nets and their subsequent maintenance **(Figure 3).**

Figure 2. Salmar's Ocean Farming 1 moored in Frøya, Norway in September 2017.

Figure 3. Nets waiting for service in a net service station in Shetland, UK.

Among several approaches, on-site cleaning emerged as a promising alternative a few years ago and after different attempts it is currently well implemented in the salmon industry and has been introduced in Mediterranean aquaculture. Sometime after its implantation at commercial scale and as it was already pointed out when first tested in the '90s (Hodson et al., 1997), its effectiveness, effects over the environment and fish and benefits are under discussion.

In aquaculture, as in many other fields, there is a lack of research conducted at commercial scale, in an industrial environment. There is a lack of knowledge regarding net fouling management and the nouvelle on-site cleaning strategy and its drawbacks have never been assessed under real industrial conditions.

The present work will contrast the on-site cleaning strategy with the use of a commercial antifouling paint in aquaculture nets management simulating a real rearing environment. The analysis will be especially focused on the effects on fish since most of the research already done in this field are concentrated on the engineering rather than the biological side of the discipline.

Fouling in cage aquaculture

As it has been said above, the term fouling could be defined as the attachment and growth of living organisms such as bacteria, algae, bryozoans, hydroids, barnacles and molluscs among others on surfaces immersed in the marine environment. The successional development of fouling communities has been well studied (Greene and Grizzle, 2007). Its control has been pursued since long time ago (Hole, 1952). Fouling formation is a natural phenomenon that occurs globally and is implicit in any activity developed at the sea, including boats, marine offshore structures and aquaculture cages. It is highly site and seasonal dependent. The species that compose it vary to a great extent depending on the substrate and environmental conditions (Bloecher et al., 2013; Dürr and Watson, 2010; Edwards et al., 2015; Fitridge et al., 2012; Willemsen and Science, 2016; Willemsen, 2005). In cage aquaculture, it is a major problem worldwide (Bloecher et al., 2013) and as it has already been stated above, it causes several maintenance and operational difficulties that affect not only the integrity of nets and other farm structures but also fish performance and the health status of the site (Edwards et al., 2015). The industry is constantly seeking for more efficient and affordable methodologies to fight against it.

Effects on farm structures and fish

Typically, marine cage aquaculture systems consist of wire, rope or strap grids suspended on a buoy system anchored to the sea bed through a group of mooring lines **(Figures 4 and 5)**. The grids usually allocate polyethylene floating cages which support nets were fish are raised to commercial size. The effects of fouling formation in marine cage systems can result in severe economic consequences and a loss of profitability for the aquaculture industry. This is particularly dangerous in a primary sector industry in which profit margins are often really narrow as it will be shown later in this work.

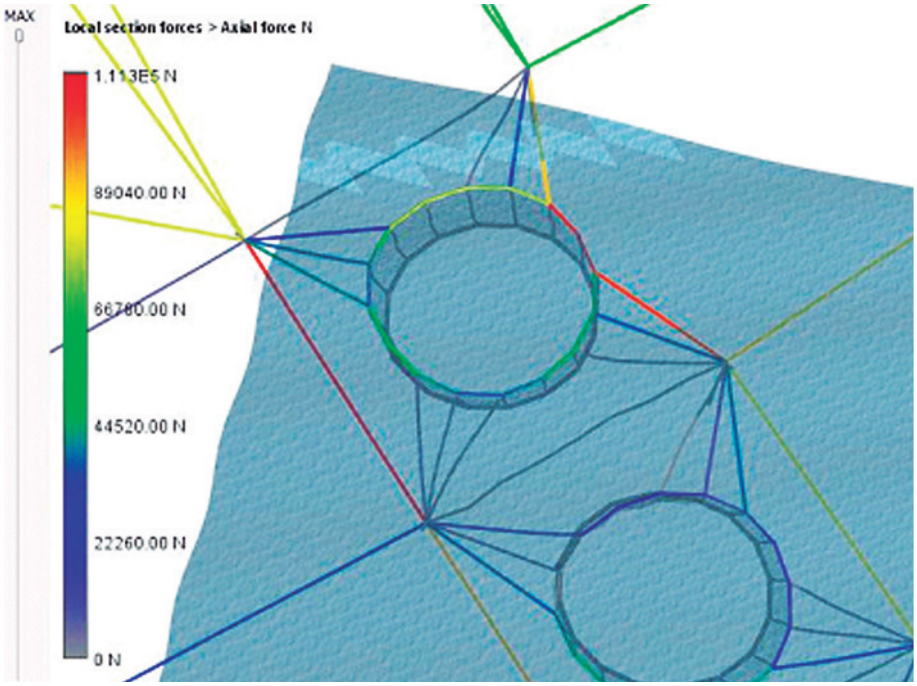


Figure 4. Heading of a row of cages in a mooring analysis. Source: Aqua knowledge, A.S., 2016.

Figure 3. Example of a typical 2x5 cages grid in Norway. Source: Aqua knowledge, A.S., 2016.

Several authors have studied the impacts of fouling in aquaculture and most of them are well reviewed (Fitridge et al., 2012). The most substantial in finfish cage aquaculture could be grouped under these three categories: (1) ***Net occlusion***, (2) ***Weight addition*** and (3) ***Disease risk***.

Net occlusion

Netting material provides an excellent surface for fouling attachment and growth (Hodson et al., 1997). Additionally, the environment surrounding aquaculture cages is rich in nutrients due to fish excretions, particles of uneaten feed and other wastes. Therefore, it can be stated that cage systems are a very suitable environment for fouling to develop. As fouling organisms settle and grow on nets, mesh openings occlude and subsequently the water exchange across the net reduces **(Figure 6)**.

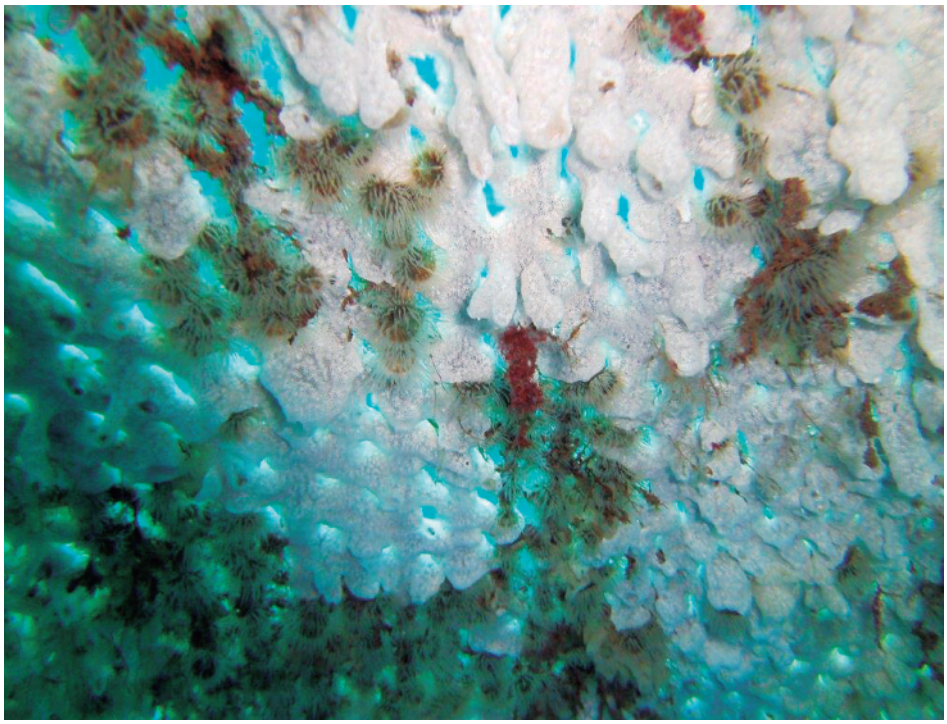


Figure 6. Heavily fouled net in a sea bream cage in the Mediterranean.

This eventually results in a reduction of dissolved oxygen levels and waste dispersal rate (Beveridge, 2004). All these consequences become more severe when the size of the cages increase as the biggest the cage, the lowest the ratio between net surface and net volume. This increase in cage size is precisely a tendency in the salmon and Mediterranean industry nowadays.

Occluded net panels increase drag forces (Bi et al., 2015, 2014; Fitridge et al., 2012; Klebert et al., 2013; Swift et al., 2006) which can cause cage deformation and a reduction of the rearing volume **(Figure 7)** that can be dramatic if nets and cages are not correctly ballasted as states Osawa et al., (1985) in (Fitridge et al., 2012). A reduction in cage volume will firstly affect fish behaviour and cause crowding stress to the fish school (Rotllant and Tort, 1997) but can eventually have much more severe consequences since it can very easily provoke abrasion and ulceration in fish skin due to friction with the net.

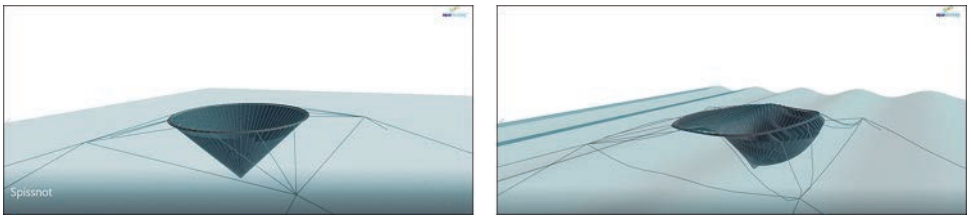


Figure 7. Simulation of the effect of current forces on a conic net. Source: Aquastructures, 2016.

Weight addition

Accumulated fouling on nets and other farm structures affects hydrodynamic performance of the system (Klebert et al., 2013). It reduces buoyancy, provokes material fatigue, mooring stress and eventually system failure (Beveridge, 2004; Fitridge et al., 2012) **(Figure 8)**. This can result in huge damages in fish and fish farm structures in episodes of severe bad weather allowing large quantities of fish to scape.

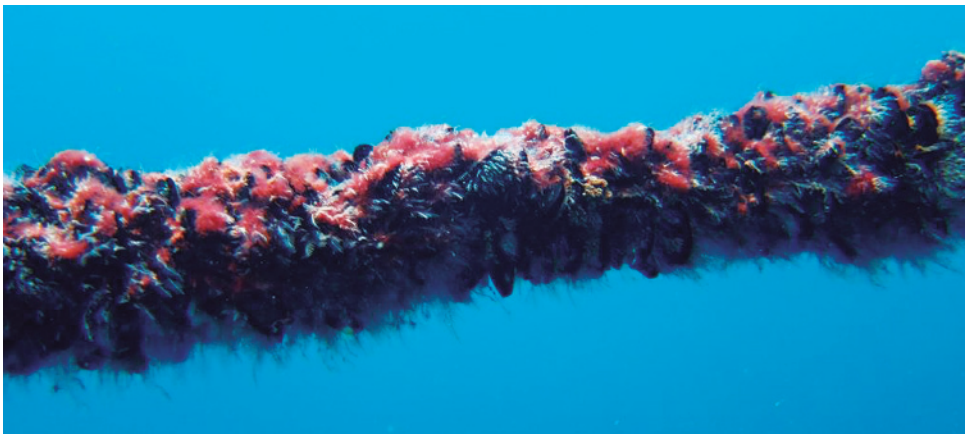


Figure 8. Fouled with *M. edulis*, and hydroids grid rope. Courtesy of T. Meyer.

Disease risk

There are several works suggesting and describing fouling as a reservoir of parasites and pathogens increasing the incidence of potential disease and reducing fish performance (Douglas-Helders et al., 2003; Edwards et al., 2015; Swain and Shinjo, 2014), thus finally increasing costs and reducing the profitability of aquaculture companies. Some of these parasitess include, among others, *Neoparamoeba pemaquidensis* (Tan et al., 2002) the causative agent of amoebic gill disease in Atlantic salmon, the sea lice *Lepeophtheirus salmonis* (Huse et al., 1990) an important salmon health problem which caused loses of US$436m in damages to the Norwegian industry in 2011 (Abolofia et al., 2017), the hydroid *Ectoplrure larynx* (Guenther et al., 2010) and the monogenean *Sparicotyle chrysophrii* (Henry et al., 2015; Sitjà-Bobadilla et al., 2010) which widely affects sea bream production performance in the Mediterranean.

Fouling control strategies in aquaculture

Human efforts to fight fouling are as old as human sailing history. The earliest direct references to fouling and antifouling paints date from 4th Century BC (Hole, 1952) regarding the effects on slowing down ships. Along the history antifouling research was

first conducted to prevent fouling formation in vessel hulls and the oil and gas industries (Dürr and Watson, 2010) and the same technology and solutions were gradually applied to marine aquaculture as it developed commercially. Historically, antifouling paints containing biocides as lime first, but arsenical, mercurial compounds, pesticides and tributyltin later have been extensively used to control fouling. Generally, these paints work by creating a toxic boundary layer at the surface of the paint as the component biocides leach out delaying and slowing down fouling onset (Fitridge et al., 2012). However, as the effects of the biocide compounds were studied it was been that many of them were detrimental for fish and shellfish survival, so their use was restricted and efforts were addressed to find alternative methods.

In aquaculture, the use of tributyltin (TBT) was very extended to control biofouling from de 60's until its restriction in the 90's decade and the posterior complete ban (IMO, 2002). Since then, antifouling paints containing cuprous oxide have proved to be highly effective against fouling formation and have been the solution widely used by the industry until nowadays among several other alternatives such as cleaning practises, net air drying,

biological control or fouling release coatings (Willemsen and Science, 2016). The properties of copper as a biocide are known since ancient times, Holzapfel, M., 1889 and Ragg, M., 1925 in (Hole, 1952) and it has been applied for disease control and water treatments by many ancient cultures (O'Gorman and Humphreys, 2012). However its use as an antifoulant was firstly suggested in 1625, Damson, M. E., 1937 in (Hole, 1952) when a patent was granted for a composition that very probably contained some form of copper.

Copper is a natural occurring element and essential in all eukaryote cells since it is a co- factor for many enzymes. However, it might also be toxic at high concentrations due to its redox nature (Grosell, 2011). Marine animals can uptake copper directly from the diet, drinking water and also across the gills. This could eventually lead to its accumulation in tissues and pose animals at risk, thus affecting consumer health and environmental balance. Today, the use of copper in antifouling paints is well regulated. As a consequence of their active ingredients, biocidal net coatings need to obtain approval from the authorities before they can be put in the market (Willemsen and Science, 2016). In Europe, the use of antifouling products including those used for aquaculture, fall under the pesticide category and are regulated by the Biocides Products Directive EC 98/8/EC (European Comission, 1998). The approval for the use of di-copper oxide (cuprous oxide) as a biocidal is regulated by the Commission Implementing Regulation EU 2016/1095(Council of European Union, 2016), (see annex I) and the maximum levels for contaminants in foodstuff by the Commission Regulation EC 1881/2006 (European Commission, 2006) where copper does not appear.

The strategy of using nets treated with antifouling paints containing copper is expensive. The cost of the antifouling product as such and posterior net treatment is high, it can represent up to 25% of the final cost of the net. Antifouling treatments clearly enlarge the life span of the nets, but they have to be replaced when eventually become occluded. Usually several nets are needed to complete a grow-out cycle both in the salmon and sea bream/ sea bass industries. Additionally, the social perception of using metals in fish farming is generally not very good for the possible bioaccumulation in fish tissues and the environmental impact. That is why although there is no evidence that copper based products will be prohibited in the near future (Willemsen and Science, 2016), the industry is looking for more sustainable, environmentally friendly and cheaper solutions to reduce operational costs, the impact to the environment and to improve the social acceptance

among consumers. In this sense, for the last two decades, some research has been done and many alternatives have been explored **(Figure 9)**, among them, and looking globally in the aquaculture industry, on-site cleaning has actually been one of the most developed. It was implemented in the salmon industry in Norway and Scotland a few years ago and more recently in Canada and it is currently being introduced to the sea bream/sea bass industry in the Mediterranean.

First attempts of on-site cleaning where done manually by scrubbing the nets, later with manually operated water pressure cleaner disks, and today cleaner disks are mounted on ROVs (remotely operated vehicle) operated from the surface. The principle of on-site cleaning is to remove fouling from nets previously coated with a product that does not prevent fouling formation but allows an easy cleaning of the net by using water pressure cleaning disks. Hence the use of copper and the number of nets needed to rise a batch of fish up to a commercial size would be reduced. Several on-site cleaning constrains were already pointed out when the technique was first tested in the '90s (Hodson et al., 1997). The discussion remains today among fish producers, net manufacturers and veterinaries. The main worries are: Cleaning efficiency and frequency, the cleaning rate achieved during cleaning operation and the frequency of cleaning required, Netting abrasion, produced by high water pressure on netting fibres. and its possible effects on netting, and lastly the Effects on fish health, with special concern on the cloud of debris produced when fouling is washed off the nets and passes through the gills.

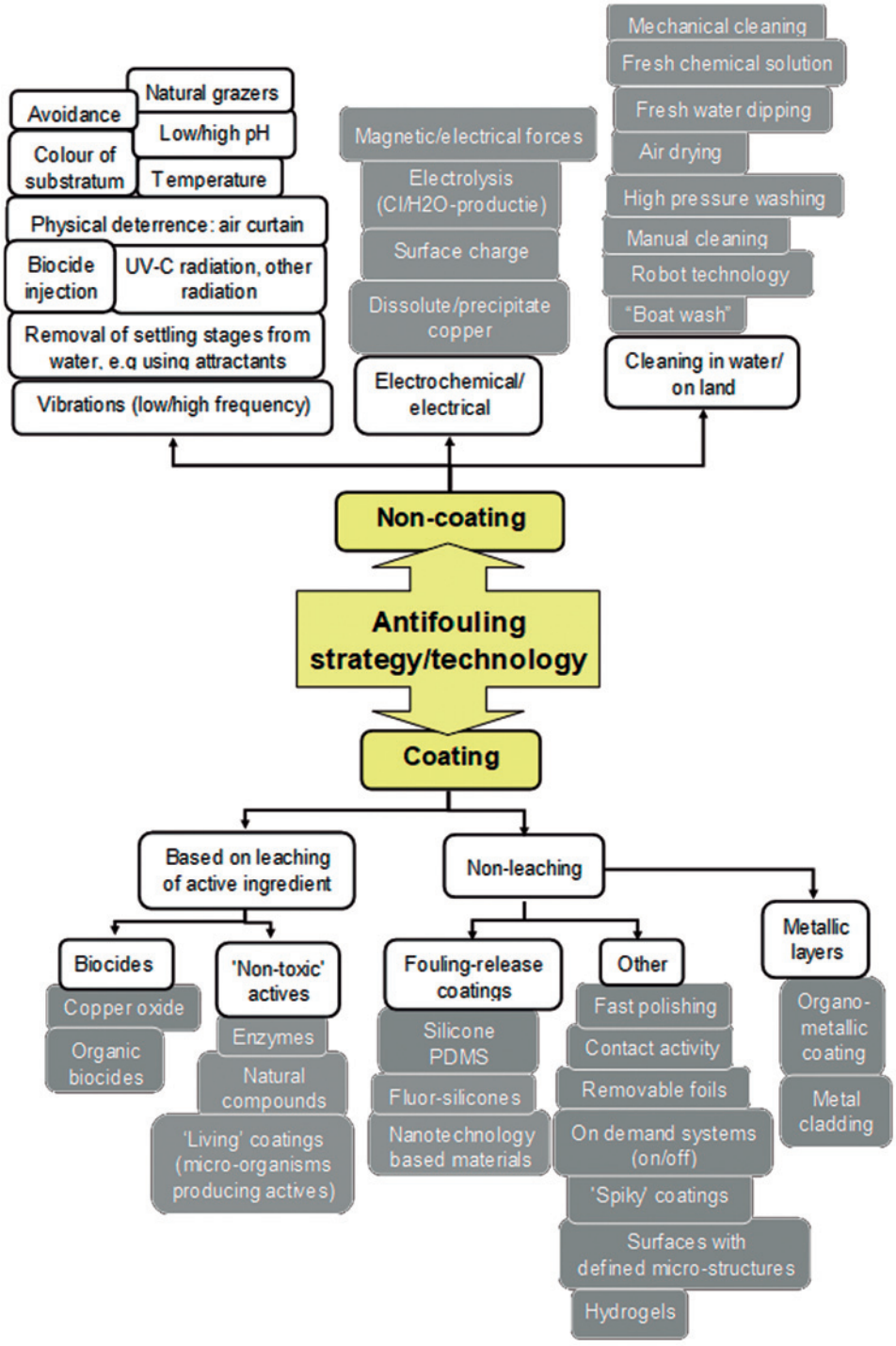


Figure 9. Summary of antifouling strategies. Source CRAB Final Activity Report, 2006.

Aims of the thesis

In this study, as a result of the existing fouling argument within the industry, two different management strategies in aquaculture nets have been compared. The use of antifouling treated nets (containing cuprous oxide) and on-site cleaning. It is necessary to remind that the frame in which this work has been carried out is an Industrial PhD program. According to that, a broad range of aspects have been assessed with the willingness to give a multidisciplinary approach to this study and to get some easily transferable results to the industry. Also for this reason, and considering the lack of works in this sense, the experimental lay out of the work as well as the experimental conditions and factors have been thought and applied simulating real rearing conditions. As already said in the preface of this thesis, working in the field rather than in the laboratory often complicates things because some variables are difficult to control. However the benefit is that the obtained results tell what actually happens at commercial scale, in real conditions.

As the field work has been performed in the Occidental Mediterranean coast of Catalonia, in the north-east of Spain, the fish species selected for this study has been the gilthead sea bream, which is a major species in the Mediterranean aquaculture with almost 160.000 tons produced in 2014 (FAO, Fishstat) and is nowadays reared under both fouling management strategies, at least in Spain.

Most of the research conducted on aquaculture nets and antifouling products has focused more into the engineering side of the activity, developing stronger and lighter materials, better designed nets and more efficient antifouling products. Despite the fact that there are several studies describing the effects of fouling accumulation in farm on nets, farms and even on fish, there is a lack of works on the effects of the fouling management strategies on fish, and those, under our point of view are very likely to happen at different levels including stress and immunological response due to the intimate relationship between fish and environment they are reared in. For this reason, in this work the focus will be put precisely in the content more than in the container, or in the fish rather than on the nets. Both replacement and net cleaning operations might cause stress or damage to the animals (Swain and Shinjo, 2014). If handling, is not done properly, the activity disrupts the protective mucous coating and fish scales, thereby increases susceptibility to parasitic or pathogenic invasion (Post, 1987).

To elucidate all this, the newly arrived and commercially implemented on-site cleaning strategy for aquaculture nets will be compared with the commonly established strategy of using antifouling (containing cuprous oxide) treated nets. Either one or another of these two strategies may have different effects on nets and fish which in the industry are reflected in the balance sheets of the companies. Along the different discussions within this work, relevant opinions from professionals in the aquaculture industry such as farm managers, farm maintenance operators, aquaculture veterinaries, etc. have been taken into account after formally recorded (see annex II) since these people have a direct and worthy field experience. The reader is also invited to fill in the form at: *https://docs.google.com/forms/d/e/1FAIpQLScdm6ooTU2Sf3c2d03PpWqacJuibSzOzGBj8iD44e 2Vm-rgLg/formResponse*

The ***working hypothesis*** of this work is to confirm whether or not on site cleaning is a better alternative to the use of antifouling in aquaculture nets. The final purpose of this work is to broadly analyze from an applied point of view the implications and constrains of this strategy. The final goal of this project is to contribute to better decision making for the industry in fouling control strategy.

The following areas of interest will be assessed in the following chapters.

The effects on nets

Effects and performance of the experimental coatings on aquaculture nets.

Effectiveness of the on-site cleaning strategy.

Effects of on-site cleaning on net integrity. The loss of tensile strength.

The effects on fish

Effects of the on-site cleaning strategies on sea bream growth.

Gill parasites occurrence and fish skin microbiota diversity.

Copper bioaccumulation in tissues in reared sea bream. Gill, liver and muscle.

Effects of the on-site cleaning strategy on gill integrity.

Effects of the on-site cleaning strategy on welfare and stress response at physiological and gene expression level. Primary stress response, biochemical response, immune response and antioxidant response.

Economic implications

Economic impact of the on-site cleaning strategy on production cost. A very basic approach.

Materials and Methods

Data obtained in this study has been base on a single, complex and long term experiment which has simulated part of a sea bream growth cycle in cages. This chapter will first explain all those procedures that were common among the different sections within the complete work and in the different sections specific methods will be further detailed.

Study area

The geographic area where the field work took place was the western Mediterranean. The trial itself was carried out in a 1 hectare shallow earthen sea water pond (see annex III) similar to those used for sea bream culture inland. The pond was located in the coast of Catalonia, north-east Spain, in the Ebre river delta (40º37'38"N; 0º39'41"E). Sea bream was usually cultured in similar ponds in the area not many years ago. Nowadays this culture system has been replaced by sea cages. There are two sea bream farms in the nearby area.

Experimental conditions

The experiment lasted 7 months, from December 2015 to June 2016. There was constant water circulation between the pond and open sea. It simulated real rearing conditions of seawater cages. It was performed under natural photoperiod and temperature profile. Water quality parameters during the experiment were well within a suitable range for sea bream culture **(Table 1)**.

Temperature ºC	O_2 ppm	O_2 SAT. %	$N0_2$- mg L^{-1}	NH_4+ mg L^{-1}	pH	Salinity ‰
16,61 ± 3,95	11,99 ± 4,31	124,06 ± 40,05	0,19 ± 0,18	0,05 ± 0,07	8,32 ± 0,19	28,99 ± 4,11
8,7	*5,2*	*70,0*	*0,00*	*0,00*	*8,00*	*14,40*
24,4	*24,2*	*249,0*	*0,80*	*0,70*	*8,80*	*34,80*

Values are means ± SD. Rows in italics show minimum and maximum values recorded during the experiment.

Table 1. Water parameters monitored in the pond during the experiment.

During winter months, though temperature was below the optimal range for the culture of this species, it did not pose any risk for the animals' health (Blancheton, 2000). Sea bream culture in ponds in the area often experiences the same low temperatures profile during winter. Temperatures below 10ºC were seldom reached and temperatures were below 12ºC for two weeks during February-March **(Figure 10)**. Some authors consider 12ºC as the threshold below which winter syndrome in sea bream appears (Ibarz et al., 2010; Tort et al., 1998). A peak in nitrites and a low peak in salinity were detected for a very short period of time.

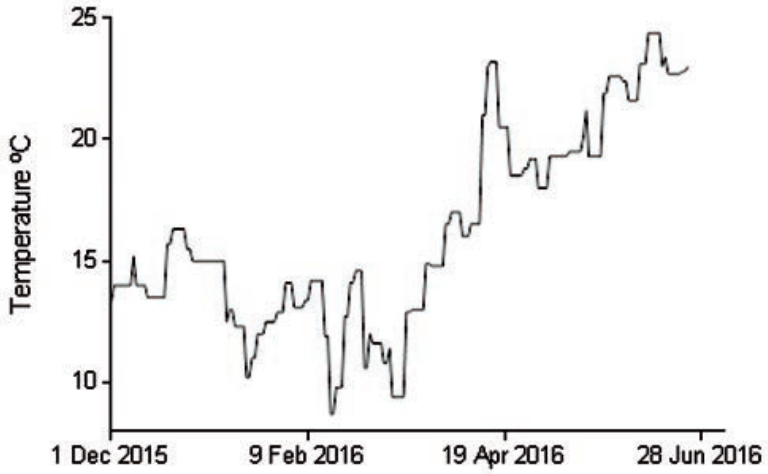


Figure 10. Registered pond temperatures during the experiment.

Experimental set up and system description. System lay out

Three experimental conditions or groups (C1, C2 and C3) with three replicates each were settled. Each replicate was allocated in a square 2.25 m^2 polyethylene floating cage supporting a nylon® net 1.3 m deep which provided a rearing volume of 2.92 m^3 **(Figure 11)**, (see annex III).

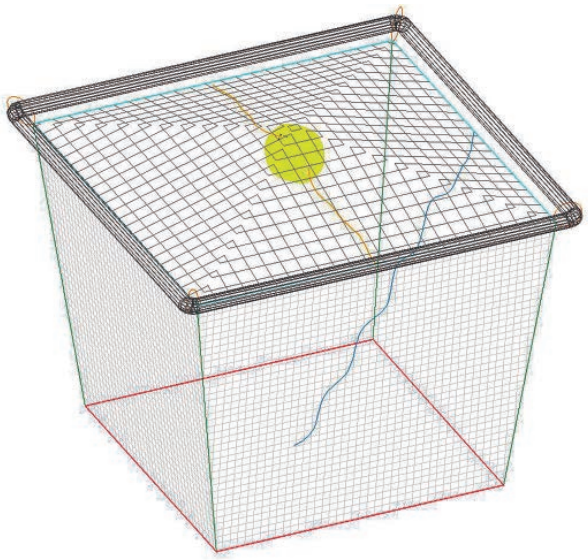


Figure 11. Technical 3D view drawing of an experimental cage. Mørenot, 2015

The system was laid in a way that the replicates of each condition followed the water flow across the pond. There was a distance of three meters among different cages from different conditions **(Figure 12)**.

Figure 12. View of the experimental lay out.

Experimental materials and treatments

Knotless Nylon netting is by far the dominating material in aquaculture net cages (Moe et al., 2007). In this experiment, the netting used was a (Mørenot, Norway) superknot 210/96, 18 mm half mesh (ISO 1107:2017) knotless nylon® (PA6 light protected, Nexis, Slovakia) which is a common intermediate mesh used in both the sea bream and salmon industry.

In C1, nets were treated with a commercial copper based antifouling (Netkem, Norway) paint containing 11% v/v of cuprous oxide Cu_2O. In C2 and C3 nets were coated with a polyurethane layer (Taytech, UK) (see annex IV). In C2 the polyurethane solution contained 3% v/v of Cu_2O, whereas in C3 there was no cuprous oxide in the mix **(Figure 13)**.

After having previously homogenized it using a paint mixer, the ready to use (obtained from the manufacturer) antifouling treatment was applied to C1 nets through a vacuum system allowing the paint to impregnate the netting. In C2 and C3 nets were dipped for 2 hours in a liquid polyurethane diluted solution (2:3 dilution). In C2, copper was already incorporated in the polyurethane solution when treating the net. These procedures are those usually followed by the net manufacturing industry. In all three conditions nets were air dried after treatment.

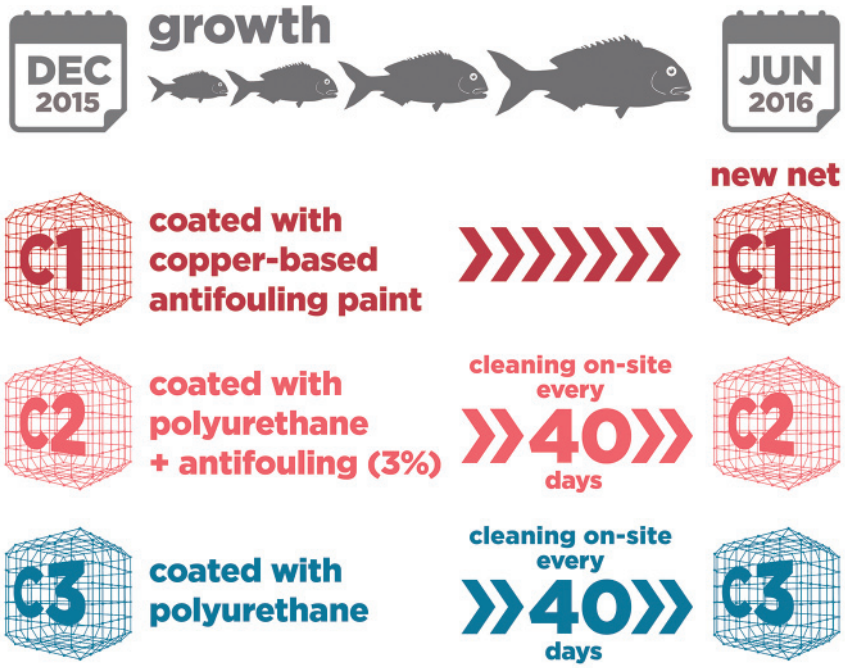


Figure 13. Experimental groups.

Fish husbandry and feeding

A total of 810 gilthead sea bream (*Sparus aurata L. 1758)* juveniles 75.50±7.31g provided from a local hatchery (Piscimar, Borriana) were acclimatized in the pond for fifteen days prior to initiate the experiment. Fish were afterwards stocked into each cage at a rate of 90 fish per cage (2.34 kg m^{3-1} roughly) and maintained under natural conditions. Fish were manually fed *ad libitum* 5 days a week with a commercial diet (Skretting, Norway 46% crude protein 19% fat). Fish were starved 24 hours before every sampling point until the end of sampling process. From this batch of fishes 252 were used for analytical purposes.

Water quality monitoring and copper determination in water

Temperature and dissolved oxygen were daily measured at dawn and afternoon in the pond using a WTW Oxi 3205 portable oximeter calibrated to measure marine water. The same measurements were also weekly taken inside each cage. Nitrites, ammonia, pH and salinity were weekly measured in the pond. All values except for punctual temperature episodes were always

within the optimal range described for the culture of this species. Water chemical parameters were analyzed using a HACH (USA) colorimeter. Salinity and pH were measured in an Inolab_IDS Multi 9310 station.

In order to experimentally assess copper transfer from experimental paints to water, five 50 liter tanks containing: tank 1: pond water, tank 2: pond water + untreated netting, tank 3: pond water + C1 netting, tank 4: pond water + C2 netting and tank 5: pond water + C3 netting were kept and agitated regularly for the same period of time that the experiment lasted. Afterwards, copper content in the water was determined. Water was analyzed after up to 200 fold dilution of sea water to avoid salt interference. Water samples were acidified with HNO_3 (Merck Suprapur, Germany) to reach a final sample medium HNO_3 1% (v/v). Copper determination was performed with inductively coupled plasma optical emission spectrometry (ICP-OES) in a PerkinElmer (USA)-Optima 4300DV spectrometer.

Cage and net operations

Depending on the experimental condition, during and/or at the end of the experiment, after the seven months, different procedures were used to check the effect of each fouling management strategy on the nets and fish.

At the end of the experiment, nets in C1 were changed by a new one in a procedure that took about 2 hours following the same methodology used in commercial cages for this operation. This consisted on lifting the bottom center of the net up with a small crane, positioning an outer new net hanging from the floating pipes and lastly removing the old net from the inside out **(Figure 14)**. Used nets were washed in a commercial washing machine.

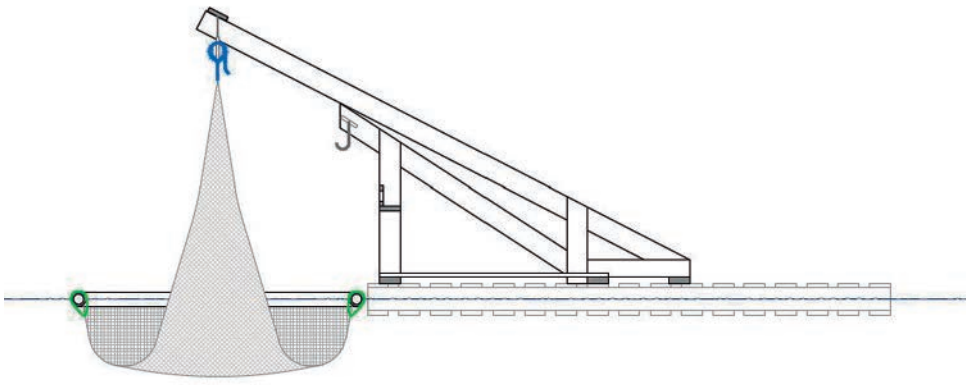


Figure 14. Net bottom center lifting during net replacing procedure.

Overall, nets in C2 and C3 were cleaned on-site every 40 days approximately since the beginning to the end of the experiment. The frequency was lower from December to May and higher from May to the end of the experiment when fouling production was higher due to higher temperatures and longer days. Cleaning operation was performed at 250 bar using a diesel water pressure washer with a modified ending simulating the cleaner disks used in fish farming. This operation lasted about 20 minutes per cage depending on the amount of fouling present on the net panels **(Figure 15)**.

Figure 15. On-site cleaning operation in a cage during the experiment.

Sampling schedule

Sampling points included net panel pictures and fish tissue including gills, liver, spleen, muscle, skin mucus and blood. Detailed sampling procedures will further be explained in the appropriate chapters, but generically initial values of all analyzed parameters were obtained in an initial sampling at the beginning of the experiment. At the end of the trial sampling points were set right before, right after, and 24 hours after net maneuver for all three conditions. In C2 and C3 four intermediate samplings were also taken again right before, right after and 24 hours after net cleaning from both net panels and fish **(Figure 16)**, but only data from net panels in those intermediate sampling points have been analyzed in this study.

Figure 16 Experimental lay out.

Skin mucus, blood collection, and tissue sampling

Skin mucus, blood and tissues (liver, spleen and gills) were obtained from nine animals (3 animals per group) at the beginning of the trial and eighty one animals, (3 animals per cage, 9 animals per group per each sampling point) at the end of the experiment. Fish were sacrificed with 200 mg^{-1} MS222 (Sandoz, Germany). Mucus samples were collected in Petri dishes gently scraping the dorso-lateral skin surface of the fish from head to tail using sterile microslides trying to avoid contamination with blood or urino-genital and intestinal excretions (Xu et al., 2013). In order to get enough mucus quantity, each fish was re-immersed for 10 seconds into pond water after having been scraped to promote more mucus production and allow a second scraping. Mucus samples were transferred into 15 mL tubes and stored in ice for a few minutes.

Once in the laboratory mucus samples were homogenized using a 1000 µL micro pipette and centrifuged at 1500 rpm for 10 minutes at 4°C to remove cells and debris. To separate skin bacteria from mucus, the cell-free supernatant was thereafter centrifuged at 10,000 rpm for 10 minutes at 4°C. The resulting supernatant (containing skin mucus) was harvested and stored at −80 °C in Eppendorf tubes for other analysis, whereas the pellet (containing skin bacteria) was re-suspended with PBS (pH 7.2) and centrifuged again at 10000 rpm for 10 minutes at 4°C. The resulting pellet, containing the microbiota, was collected and stored at -80°C for further analysis.

After mucus collection a blood sample was taken from each anesthetized fish by puncture in the caudal vein with a 2 mL heparinized syringe. Samples

were stored in Eppendorf tubes heparinized with sodium heparin (Hospira, Spain), stored in ice and shortly after centrifuged for 10 minutes at 2500 rpm at 4ºC. Plasma aliquots were frozen at -80°C for posterior analysis.

After blood extraction fish were dissected and liver, spleen, gills and muscle were excised and immediately frozen at -80ºC for posterior analyses. Liver and spleen were rapidly weighted for somatic indexes determination.

On-site cleaning strategy. Cleaning efficiency and effects on nets

Introduction

Fouling formation and its accumulation in marine farms and aquaculture nets can result in very detrimental consequences for the integrity of fish farms and reared fish, therefore it requires reliable control strategy. Today, fouling control strategies of the aquaculture industry involve a large quantity of resources. Practically speaking it means time and money. Briefly, this fouling accumulation results in mesh occlusion and weight addition to the farm structures. This negatively affects the hydrodynamics of the systems and reduces cage rearing volume. Especially if farms are not well designed and dimensioned (Bi et al., 2015; Fredriksson et al., 2007; Klebert et al., 2013) this may provoke system collapse or failure during current or storm episodes, producing severe damages and loss among livestock.

In the general introduction of this study it has already been explained that the industry is constantly seeking for alternatives to the use of copper in antifouling paints despite its biocide effectiveness. The main reasons are the high cost, and the bad social perception as a result of its possible effects as a pollutant on the environment. With time, copper present in antifouling paints leaches out of the nets and releases into the surrounding media from which it easily spread out. In addition, net washing plants have problems dealing with the copper containing waste and sludge since it must be specifically disposed of and such safeguards evidently increase costs (Willemsen, 2005).

The environmental effects of copper coming from the aquaculture industry have been extensively studied (Ahmad et al., 2014; Casado-Martinez et al., 2013; Nor, 1987) and will not be discussed in this thesis. It is a fact that the aquaculture industry worldwide contributes in releasing copper into the environment, but it is far away from being one of the main sources of this kind of pollution after fossil fuel combustion, waste waters, manure, pesticides and fertilisers used in agriculture, forestry and mining and boating and *marinas* activity (NOAA, 2017).

Among several approaches already cited in this work and after many attempts during the last twenty years, on-site cleaning is the alternative that has successfully been globally implemented in the aquaculture industry. However, after assessing its performance in commercial fish farms, its relia-

bility is under debate amongst fish farmers because of several weak points. Essentially its effectiveness, effects on netting and fish health and cost.

Looking at the effects of net management on the netting it is easy to assume that different fouling control strategies should have different effects on net physical properties since they imply different treatments and operations.

The most obvious difference between the use of antifouling treated nets and on-site cleaning strategy is that while the former are replaced every certain time and cleaned in land as they become occluded, the latter are cleaned *in situ* using water pressure cleaner disks, and ideally only one net is used to raise a batch of fish to commercial size. In the case of on-site cleaning, additionally, it implies changes in net construction. Net panels need to be more rigid than usual to ensure effective cleaning, and for that purpose they are constructed with less slack, usually 2%-3% than ordinary nets.

Two desired characteristics of netting are resistance to strain and resistance to abrasion. Extra weight produced by fouling accumulation causes strain and increases drag forces. Friction between net panels or between nets and other farm structures can cause abrasion. In order to protect twine from abrasion during the cleaning operations, nets are usually coated with different materials. In this thesis the coating tested has been polyurethane in a 2:3 dilution. This product is already implemented at industrial scale in Scotland and Norway. Polyurethane does not prevent fouling formation but allows an easy cleaning of the net.

In this chapter, the effectiveness of fouling control of both strategies will be analysed by calculating the Percentage Net Aperture (PNA) and Percentage Net Occlusion (PNO) along the experiment. The effects of net treatment and cleaning strategy on netting tensile strength will be assessed by comparing breaking strength (BS) under the different experimental conditions.

Materials and Methods

Net occlusion and on-site cleaning efficiency

Fouling formation on the nets and the efficiency of on-site cleaning were measured according to the works previously done by (Braithwaite et al., 2007). To analyze PNA and PNO, pictures of 8 panels ($n = 8$) per experimental condition and also pictures of 8 panels of experimental untreated netting were taken at the beginning of the experiment.

Along the experiment, pictures of two netting panels of each cage (the ones north and south oriented) ($n = 6$) were also taken before and after every

cleaning operation in C2 and C3. At the end of the experiment pictures of two netting panels of each cage (the ones north and south oriented) *(n = 6)* were also taken from C1 cages right before net changing.

In all cases images were captured with a 13 MP digital camera. To obtain them, nets were carefully lifted up and a white background was used to increase contrast. Pictures were always taken at the same distance of 28 cm **(Figure 17)**.

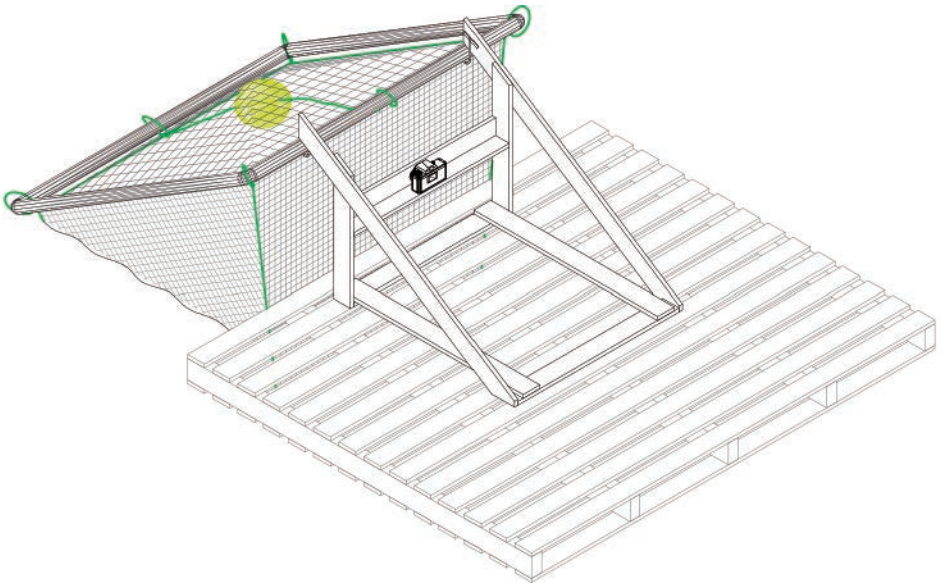


Figure 17. Net panel lifting maneuver for panel picture capture.

Afterwards, images were digitally analyzed with the open source ImageJ 1.50i (*https://imageJ.nih.gov/ij/*) (Schneider et al., 2012). Image processing was performed by thresholding in HSV (Hue, Saturation, and Value) format.

The percentage net aperture (PNA), is defined as the area of netting not covered by twine or fouling and was calculated as the ratio of the number of pixels which represented mesh holes to the total number of pixels of the image **(Figure 18)**. Percentage Net Occlusion (PNO) at a given moment was calculated by using the equation suggested by Braithwaite et al., (2007).

$$PNO = 1 - \frac{PNA\ day\ \chi}{PNA\ day\ 0}$$

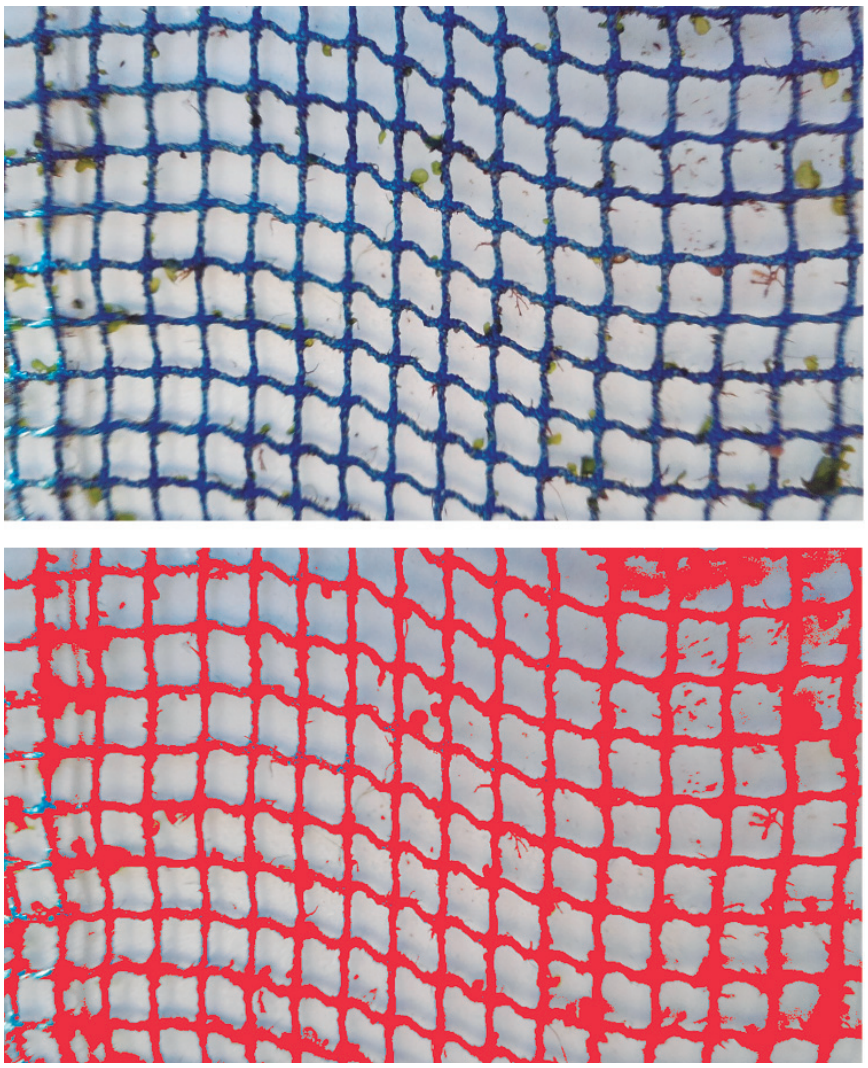


Figure 18. Example of image thresholding process with imageJ software prior to PNA analysis.

Effects of coatings and cleaning systems on breaking strength

In order to determine changes in the netting tensile strength under the different experimental conditions, netting breaking strength (BS) was measured according to Nørsk Standard 9415-NYTEK and ISO 1806/2002 (The International Organization for Standadization ISO 1806, 2002) using a DYNA 300D/P dynamometer (Buraschi, Italy).

To assess the effects of the experimental treatments (antifouling and coating) on the netting in rearing conditions, a total of 180 BS tests were performed at the beginning of the experiment on different pieces of experi-

mental wet untreated netting. A portion of every experimental treated and untreated nettings were placed into the experimental pond. At the end of the experiment 45 BS tests were performed on each one of them. To compare the effect of the different cleaning strategies on netting tensile strength, at the end of the experiment 10 BS tests were performed in each net replicate. Results were contrasted with those obtained in the BS tests performed at the end of the experimental period in the treated netting portions laid in the pond but not subjected to any cleaning process.

Statistical analysis

Statistical analyses were performed using the statistical package MINITAB (version 17) (Minitab, Inc. Pensylvannia). After checking normality with the Anderson-Darling test, net aperture, net occlusion and netting breaking strength were compared at the end of the experiment with one way ANOVA considering "net treatment + net operation" as a single factor effect since one always depends on the other. Tukey's Test was used to see specific differences between treatments. Significant differences were considered when P values were <0.05.

Results

Netting treatments and fouling control strategies tested in this work had different effects and displayed different results on netting tensile strength and fouling presence in net panels.

Figure 19 shows how in all 3 experimental conditions, netting PNA slightly increased after treatment with the antifouling paint or coating treatment. This agrees with the observations previously done by other authors (Swain and Shinjo, 2014) and occurs due to twine contraction after antifouling or coating treatment. It can also be seen that at the end of the experiment, and right before net operation (net changing or cleaning), PNA was significantly lower in C2 and C3 compared to C1. Subsequently PNO was significantly higher in those conditions. On-site cleaning allowed PNA recovery in C2 and C3 (cleaning effect) to levels similar to those base line values but there was a remnant 10% which was impossible to recover. It is not shown in the graph but cleaning efficiency in C1 (in a commercial washing machine) was 100%. Within each one of the experimental conditions C2 and C3, no significant differences were found in the degree of mesh occlusion recorded right before cleaning in all intermediate cleaning operations. In **Figure 20** the actual appearance of net panels at the end of the experiment can be visually assessed.

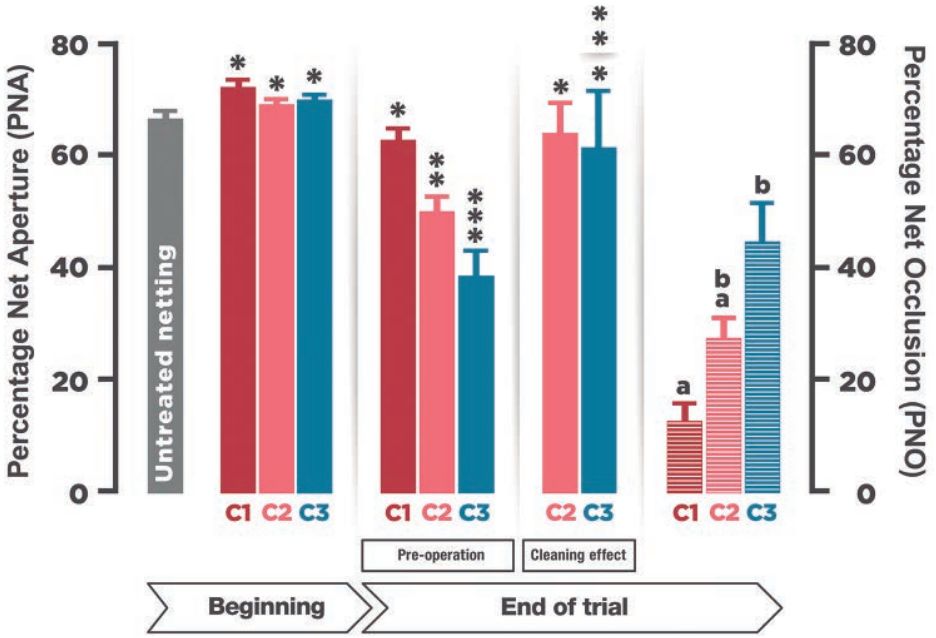


Figure 19. Effect of netting treatment in PNA and PNA/PNO changes during the experiment.

Bars represent PNA and patterned bars represent PNO (plotted on the right Y axis). At the beginning of the experiment *(n = 8)* there were no significant differences in PNA between treatments. Significant differences in PNA and PNO were recorded at the end of the experiment *(n = 6)* before cleaning operations between C1 and C2, C3 and within C2 and C3. Cleaning effect in C2 and C3 promoted PNA recovery to similar values at the end of the experiment. Values are means of three replicates. Different numbers or letters denote significant differences (P< 0.05).

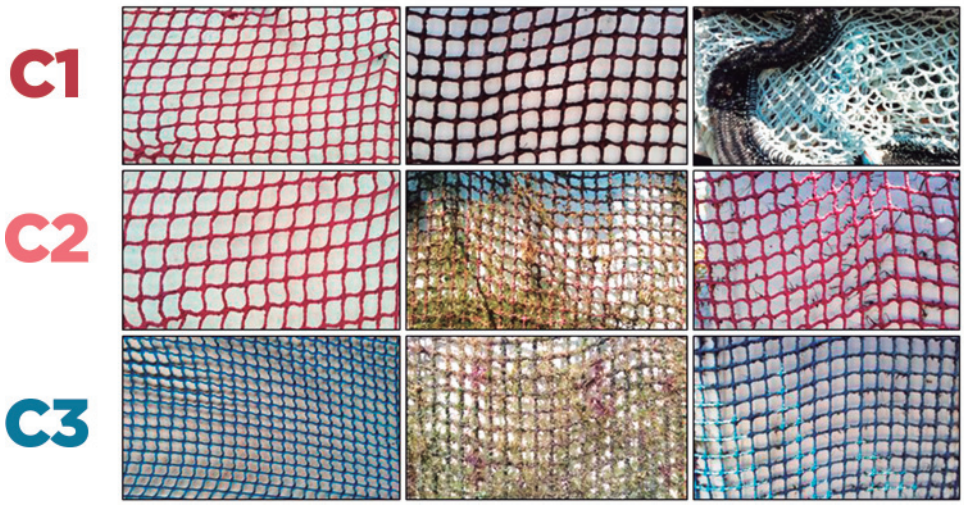


Figure 20. View of panels from C1 (top), C2 (center) and C3 (bottom) along the experiment.

Regarding netting tensile strength, at the beginning of the experiment BS tests performed on untreated wet netting suggested a lower initial tensile strength of the netting than the expected according to material specifications (Mørenot, 2015) (see annex V). At the end of the experiment BS tests on untreated netting denoted a significant loss of resistance. Under the same pond conditions, the antifouling paint and polyurethane coatings seemed to provide a substantial degree of protection, especially in C3 nets **(Figure 21)**.

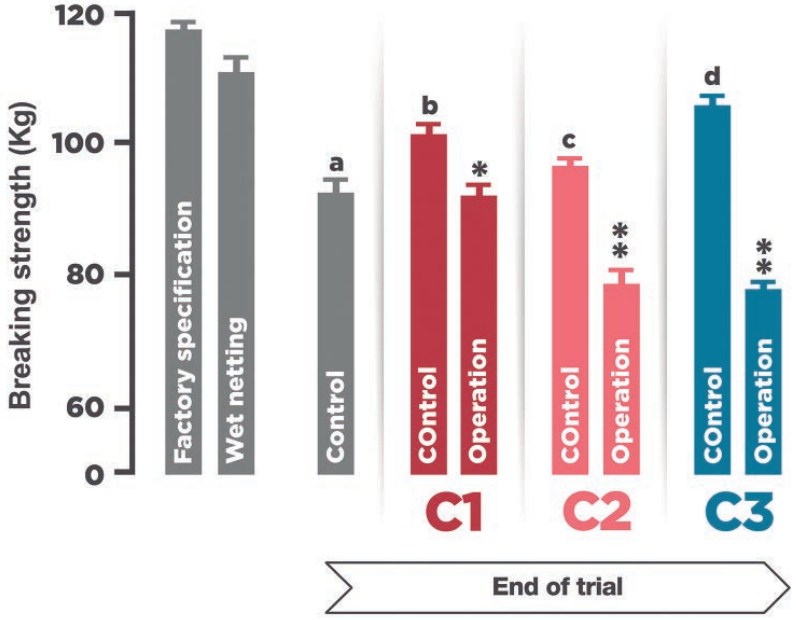


Figure 21. Results of BS tests performed during the experiment.

Bars represent BS. During the experimental time treated netting displayed significantly better resistance than the untreated netting (control). Cleaning effect along the experiment produced a loss of tensile strength in all three conditions. This was especially noticeable in C2 and C3. Values are means of three replicates, in Control columns (Control, Co) $n = 45$, in Operation columns (Op) $n = 30$. Different numbers and letters denote significant differences, ($P<0.05$).

According to the results obtained, cleaning operations clearly and significantly reduced netting tensile strength regardless the strategy used, but it seems that the effect of on-site cleaning at 250 bar caused much more damage on the netting than ordinary cleaning in a washing machine. In this experiment, after a rearing period of seven months breaking strength of those nets in C2 and 3 were significantly lower than breaking strength in C1. No differences were observed between C2 and C3. In C1 nets were washed in a washing machine once being changed by new nets and nets in C2 and C3 were cleaned on-site six times.

Discussion

In cage aquaculture, nets are the strongbox which contain a tremendous value in livestock. For this reason, issues regarding net management and their maintenance status have to be very carefully taken into account. It is mandatory to keep the batch of nets in perfect conditions in a fish farm and to know how net operations affect net properties and performance. It is especially necessary to assess nets behavior under real working conditions and over long periods as (Sala et al., 2004) state since many of the factors affecting their performance are impossible to reproduce in laboratory tests.

As it was already known and it has been described for other copper based paints (Braithwaite et al., 2007; Douglas-Helders et al., 2003; Edwards et al., 2015; Swain and Shinjo, 2014), antifouling treatment applied in C1 resulted to be highly effective. After seven months, at the end of the experiment, PNA values in C1 reduced only from 72% to 63% (PNO 13%).

According to the statement by Bloecher et al., (2013) washing off nets is generally conducted every eight weeks in winter to two weeks in summer, but it can occur as often as weekly during periods of high biofouling pressure. Considering the amount of resources needed at industrial scale and the necessary cost-effectiveness of the operation, in this study, cleaning in C2 and C3 was performed approximately every 45 days from December to April when temperatures were below 18ºC and approximately every 30 days from May to the end of the experiment, with higher temperatures, longer days and consequently more fouling production.

After image analysis the results suggest that in order to maintain low levels of net occlusion, on-site cleaning in C2 and C3 should have been performed more often as initial values of PNA ranging about 70% clearly reduced to values around 50% in C2 and 38% in C3 (PNO_{C2} 28%, PNO_{C3} 45%). However, a high cleaning frequency, might have a direct effect on fish and net performance which would eventually increase fish production cost, and the cost of operations is precisely one of the reasons the industry works to find alternatives to the use of antifouling coatings. The results show a very clear antifouling effect of the cuprous oxide incorporated in the C2 coating. This treatment clearly appears as a promising option which would be worthy to further study in order to reduce both the use of copper and cleaning frequency. Again, these are two of the main purposes pursued by the fish farming industry in fouling control.

Net panels in the experimental cages were constructed according to industry procedures to avoid pockets due to excessive slack to ensure a good cle-

aning efficiency when cleaning on-site. However, complete cleaning in C2 and C3 was not achieved **(Figure 22)** as it was very difficult to completely remove fouling organisms especially in those areas next to ropes and net folds and pockets. Pre-cleaning PNA values in C2 and C3 recovered from 50% and 38% to 64% and 61% respectively. There was a remnant of about 10% PNO which on-site cleaning was not able to remove. As a consequence, fouling re-growth rate was fast in C2 and especially in C3, where the coating did not contain copper at all.

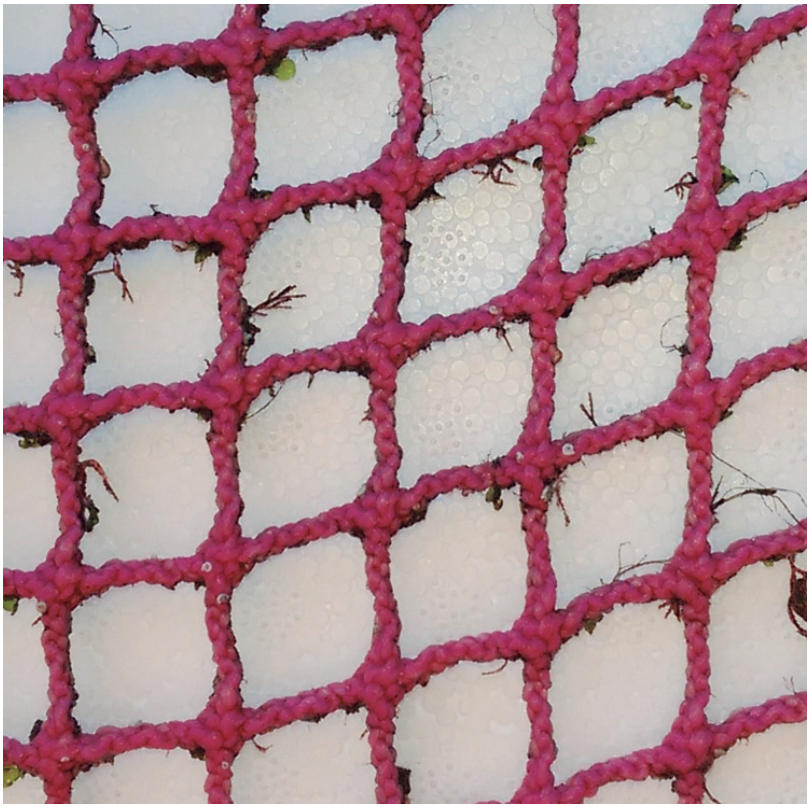


Figure 22. Portion of net panel of C2 after on-site cleaning at the end of the experiment. View from the inside out.

Although Moe et al., (2007) concluded that antifouling treatments reduce tensile strength of netting, in this work, after seven months in seawater it seems like both antifouling and coating treatment protected the netting to some extent from external agents. This discrepancy could be explained by the fact that being probably true that the direct effect of a coating or paint as such on polyamide fibers' tensile strength can be detrimental for the direct effect of the paint/coating on the fibers, in actual working conditions the layer net coating could protect it from external agents such us UV radiation, abrasion or temperature changes.

In fact, the industry uses coatings to protect net fibers from abrasion produced by high water pressure disks in those nets designed to be cleaned on-site. In this work, after a period of 7 months under experimental conditions breaking strength on those treated panels was significantly higher than that of the untreated netting. In C3 the difference was especially noticeable increasing from 93,4 Kg to 106 kg. Similarly, in C1 and C2 BS after 7 month was also higher than the recorded on tests performed on untreated netting.

Regardless the procedure, cleaning practices on textiles produce a loss of tensile properties. A concern regarding on-site cleaning is the effect of high water pressure on netting fibers. In this experiment, the polyurethane coating used in C2 and C3 did not provide enough degree of protection and there was a dramatic loss of tensile strength after only six episodes of *in situ* cleaning. BS values recorded on non-washed panels were 97 Kgs and 106 Kgs in C2 and C3 and those recorded on washed panels situated around 78 Kgs. On the other hand, in C1, the abrasion caused by friction between net panels when nets were washed in the washing machine caused a much less, although significant difference between panels. In this case BS dropped from 102 Kgs to 92 Kgs. This suggests that nets should have been better protected to avoid damage produced by the effect of high water pressure.

Conclusions

In fouling control, on-site cleaning seems to be still many steps behind the use of antifouling treated nets. In this experiment, the commercial antifouling used resulted to be highly effective. On the other hand, on-site cleaning performance was poor. Cleaning efficiency needs to improve as currently the cleaning rates obtained with the existing technology together with the nets design allow a rapid re-growth rate of the fouling making necessary a high cleaning frequency which damages the nettings and increases operational costs. Cleaning procedures are highly thrilling to the nets causing a severe depletion of fibers tensile strength. For this reason, nets should be better protected to withstand high water pressure abrasion or alternatively use other methodologies than high water pressure. Currently the use of low pressure, cavitation and suction cleaning alternatives and high performance coatings are being explored (unpublished data).

Growth and health features

Introduction

Good growth rates are a major goal for the aquaculture industry. As it has been said in the general introduction, both the fouling accumulation in nets and cage operations might have direct consequences on fish performance and health features. This chapter will review key performance and health indicators related to fouling and fouling management.

Parasites reservoir

A relevant factor affecting fish performance is the occurrence of parasite reservoir associated to fouling. Thus, several authors suggested the hypothesis that considers fouling as a reservoir for parasites in aquaculture nets (Douglas-Helders et al., 2003; Edwards et al., 2015; Swain and Shinjo, 2014; Tan et al., 2002). Among those parasites, the blood sucking monogenean *Sparicotyle chrysophrii* ,(Henry et al., 2015a) that according to (Sitjà-Bobadilla et al., 2010) is able to survive a whole production season and thus spread easily to newly introduced fish. Should this relationship between fouling and parasites exist, it would increase the incidence of potential disease thus finally increasing costs and reducing the profitability of aquaculture companies. Following the same principle, net fouling could also play a role as reservoir for pathogenic bacteria.

The use of copper in aquaculture nets and parasite control

For the past decades, the use of antifouling paints containing copper as a biocide has been used extensively to control fouling in aquaculture nets. Although well-regulated (Cockell et al., 2008; Council of European Union, 2016; European Commission, 2006; Hedley and Huntington, 2009), the use of copper in animal production is a concern among public opinion because of its release into the environment and incorporation in the trophic chain through bioaccumulation in tissues. As opposed to what happens with other heavy metals as mercury (Hg), lead (Pb) or cadmium (Cd) (Perugini et al., 2014), in the particular case of copper, bioaccumulation in fish tissues should not represent a major threat besides being a micro-requirement component of biological molecules. The literature reveals that copper content in both wild caught and reared fish are usually low and differences among them are small (Fernandes et al., 2009; Minganti et al., 2010). Nevertheless, under conditions of exposure it can be accumulated in tissues and pose animals at risk, thus affecting consumer health and environmental balance. Humans accumulate copper firstly in liver and secondarily in brain and kidney (Linder, 1991). Large amounts of copper accumulation can result in the formation of cirrhotic nodules and dramatic morphological changes in the organs (Buiakova et al., 1999).

Copper toxicity

Copper is a natural occurring element present in all waterbodies and essential to all eukaryote cells. Although diet is the main pathway for copper incorporation in marine animals (Watanabe et al., 1997), fish can also uptake copper directly across their skin, gut and gills (Grosell and Wood, 2002). Copper is required for normal growth and development of fish since it is a co-factor for many enzymes that require redox activity as cytochrome *c* oxidase. However, also due to this redox nature, copper can be toxic at high concentrations (Grosell, 2011). Mainly in its free ionic form ($Cu^{2}+$) it is cytotoxic and it can stimulate production of reactive oxygen species (ROS) which can damage lipids, proteins and DNA. It can also bind to protein thiol groups (Halliwell and Gutteridge, 1984) compromising protein functionality. In fish, copper metabolic imbalance causes a reduction in food intake which eventually results in growth inhibition. Due to this toxicity, organisms have developed good copper homeostatic mechanisms.

Most aquaculture environments are rich in dissolved organic matter. This is particularly advantageous in helping to prevent copper toxicity since a major factor in reducing this toxicity is the presence of dissolved organic carbon (DOC). DOC binds to copper ions with high affinity originating complexes that prevent or reduce copper binding or uptake (Playle et al., 1993). Furthermore, copper toxicity, as with other heavy metals, decreases as salinity increase (Hall and Anderson, 1995) The toxicity of copper nanoparticles on fish has also been described producing gill lamellae hyperplasia in zebrafish (Griffitt et al., 2009), oxidative stress and cell apoptosis (Wang et al., 2014).

Cage operations and growth and gill performance

In general, fish farm operations and particularly net changing or net cleaning are a source of disturbance to the animals. It is commonly assumed that feeding patterns have to be modified to allow net maneuvers. In fish farming it might take some time for a regular feeding pattern to restore after a disturbance. This can very easily result in a loss of growth.

In this sense, on-site cleaning is seen as a methodology causing a much lower intensity of disturbance than net changing, an operation during which animals are often confined to reduced water volumes for a period of time suffering stress (Ortun et al., 2001; Rotllant et al., 2001; Rotllant and Tort, 1997; Tort et al., 2001), external damages, and even causing some mortality. However, in the previous chapter it has been shown that cleaning frequency is a key factor when assessing on-site cleaning reliability as it not only damages the net but also could affect fish performance and health in several ways.

It has been seen in the field that during on-site cleaning operations, as fouling is washed off the nets, a cloud of debris is produced that can remain in and around the cages for several hours depending on the degree of fouling accumulation and

current intensity and direction. This suspended organic matter can affect gill integrity when eventually passes through the gills. Gills are a major organ for homeostasis and osmotic balance in fish, and damages in gills quickly can lead to general organism malfunction and death (Baxter et al., 2011). Gill damage could not be the only effect of the on-site cleaning strategy on fish health. A high cleaning frequency could also produce stress on fish and as already stated just a few lines above in this introduction it might also modify the feeding pattern in a fish cage.

The aim of this chapter is to assess the effects of fouling presence and the control methodologies here tested on fish performance and health features. Growth and somatic indexes will be firstly assessed among the experimental conditions. As it is a worry among fish consumers, Cu content in tissues will be determined in order to assess effects on fish and therefore the consequences on consumers' health. The presence of gill parasites and the skin microbiota dynamics will be analyzed along the experiment as skin has been described as a major entry pathway for pathogenic bacteria in fish (Bøgwald and Dalmo, 2014) and copper can interfere in parasites and bacterial proliferation as a biocide. Lastly the consequences of on-site cleaning on gill integrity will be also studied as it can directly affect fish performance.

Materials and methods

Growth and somatic indexes

To quantify growth along the experiment all the animals were weighed when setting the replicates of each condition. All surviving fish were weighed at the end of the experiment. During the experiment weight measurements were also performed at different stages in order to obtain a growth curve. Specific Growth Rate (SGR day^{-1}) was calculated using the formula reported by Hopkins (1992) where $W\chi$ refers to the weight at the end of the experiment in g, *W0* is the weight at the beginning of the experiment in g and *T* is the time in days.

$$SGR = \frac{lnW\chi - lnW0}{\Delta T} \times 100$$

Growth was also compared with the theoretical value that should have been obtained in a commercial fish farm. To do that, data from several actual sea bream batches reared and harvested in commercial cages in the same Mediterranean area and under the same temperature and feeding profile were used.

Hepatosomatic (HSI) and spleen somatic (SSI) index were calculated at the beginning and at the end of the experiment as previously described (Nikolsky, 1963). Initial somatic index was calculated using twenty seven animals, (3 animals per cage, 9 animals per condition), and at the end of the experiment somatic indexes were calculated in all surviving animals. Fish were sacrificed with 200 mg–[1] MS222

(Sandoz, Germany), dissected and their liver and spleen excised and weighed. After that, those organs were immediately kept frozen at -80ºC for other analyses.

Copper in tissues

To determine the amount of copper present in fish tissues nine animals at the beginning of the trial and twenty seven animals, (3 animals per cage, 9 animals per condition) at the end of the experiment were used. Fish were sacrificed with 200 mg^{-1} MS222 and dissected. A sample of liver, gills and muscle was obtained from each one of them and immediately frozen at -80ºC. For copper content determination, 0.2-0.6 g. of each sample was digested in a microwave oven (Milestone, Ultrawave, Italy) in concentrated HNO_3 (Merck, Germany). Copper determination was performed using an ICP-MS spectrometer (Agilent 7500ce, United States).

Skin microbiota analyses

Following the procedure described in General materials and methods for skin mucus collection, pellets containing skin from 18 fish at the beginning of the experiment and 18 fish at the end of the experiment (24 hours after net operation) were collected and stored at -80ºC. To identify the bacterial component of skin microbiota the DNA was purified using a GeneJet Genomic Purification Kit (Thermos Fisher Scientific, USA) according to manufacturer's instructions and genomic DNA was quantified in a NanoDrop-1000 spectrophotometer (Thermos Fisher Scientific, USA). Afterwards an Illumina®16s rRNA sequencing was performed as analyses of the prokaryotic 16S ribosomal RNA gene (16S rRNA) are commonly used in metagenomics studies. This gene contains nine variable regions interspersed between conserved regions that are frequently used in phylogenetic classifications such as genus or species determination in diverse microbial populations.

Gill parasites quantification

To quantify the parasites in the gills, twenty four animals were sacrificed at the end of the experiment with an overdose of anesthetic (200 mg^{-1} MS222) and individually frozen in plastic bags at -20ºC in order to avoid parasites loose or mix during the handling of samples. For parasite quantification, once the frozen fish thawed, full gill arches were carefully excised and examined under stereomicroscope to determine parasites prevalence, abundance and mean intensity (Bush et al., 1997). A manual counting was performed and Prevalence (P, infected fish/non infected fish), Abundance (A, # of parasites/total # of fish), and Mean Intensity (MI, # of parasites/total # of fish) were calculated.

Gill histology and gill score protocol

Histological observation was done in gills at the beginning and at the end of the experiment (before, right after and 24 hours after cleaning operations) to assess the effect of on-site cleaning and net changing in gills integrity. Seven animals at the beginning of the experiment and 15 at the end of the experiment were sacrificed

using 200 mg–[1] MS222, their complete gill arches were removed and fixed in 10% formalin for 24 hours and stored in 70% ethanol. To obtain tissue sections, gills were cleared in xylene in a tissue processor Leica TP1020 (Leica Biosystems, Germany) and paraffin-embedded in a paraffin embedding station Leica EG1150H following standard procedures. A microtome LEICA RM 2255 was used to obtain 5 µm sections which were afterwards stained in Hematoxylin-Eosin (Harris hematoxylin - Sigma HHS16 - Eosin Y-Sigma HT110-216) for 90 seconds. The microscopy examination was done with a NIKON Eclipse 80i (Nikon, Japan) microscope at 200x and 400x magnification.

A semi quantitative analysis was done on gill damage according to the gill scoring protocol developed by Mitchell et al. (2012) (see annex VI) slightly modified. The score for the index parameters was based on the presence and extent of the following four primary parameters*: lamellar hyperplasia, lamellar fusion, cellular anomalies (including degeneration, necrosis and sloughing)* and *lamellar oedema* with a score ranging from 0 to 3 (none, mild, moderate or severe) being assigned to each parameter. We also measured the presence (1) or absence (0) of additional alterarions: *cellular hypertrophy, inflammation, excessive numbers of mast cells, circulatory disturbances (haemorrhage, telangiectasis, congestion) and the excessive number of mucus cells*. In this analysis the presence of pathogens and parasites was not assessed. Total score assigned to each sample ranged from 0 to 17 according to **Table 2**.

Score	Gill damage
0 to 3	No damage
4 to 6	Minor damage
7 to 9	Moderate damage
≥ 10	Severe damage

Table 2. Gill score for gill scoring protocol.

A total of 10 fields of gill sections from different animals of the 3 conditions were analyzed at the beginning of the experiment to obtain a control score. At the end of the experiment 10 fields of gill sections from animals of C2 and C3 just before net cleaning were also analyzed. Immediately after net operation, gill analyses were performed on 10 fields of gill preparations from fish of each one of the experimental conditions. Finally, the gill status 24 hours after net maneuver was conducted on 10 fields of gill samples from C2 and C3 animals.

Statistical analysis

Statistical analyses were performed using the statistical package MINITAB (version 17, Minitab Inc., Pennsylvania). All analyzed data were normally distributed.

Normality was checked with the Anderson-Darling test. Growth, somatic indexes, copper bioaccumulation, and skin mucus richness differences were compared with repeated one way ANOVA. Tukey's tests were used to see specific differences between treatments at different moments considering significant differences values of $P<0.05$. Gill alterations were analyzed to see differences between the different experimental treatments at different moments using the General Linear Model with treatment and operation as factors. One-way ANOVA and Tukey's tests were used at the end of the experiment to see differences between treatments. Statistically significant differences were assumed when P values were <0.05. When analyzing skin microbiota data were log transformed to give more consistent results. Descriptive statistics were used to show differences in parasite presence in gills.

Results

Growth and somatic indexes

At the end of the experiment, growth achieved in C1 was significantly higher than that achieved in the other two experimental conditions **(Figure 23)**. In all cases growth attained was lower than that showed by the model to which it was compared. This was especially evident in C2 and C3, where mean weight of the fish was always below the model along the experiment. On the other hand in C1, the weight of the animals was always above the model except in the very last part of the experiment. Accordingly SGR % day^{-1} was also lower in C1, C2 and C3 compared to the growth model. SGR % day^{-1} in C1 was significantly higher than in the other two groups **(Figure 24)**.

Looking at the evolution of growth C2 and C3 display the same growth rate than the model since the beginning of the experiment until mid-May, when there was an increase of the temperature reflected in the model with a much higher step of the slope in SGR. During all winter growth in C1 was better than the showed by the model and from March to mid-May it equals the model. Again the increase of temperature corresponding to a much better SGR predicted in the model from May to the end of the experiment was not performed by any of the experimental groups.

At the end of the experiment, both organic index, hepatosomatic index (HSI) and spleen- somatic index (SSI), displayed significant differences between the different strategies. At the end of the trial, in the groups under the on-site cleaning strategy those organs were bigger. However, while along the experiment, in the case of SSI the spleen did not significantly change in C1 and increased in size in C2 and C3, in the case of HSI the size of the organ of all three groups proportionally and significantly decreased compared to the size at the beginning of the trial **(Figure 25)**.

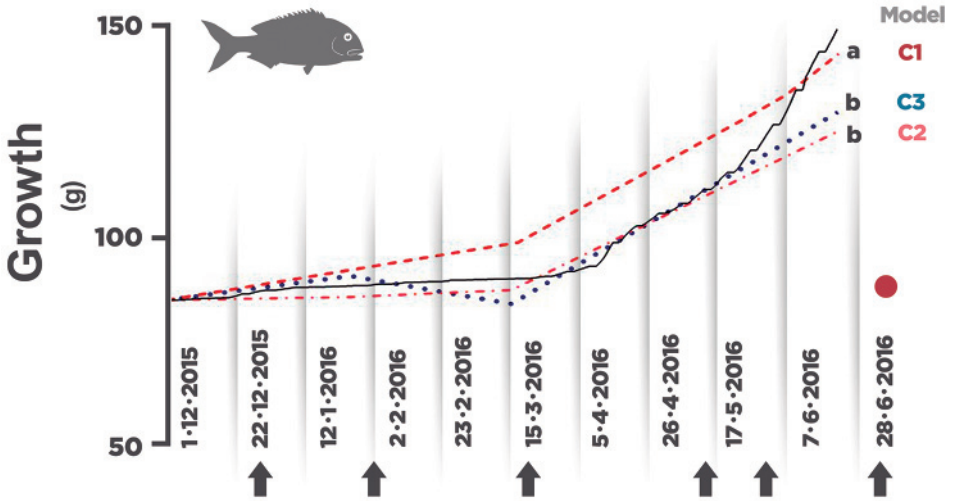


Figure 23. Growth evolution during the experiment.

Continuous line represents theoretical growth according to growth model. Stapled line represents C1, stapled-dotted line C2 and dotted line C3. Arrows are on-site cleaning episodes in C2, C3 and spot stands for net changing in C1. Letters denote significant differences P<0.05.

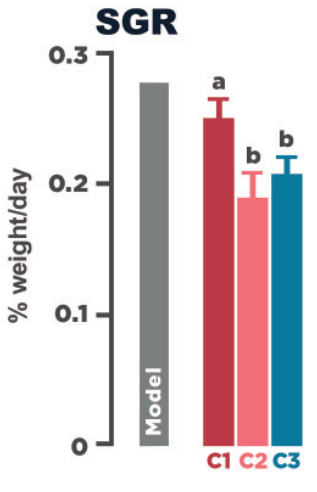


Figure 24. SGR at the end of the experiment compared to expected values.

C1, C2 and C3 values are means of three replicates (n=40). Letters denote significant differences between groups (P<0.05).

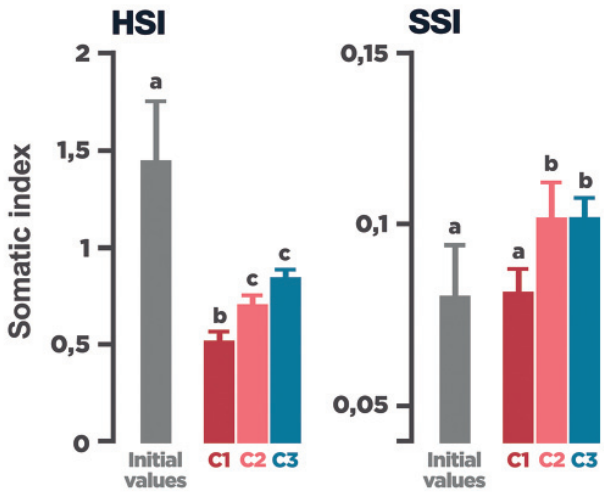


Figure 25. Initial and final values of hepatosomatic (HSI) and spleen-somatic (SSI) indexes.

Initial values were obtained from a pooled population of animals from the experimental groups. Final values are means of three replicates (n=27). Error bars represent SE. Letters denote significant differences (P<0.05)

Copper in tissues

At the end of the experimental period the results suggest that there was some copper release from the nets which eventually may accumulate in tissues. The small test performed in order to assess copper release from experimental paints to the water (see general materials and methods), showed that indeed, it happened. While no copper was detected in the tanks containing pond water or pond water + untreated netting, a concentration of 268 mgL^{-1} was registered in the tank containing pond water + C1 netting. Whereas a much lower concentration was recorded in tanks containing water + C2 netting and water + C3 netting, 15 5 mgL^{-1} and 13.9 mg^{L-1} respectively.

At the end of the experimental period, the amount of copper found in tissues was higher than the control values obtained from tissue samples at the beginning of the experiment. Significant differences were found in C1 when comparing control values of copper to those at the end of the experiment in both liver and gills. The differences were more evident and significant in liver than in gills. In C2 and C3 copper content in liver at the end of the experiment was also significantly higher than control values, but in contrast copper concentration in gills did not significantly increased. There were no significant differences between treatments at the end of the experiment **(Figure 26)**. Although not represented here, copper content in muscle at the end of the trial was lower than 0.25 µg g^{-1} in all three groups, below the technique detection threshold. It is relevant to mention that quantities of copper accumulated in tissues were far below safety recommendations for human consumption in Europe. (European Commission, 2006).

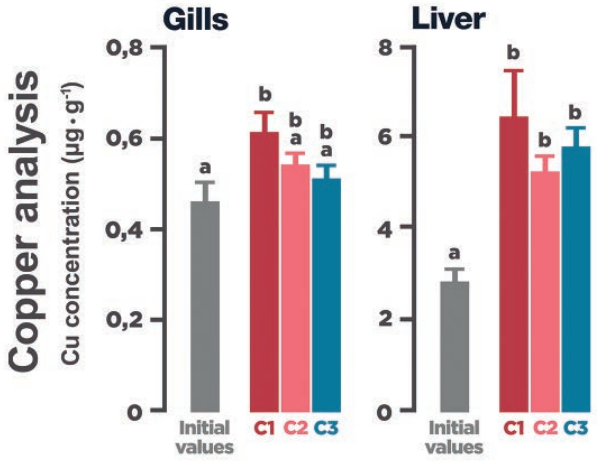


Figure 26. Copper content in liver and gills at the beginning and at the end of the experiment.

Liver values are plotted on the left Y axis while gill values are plotted on the right Y axis. Significant differences are shown in lower case letters in liver and in uppercase letters in gills. Values at the beginning of the experiment come from a pool of 9 samples from animals of all groups. Values at the end of the trial are means of three replicates (n=9). Error bars represent SE. Values not sharing letters are significantly different ($P<0.05$).

Gill parasites

After seven months of rearing period in the pond, the presence of the parasite *Sparicotyle chrysophrii* among the experimental groups was low, but while no evidence of monogenean was found in C1, a few adult specimens were found both in C2 and C3 gills **(Table 3)**.

	Prevalence (%)	Abundance	Mean Intensity
C1	0,0	0,0	0,0
C2	25	0,83	3,3
C3	40	0,8	2

Table 3. Presence of *Sparicotyle chrysophrii* at the end of the trial.

Skin microbiota

The tendency in the evolution of skin microbiota richness along the experiment behaved differently in C1 compared to C2 and C3. At the beginning of the trial, looking at the three conditions separately, C1 showed a slightly higher microbiota richness than the other two conditions although this difference was not statistically significant. However, at the end of the experiment there were significant differences between C1 and C2, C3. While in C1 microbiota richness significantly reduced over the experimental period, in C2 and C3 it significantly increased **(Figure 27)**.

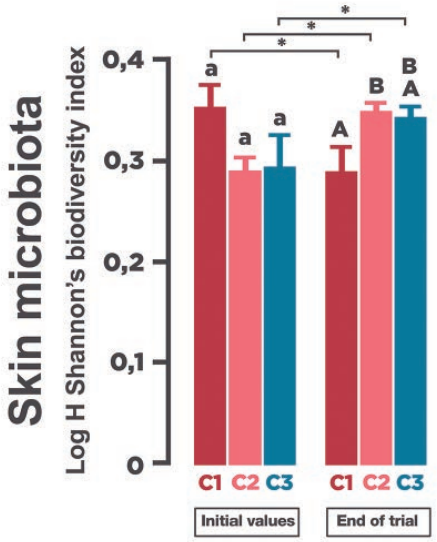


Figure 27. Skin microbiota richness at the beginning and at the end of the experiment expressed as log H (Shannon's index H).

Values are means of three replicates (*n=18*). Error bars represent SE. Shared lowercase letters at the beginning and upper case letters at the end of the experiment, denote not significant differences. Asterisks denote significant differences within treatments between initial and final values, *P<0.05*.

Gill histology

The results of this experiment indicate a very clear effect of net operations in gill health. Score values of analyzed gill arches ranged from 0 to 14 In C1, slightly significant differences were found right after net changing at the end of the experiment after seven months. In this group, the control gill score of 2.78 (no damage) increased to 4.89 (minor damage). The level of branchial tissue alteration was general but mild, and mainly in those primary characters. Damage was specially observed in the very apical areas of secondary lamellae. No relevant lesions were detected **(Figure 28)**.

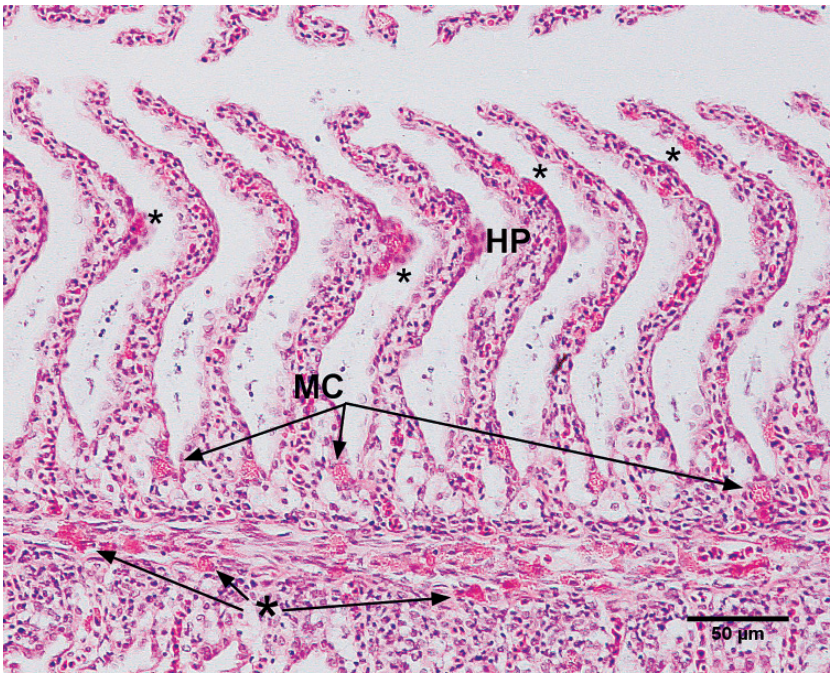


Figure 28. Example of a scored "6" gill arch of C1 after net changing at the end of the experiment. Hyperplastic lesions (HP) can be seen in secondary lamellae as well as mucous cells (MC) and MAST cells (asterisk) proliferation.

On the other hand in C2 and C3 very relevant and significant differences were found between initial and final status of gill integrity. Gill damage after net cleaning was scored and 9.20 in C2 and C3 respectively. The time lapse between cleaning episodes allowed a partially but not significant damage recovery in gills caused by the regularly performed cleaning operations in C2 and C3 along the trial. Averaged gill score just before cleaning in C2 and C3 was 8.22, which is considered a moderate damage. No significant differences were found within and between groups when comparing gill damage just after and 24 hours after on-site cleaning in C2 and C3 **(Figure 29)**.

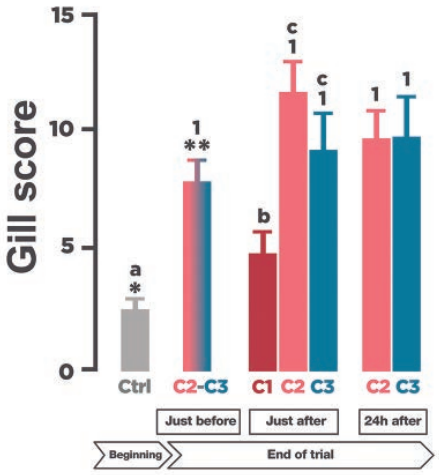


Figure 29. Gill score at the end of the experiment.

Effect of on-site cleaning in gill disorders. Grey bar represents control. Patterned bar represents mean value for C2 and C3 at the end of the experiment (after five cleaning episodes and before the last one).Error bars are SE. Different letters, numbers and asterisks denote significant differences. P<0.05.

At the end of the experiment, gills in both C2 and C3 showed evidences of severe gill alterations with high levels of damage and deterioration, including, primary and secondary lamellae hyperplasia, edema and fusion, circulatory disturbances and an increase in the presence of MAST and mucous cells **(Figure 30)**.

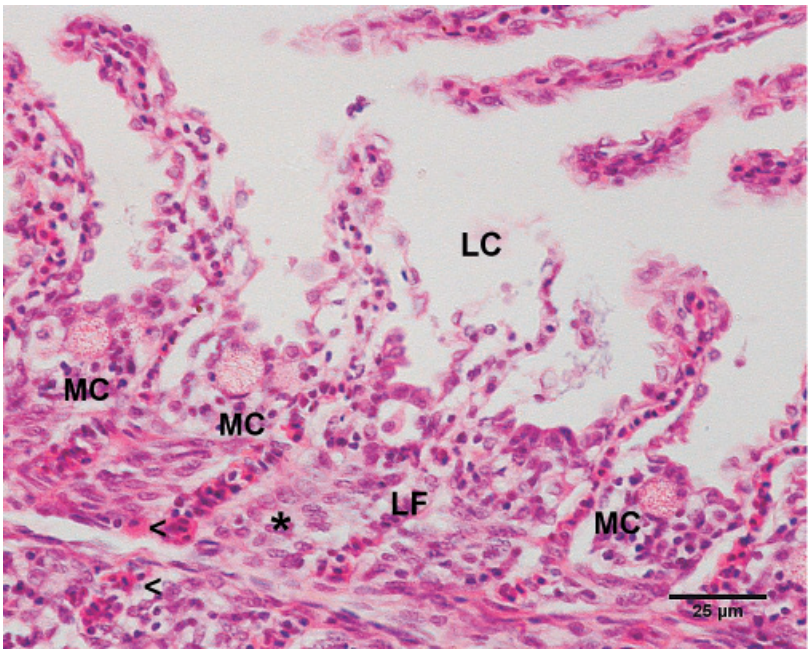


Figure 30. View of a seriously damage gill in C3.

Proliferation of mucous cells (MC) and MAST cells (<), lamellar fusion (LF) and cellular hyperplasia (*) and complete loss of structure (LC) in secondary lamellae.

Discussion

Attaining maximal animal growth rates is a major goal for aquaculture industry. In terms of production performance, handling and farm operations can generate stress and often have a direct impact on fish (Conte, 2004; Rotllant and Tort, 1997). The on-site cleaning strategy was originally thought to be a low invasive practice that would not cause stress in fish.

In this trial, growth in all groups was lower than the expected when compared to the one that should have been attained in a commercial cage in the area. A factor that could have modulated this result is temperature. The fact that the trial was carried out in a shallow pond involved higher temperature variations than those at open sea. During the experiment these temperatures variations, which sometime were up to 4ºC, substantially reduced animals feed intake. The negative effects of temperature variations on fish growth have already been described (Tort et al., 2004). In fact, the feeding response during those high temperature fluctuation periods was very poor in all cages. Another factor that could have produced a lack of growth was a lymphocystis infection that fish in all groups underwent during the coldest months.

On the other hand final weight achieved in C2 and C3 was significantly lower than in C1. Cleaning operations may also affect feeding pattern and behavior of fish, and thus growth rate. In this trial fish were fed before net cleaning, but feeding response was very poor after cleaning operation, even when the next meal was the following day. This would also explain the lack of growth.

Looking at the evolution of growth along the experiment it is easy to see that fish in all conditions did not respond to the increase of temperature recorded in May as the predicted model, probably due to the daily temperature variations recorded in the pond. On the other hand, it seems that on-site cleaning operations during the experiment could effectively have exerted an effect on fish growth, and this would be in contraposition with the initial hypothesis.

As described before (Vera and Vergara, 2016), in this work, on-site cleaning operations generated a thick cloud of debris also in this work, when fouling particles were washed off the nets as the amount of fouling in net panels was substantial. This suspended organic matter remained in and around the cages for a time up to 2 hours and very likely passed through the gills causing severe damage. Gill alterations observed in C2 and C3 were much more severe than those observed in C1 after net changing **(Figure 31)**.

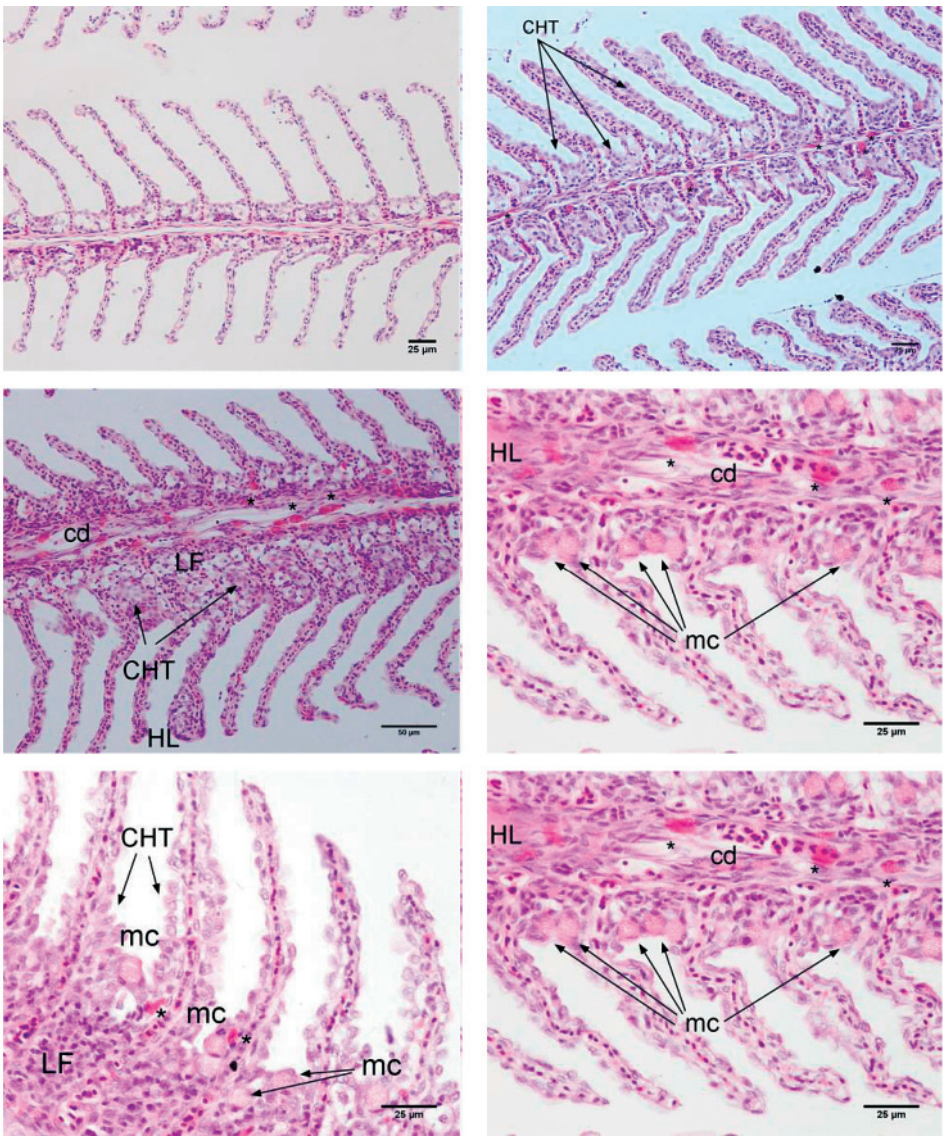


Figure 31a. Gill damaged produced in gills in C2 and C3.

This result would reinforce the position of those against the use of this cleaning methodology. Mild damage in C1 gills could also respond to the chronic exposure to copper (Dalum et al., 2015).

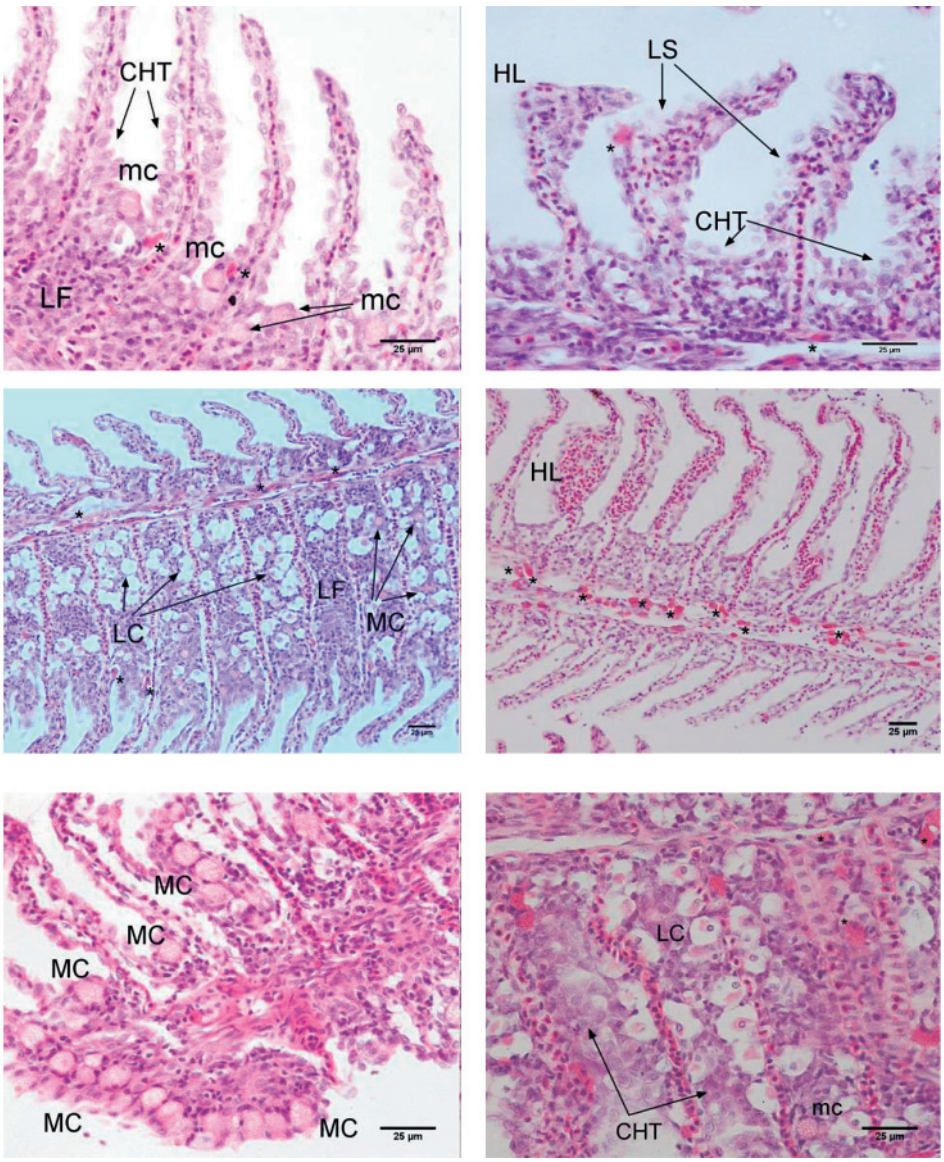


Figure 31a. Gill damaged produced in gills in C2 and C3.

Though, it should be borne in mind, that the ratio between net surface and net volume reduces as the cage/net size increases, and so the effects of net cleaning could possibly be lower in larger commercial cages than those obtained in this work. In this study, on-site cleaning caused the complete array of lesions and alterations as well as the cellular mobilization cited in previous studies (Alsafy, 2013; Bosch-Belmar et al., 2016a, 2016b; Chalmers et al., 2017; Dezfuli et al., 2011, 2010; Dezfuli and Giari, 2008; Lauriano et al.,

2012; Taylor et al., 2009). Literature review suggests that fouling in aquaculture cages and nets may act as a reservoir for pathogens and parasites increasing disease risk (Andersen et al., 1993; Douglas-Helders et al., 2003; Edwards et al., 2015; González, 1998; Henry et al., 2015b; Sitjà-Bobadilla et al., 2010; Tan et al., 2002). However, Sitjà-Bobadilla et al., (2010) states that under the conditions in which this trial was carried out, the presence of the gill parasite *Sparicotyle chrysophrii* is very unusual suggesting that factors like temperature oscillations and turbidity do not promote its proliferation. That is why the evidence of some specimens in C2 and C3, despite being scarce was very noticeable and would confirm the above hypothesis. The same principle, together with the biocide effect of copper would explain why skin microbiota behaved differently in C1 than in C2 and C3 along the experiment. Biodiversity richness tended to decrease at the end of the experiment compared to the start in C1. On the other hand, in C2 and C3 microbiota richness was higher at the end than at the beginning of the experiment. Bacterial communities in skin may contain pathogens and some works performed in fish assessed the status of fish health through those communities, following the works by Reed and Spence (1929) among others. Very scarce data are available on sea bream. Regardless that bacterial proliferation around the cage and in the skin could have positive or detrimental effects on fish depending on bacterial populations, it could be deducted that the presence of fouling enhances the chances for bacterial proliferation in the aquatic environment surrounding fish production. In this work no pathogenic bacteria were found among the components of the microbiota, being the most present genus *Faecalibacterium*, *Bacteroides* and *Sarcina*.

The presence of parasites, the higher degree of bacterial proliferation and the effects of on-site cleaning operations could explain the fact that liver and spleen organo-indexes where significantly higher in C2 and C3 compared to C1 at the end of the experiment. These organs are highly involved in inflammatory and immune responses. Consequently conditions above could promote an initial response to the rearing conditions (De Vico et al., 2008; Mauri et al., 2011; Monshouwer and Hoebe, 2003; Wiens and Glenney, 2011). Spleen is a major lymphoid organ involved in innate immunity and in antibody synthesis (Uribe1 et al., 2011). Looking specifically to the liver, results show a reduction in size in all three conditions comparing final with initial weight. This could be associated to glycogen reserves mobilization during winter period suggesting that resources from feed intake would not entirely cover the energetic demand. In this experiment, feeding was *ad*

libitum 5 over 7 days per week, but as stated before, daily temperature variations affected appetite. However, in C1 growth achieved after seven months was only 6% lower than the expected rates in a commercial batch. Visceral fat levels were reduced compared to those in reared animals. Hepatosomatic index was also low compared to reared sea bream (Benedito-Palos et al., 2016; Chatzifotis et al., 2008; Roncarati et al., 2006) Since the final growth under our experimental conditions were close to what should be expected under commercial standards, the results suggest that an over-feeding is generated during or before winter in sea bream fish farms that is not used for protein growth and is accumulated as visceral fat. These results would match with the metabolic and physiological studies performed in sea bream under the episodes of winter syndrome (Ibarz et al., 2010).

Heavy metals bioaccumulation in fish tissues is a concern as it could affect consumer's health. Several guidelines, regulatory frameworks and government regulations control the content and use of heavy metals in edible fish and aquaculture practices (Cockell et al., 2008; Council of European Union, 2016; European Commission, 2006; Hedley and Huntington, 2009). It is true that copper is an essential element for eukaryotic cells, but could suppose a risk to organisms at very high concentrations due to its redox nature (Grosell, 2011) and several works have been conducted to describe different consequences of copper toxicity (Blanchard and Grosell, 2006; Grosell et al., 2007, 2004; Kamunde et al., 2002; McGeer et al., 2000; Sanchez et al., 2005; Varo et al., 2007) Commonly, copper content in fish is not a major issue since levels either in wild or reared fish are low. Furthermore, fish have very well developed copper homeostatic pathways (Grosell, 2011). Several authors have assessed and compared copper bioaccumulation in wild caught and aquaculture reared fish. In 2010 (Minganti et al., 2010) reported even less copper content in muscle of farmed *Sparus aurata*, (1.3 ± 0.3 μg g^{-1}) than in wild caught specimens (1.6 ± 0.4 μg g^{-1}) in the Ligurian sea.

Referring to copper accumulation in tissues, the results of this chapter suggest that this should not be a major concern for consumers' health, since looking at the content of copper especially in muscle values were lower than 0.25 μg g^{-1} below the technique detection threshold in all three conditions being those completely safe for human consumption.

Fish can uptake waterborne copper through the skin and gills (Grosell and Wood, 2002) and incorporate it directly from the diet. Liver is the organ where the metal can be found at higher concentrations due to the major role of this organ in copper homeostasis (Grosell et al., 2003). Confirming this,

the quantity of copper found in liver in this experiment was much higher than the quantity measured in gills. In all three experimental groups copper content at the end of the experiment was higher than control values, thus some accumulation occurred. Even in C2 and C3 some of this bioaccumulation was significant despite the fact that net treatments in those groups contained a small amount of copper (C2) or even did not contain copper at all in the case of C3. This presence of copper could be explained by the fact that although cages layout followed the flow across the pond and there was a distance of three meters among one and another row of cages, copper contained in the antifouling paint in C1 probably may have reached the other cages. Moreover, the intimate relationship between the animals and the aquatic medium in which they are reared, could have facilitated this contact. On the other hand, sample contamination is very difficult to avoid in copper analyses since copper as a natural element, is present almost everywhere including dust. In all cases, amounts of copper detected in tissues in this work were well below base line (Ghedira et al., 2010) values reported previously for this species.

Conclusions

Apart from the mechanical implications of fouling accumulation seen in the previous chapter, the results of this section also seem to confirm biological interactions between fouling formation and fish health. Broadly looking at the results of this chapter it can be concluded that today on-site cleaning does not represent any major advantage compared to the traditional strategy in terms of fish growth and health features.

The high cleaning frequency needed to keep fouling growth under control is not always achieved in the real life and this fact was also reflected in this experiment. As a consequence of that, fouling develops on net panels and when it is eventually eliminated causes severe gill damage. Furthermore, the cleaning operations as such, seem to affect feeding response, thus fish growth.

As a result of the observations in this experiment it seems that indeed a relationship between fouling production and parasites occurrence exists, as well as bacterial proliferation. Apart from the shelter that fouling can provide to these organism, the action of copper as a biocide could also explain why in those groups a higher degree of fouling parasites and bacteria was observed. The existence of copper in the nets, should not represent a concern in terms of fish and consumer's health since the amounts accumulated in tissues were very low or even undetectable (in muscle).

The intermediate alternative presented in this work (C2), arises as a promising option, since the results show a clear antifouling effect of the smaller amount of cuprous oxide contained in the polyurethane coating which would allow both, a lower cleaning frequency and a reduction in the use of copper. Overall, on-site cleaning technology as well as operational procedures must improve to be considered a future alternative.

Fish welfare, primary and secondary stress responses

Fish welfare

In aquaculture, as in other animal productions, fish welfare should not only be considered from the moral, ethical or legal point of view but also in terms of production efficiency and quality, marketing and social perception which eventually means profitability. It is easy to understand that preserving fish health and well-being within production systems should have obvious benefits since animals will perform better, thus increasing profit margins.

The term welfare was possibly first applied to fish by Shelbourne, (1975) when discussing acclimation to the captive environment of wild caught broodstock of plaice *Pleuronectes platessa*. The concept of fish welfare was first legitimized in 1986 when fish were included within the UK's animal experimentation legislation (Parliament of the United Kingdom, 1986). Since then, the concept of fish welfare has spread mirroring the welfare avenues for mammals and birds. This, has occurred despite continuing dispute within the scientific community about whether fish do have the psychological capacity to suffer (Huntingford, 2006; Rose, 2007, 2002). Today, in Europe, animal welfare is regulated by Council Directive 98/58 (Council of European Union, 1998). The Lisbon Treaty introduced in 2009 the recognition that animals are sentient beings in its Article 13, and so fish reactions to stimuli would therefore probably not only be a physiological response but also cause an associated psychological experience that requires cognitive capabilities (Chandroo et al., 2004).

Various definitions of welfare have been proposed from philosophical arguments to those concerned purely with empirically measurable biological factors. The subject is a highly complex one, with no clear consensus on how welfare should actually be defined or measured. Many authors have suggested simple definitions such as whether the animal appears in good health or well-being and whether they have what they need or not (Dawkins, 2012; Stanley, 1985). Others suggest that the welfare of the animal can be assessed either in regards of its own subjective feelings, emotions and mental state; its physical functioning, health and capacities; or by the ability the animal has to express its natural behavior and lead a natural life (Fraser et al., 1997).

Anyway, fish as animals, present interesting challenges for the measurement of welfare due to the differences in their behavior and physiology. Fish welfare is an area that is investigated scientifically nowadays, in spite of the difficulties in identifying and measuring it. Several approaches have been proposed to measure the welfare of animals, although three main methodologies have been extensively developed (Dawkins, 2012). Specifically, these definitions are feelings-based, function-based, or nature-based (Huntingford, 2006).

In common with all vertebrates, in case of welfare impairment, fish respond with a series of adaptive neuro-endocrine adjustments that are collectively termed *the stress response*. These adjustments in turn induce reversible metabolic and behavioral changes that make the fish better able to overcome or avoid the challenge. However, when the source of welfare impairment cannot be successfully overtook or avoided it leads to a chronic stress situation that will have negative consequences. Stress is considered a primary contributing factor that leads to impaired health in cultured fish (Iwama, et al., 1997).

Production systems including aquaculture are susceptible of causing acute (Pickering, 1981) and chronic(Montero et al., 1999) stress and as such may impair fish welfare. In **table 4** (Conte, 2004) summarizes sources of stress and their studied effects in aquaculture.

The growth period is the longest stage for most fish in aquaculture, hence possibly the most important one from a welfare perspective. In aquaculture, welfare is an issue that no longer can be avoided.

Transportation	Transportation induces physiological stress requiring prolonged recovery (Bandeen & Leatherland, 1997; Iversen et al., 1998; Rouger et al., 1998; Barton, 2000; Sandodden et al., 2001; Chandroo et al., 2005).
Handling and netting	Physical disturbance evokes a neuroendocrine stress response in many species of farmed fish (reviewed by Pickering, 1998) and reduces disease resistance (Strangeland et al., 1996). Handling stress increases vulnerability to whitespot in channel catfish (Davis et al., 2002).
Confinement and short-term crowding	Physical confinement in otherwise favourable conditions increase cortisol and glucose levels and alters immunological activity in various species (Garcia-Garbi, 1998). Carp (*Cyprinus carpio*) show a mild, physiological stress response to crowding that declined as the fish adapted, but crowded fish are more sensitive to an additional acute stressor (confinement in a net; Ruane et al., 2002). Crowding during grading increases cortisol levels for up to 48 h in Greenback flounder *Rhombosolea tapirinia*, Gunther (Barnett & Pankhurst, 1998).

Table 4. Actions susceptible to produce stress in farming conditions and works assessing their effects (Conte, 2004).

Inappropriate densities	High densities may impair welfare in some species (trout andsalmon, Ewing & Ewing 1995; sea bass, Dicentrarchus labrax L., Vazzana et al., 2002; red porgy, *Pagrus pagrus*, Rotllant & Tort, 1997; seabream Sparus auratus, Montero et al., 1999), but enhance it in others (Arctic charr, Jergensen et al., 1993). Halibut suffer less injury at high densities (Greaves, 2001) but show more abnormal swimming (Kristiansen & Juell, 2002; Kristiansen et al., 2004). The relationship may not be linear (in salmon negative effects begin to kick in at a critical density, Turnbull et al., 2005) and density interacts with other factors such as water quality (Ewing & Ewing, 1995; Scott et al., 2001; Ellis et al., 2002). Genes coding for heat shock proteins are over-expressed in sea bass held at high densities (Gornati et al., 2004). An enolase gene is sup-regulated in sea bream held at high densities (Ribas et al., 2004).
Enforced social contact	Aggression can cause injury in farmed fish, especially when competition for food is strong (Greaves & Tuene, 2001). Subordinate fish can be prevented from feeding (Cubitt, 2002), grow poorly and are more vulnerable to disease (reviewed by Wedermeyer,1997).
Water quality deterioration	Many adverse effects of poor water quality have been described, with different variables interacting, e.g. undisturbed salmonids use c. 300 mg of oxygen per kg of fish per hour and this can double if the fish are disturbed. For these species, access to aerated water is essential for health (Wedermeyer, 1997). Immunoglobulin levels fall in sea bass held at low oxygen levels (Scapigliati et al., 1999). Poor water quality mediates density effects on welfare in rainbow trout (Ellis et al., 2002).
Bright light and photoperiod manipulation	Atlantic salmon avoid bright light at the water surface, except when feeding (Ferno et al., 1995; Juell et al., 2003). Continuous light is associated with increased growth in several species (e.g. cod: Puvanendran & Brown, 2002).
Food deprivation	Dorsal fin erosion increases during periods of fasting in steelhead trout (Winfree et al., 1998). Plasma glucose increased in Atlantic salmon after 7 days without food, but other welfare indices were unaffected (Bell, 2002). Atlantic salmon deprived of food for longer periods (up to 86 days) lost weight and condition, but this stabilized after 30 days (Einen et al., 1998). Farmed Atlantic salmon swim slower and fight less during feeding bouts when fed on demand (Andrew et al., 2002).
Disease treatment	Therapeutic treatments themselves may be stressful to fish (e.g. Yildiz & Pulatsu, 1999; Griffin et al., 1999, 2002; Thorburn et al., 2001; Sørum & Damsgard, 2003).
Unavoidable contact with predators	Brief exposure to a predator causes increased cortisol levels and ventilation rate and suppressed feeding (e.g. Metcalfe et al., 1987). Mortality and injury due to attacks by birds and seals can be high among farmed fish (e.g. Carss, 1993).
Slaughter	All slaughter methods are stressful, but some are less so than others (Robb et al., 2000; Southgate & Wall, 2001). Sea bass killed by chilling in ice water had lower plasma glucose and lactate levels and showed less marked behavioural responses than those killed by other methods, in particular by asphysia in air and electro-stunning (Skjervold et al., 2001; Poli et al., 2002), see Robb & Kestin, 2002; Lines et al., 2003;(Lambooij et al., 2007).

Table 4 (cont.). Actions susceptible to produce stress in farming conditions and works assessing their effects (Conte, 2004).

Specially, cage systems require a high degree of operations to keep them running and those may cause a large number of disturbances to the stock. The industry needs to carefully review procedures in order to maximize their livestock welfare, which in turn will reflect a good health status of the accounts.

Fouling and fouling management cause changes in rearing conditions and involve cage operations that might impact in different ways to the fish. The strategy using antifouling treated nets involves a few net changes along the growth cycle of a batch of fish. On the other hand, on-site cleaning requires periodical operations. The initial hypothesis was that, although more frequent, disturbances caused by on-site cleaning should be less intense and so their effects on fish welfare should be lower.

The welfare approach in this work was associated to the measurement of those biological functions and parameters resulting from the stress response and their effects, as they provide empirical measurements. Specifically, we analyzed the effects on metabolic, immune and antioxidant response and the subsequent expression pattern of those involved genes that together with the stress response will constitute four large sections in this chapter. All this in agreement with (Dawkins 1980), assuming that only by this approach a complete picture of the situation cannot be provided. It is also important to remind, when analyzing the results, that the context in which biological functions and changes have been measured in this experiment, should be cautiously interpreted (Jensen, 2002) due to the fact that the trial was carried out under natural conditions where many uncontrollable factors may have exerted an influence on fish.

Materials and methods

In the General Materials and methods chapter were already described the procedures through which blood plasma, skin mucus and tissues were collected and stored. In every section within this chapter we will explain which analyses were performed in those tissues to determine symptoms of welfare impairment and the subsequent physiological responses. However, a common trait for almost all the sections will be the modulation of the gene expression associated to the different responses. The methodology followed for gene expression analysis will be explained only in this section and the obtained results and discussion will be further exposed in the corresponding chapter.

Total RNA extraction and cDNA Reverse Transcription

Approximately 50 -100 mg of each tissue were used for total RNA extraction using 1mL Trireagent (Sigma-Aldrich, USA) following manufacturer's instructions. RNA was precipitated using isopropanol. Total RNA quantification and quality were determined using a NanoDrop-1000 spectrophotometer (Thermos Fisher Scientific, USA) and to avoid protein or phenol contamination only those samples with an A260/A280 ratio around ≥ 1.8 (McKenna et al., 2000) were used for the analysis. The integrity was measured by an Agilent 2100 Bioanalyzer (Agilent Technologies, USA).

Reverse transcription (RT) to obtain cDNA was performed with 1µg of total RNA in a final volume of 20 µL using a High Capacity cDNA Reverse Transcription Kit (appliedbiosystems, Thermo Fisher Scientific, USA) according to manufacturer's protocol. Amplification was performed in a S1000 Thermal Cycler (BIO RAD, USA). After checking primers efficiency, which was around 100%, cDNA was diluted 1:10 to determine expression of target genes and 1:1000 to determine expression reference genes.

Transcriptomal analysis

Quantitative reverse transcription PCR (RT-qPCR) was performed in liver, spleen and gills. The set of genes chosen for the analysis included indicators of: innate immune function ***(il1β, il6 and c3)***, adaptive immune function (*igm)*, antioxidant status (*cat, gpx1, sod2)*, acute phase stress proteins *(hsp70)* and toxicity *(mt, cyp1a1)* plus two housekeeping genes (*18s* and *act)* **(Table 5)**.

PCR analysis was performed in triplicates in a Bio-Rad CFX384 Real-Time PCR Detection System (Bio-Rad Laboratories, USA). Reactions were done using iTaqTM Universal SYBR® Green Supermix (Bio-Rad Laboratories, USA) according to the manufacturer's instructions. After the analysis those samples that showed unspecified bands were rejected.

Gene name	Acronym	Endpoint	Genbank ID	Forward	Reverse
18S ribosomal RNA gene	*18s*	Housekeeping	AY993930	GCATTTATCAGACCCAAAACC	AGTTGATAGGGCAGACATTCG
β-Actin	*act*	Housekeeping	X89920	TCCTGCGGAATCCATGAGA	GACGTCGCACTTCATGATGCT
Superoxide dismutase [Mn]	*sod2*	Antioxidant response	JQ308833	CCTGACCTGACCTACGACTATGG	AGTGCCTCCTGATAT TTCTCCTCTG
Catalase	*cat*	Antioxidant response	JQ308823	TGGTCGAGAACTTGAAGGCTGTC	AGGACGCAGAAATGGCAGAGG
Glutathione peroxidase 1	*gpx1*	Antioxidant response	DQ524992	GAAGGTGGATGTGAATGGAAAAGATG	CTGACGGGACTCCAAATGATGG
Interleukin 1β	*il1β*	Immune response	AJ277166	GGGCTGAACAACAGCACTCTC	TTAACACTCTCCACCCTCCA
Interleukin 6	*il6*	Immune response	AM749958.1	AGGCAGGAGTTTGAAGCTGA	ATGCTGAAGTTGGTGGAAGG
Complement component c3	*c3*	Immune response	HM543456.1	GTTCCACAACAACCCACAGC	ACATACGCCATCCCATCCAC
Immunoglobulin M	*igm*	Immune response	JQ811851.3	GATCGTGACATCGTCTGAGG	TGTTGGGTTGTGGTTGTAGG
Cytochrome P4501A1	*cyp1a1*	Oxidative enzyme	AF011223.1	GCATCAACGACCGCTTCAACGC	CCTACAACCTTCTCATCCGACATCTGG
Heat-shock protein 70	*hsp70*	Stress protein	EU805481	AATGTTCTGCGCATCATCAA	GCCTCCACCAAGATCAAAGA
Metallothionein	*mt*	Detoxication	U93206	CTCTAAGACTGGAACCTG	GGGCAGCATGAGCAGCAG

Table 5. Sequences and of primers used for quantitative real-time PCR analysis.

The primary stress response

Hans Seyle proposed in 1953 (Seyle, 1953) the term stress when studying animal's responses to situations that could endanger their integrity. Since then many authors have described and reviewed the stress response in fish (Barton, 1997) and a short definition of stress response in one single sentence we could be "The set of adjustments to overcome challenges causing welfare impairment".

The stress response is a general, widespread and integrated reaction that shows common traits along the phylogenetic tree both in physiological mechanisms and especially in the involved molecules. Its outcome depends on the duration and intensity of the stressor, it occurs at cellular and organism level and involves all three regulatory systems: neural, endocrine and immune (Tort, 2011). The stress response is broadly categorized into **primary** (neuroendocrine level), **secondary** (physiological level) and **tertiary** responses (organism and performance level) (Iwama, et al., 1997; Mazeaud et al., 1977, Carl B. Schreck, Lluís Tort, Anthony P. Farrell, 2016; Wendelaar-Bonga, 1997).

The primary response is initiated by the activation of the Hypotalamic-Pituitary-interrenal (H-P-I) and the sympatico-chromaffin (SC) axis and controlled by these two hormonal systems, leading to the production of corticosteroids, mainly cortisol and catecholamines such as adrenaline and nor adrenaline (Mommsen et al., 1999; Pickering, 1981). The release of these hormones mainly regulates the secondary stress response which involves a metabolic arrangement to make energy and oxygen available to vital areas of the body, with the consequence of compromising the osmotic balance and also the full efficiency of the immune system. It has also been shown that the initial response to stressors is characterized by the close interaction between the three main regulatory systems (neural- endocrine-immune) in order to build an integrated response (Schreck and Tort, 2016; Tort, 2011).

If the exposure to a stressor is acute, the subsequent primary and secondary responses will increase the homeostatic capacity of the animal, allowing to overcome the situation in a fight or flight reaction to a certain extent (Cannon, 1915). If the new homeostatic arrangement is not overcome the animal will die. Sometimes though, the stressor intensity is lower but the exposure is prolonged or repeated driving the animal to a chronic stress situation. This has consequences that negatively affect other necessary life functions of the organism as growth, development, disease resistance, behavior and reproduction (Schreck, and Tort, 2016) due to the continuous energy bu-

dget readjustment to withstand the stress situation. This whole organic involvement conforms the tertiary stress response. Classically, the approach to measure the magnitude of the stress response has been at tissue level by determining changes in HPI axis. Catecholamine's release is nerve triggered and therefore circulatory levels increase very fast after stress exposure making very difficult to determine basal levels. On the other hand, cortisol release from the interrenal tissue in the head-kidney occurs as a consequence of a neuroendocrine cascade in a process that takes some minutes, furthermore cortisol action is endocrine and its effects are exerted over the vast majority of tissues within the organism which have corticosteroid receptors. These facts have suggested cortisol levels as a classical measuring signal of stress.

Cortisol was already identified as the primary corticosteroid hormone in fish fifty years ago (Donaldson, 1981). It is released from the inter-renal tissue, located in the head kidney, as a consequence of the secretion of pituitary hormones, mainly adrenocorticotrophic hormone (ACTH) (Balm et al., 1994) and diffuses freely into the plasma (Mommsen et al., 1999). Some authors maintain that a more reliable approach for measuring stress is to see what happens at cellular level by measuring molecular indicators (Cordeiro et al., 2012). Most cortisol actions are predominantly genomic. These actions are mediated by specific nuclear receptors (GR and/or MR in fish) and it involves gene transcription and protein synthesis (Mommsen et al., 1999; Vijayan et al., 2005). In marine organisms, a common indicator to measure the cellular response to stress is the expression of the heat shock proteins (HSP) family. HSPs are excellent indicators of cellular stress response as they are very easily identifiable and ubiquitously expressed in almost every cell type (Deane et al., 2004; Hil and Vi, 1998). HSPs up regulate accordingly to the proportion of stress (J. Jungmann, H.-A. Reins, C. Schobert, 1993). During stress episodes, HSPs provide cytoproteccion by stabilizing the amino acid chain and preventing the protein unfolding induced by stressors that might challenge the protein homeostasis of cells (Basu et al., 2002; Hofmann, 2005; Iwama et al., 1999; Tomanek, 2010). HSP70 is one of the most highly conserved HSPs and there are a few studies on its expression in farmed sea bream (Alves et al., 2010; Deane et al., 2004) although very scarce data under natural conditions as indicators of stress. At molecular level, as the HSP70 is regulated in response to the HPI axis activation, its gene expression has been assessed in gills, liver and spleen.

In this study, cortisol levels have been used as an indicator of animals' welfare and also to see the effect of sampling in cage-net operations right before,

right after and 24 hours after the maneuver. Over the last years, it has been shown that the peripheral tissues, mainly the mucosal surfaces, may also be relevant in terms of the overall stress and immune response (Beck and Peatman, 2015). As data on skin mucus secretions are scarce and stress induces mucus secretion (Shephard, 1994), cortisol levels been determined not only in plasma but also in skin mucus exploring a non-invasive way to determine the welfare status of a batch of fishes.

Materials and methods

Blood serum, skin mucus and tissues were obtained as described in chapter 2 from nine animals at the beginning of the experiment and eighty one animals, (3 animals per cage, 9 animals per group per each sampling point) at the end of the experiment. Initial values of plasma and mucus cortisol were determined and the expression of *hsp70* gene was determined in pooled samples each one of the tissues.

Cortisol analysis

Aliquots of 20 μl of plasma and 100 μl of skin mucus were used to perform cortisol determinations measured by radioimmunoassay. The hormone was quantified using a liquid scintillation counter (Scintillation Counter Wallac 1409, PerkinElmer). The anti- cortisol antibody (ref. 07-121016, MP Biomedicals, Solon, OH, USA) was used for the assay at a final dilution of 1:4500. Antibody cross-reactivity with cortisol is 100% (manufacturers specification), and the lower detection limit of the assay was 0.16 ng mL^{-1}. Possible cross-reactivity with other steroid hormones varied from 1.6% for corticosterone and was inferior to 0.7% for 17α-hydroxyprogesterone, cortisone, desoxycorticosterone, 17α-hydroxypregnenolone, progesterone, cholesterol, estradiol and testosterone. The methodology for *hsp70* gene expression has been already detailed in the introductory section of this chapter.

Statistical analysis

After checking normality using the Anderson-Darling Test, General Linear Model (GLM) was used to see differences between baseline values and the experimental groups at different moments. One-way ANOVA and Tukey's test were used to see differences within groups in each specific time point. In all cases significant differences were considered when ($P<0.05$).

For the expression on *hsp70* gene, differences among groups were analyzed separately with one-way ANOVA and Tukey's test comparing initial values of expression with those seven months later just before cage operation, and

at the end of the experiment just before, just after and 24 hours after cage operation. At the end of the experiment General Linear Model (GLM) and Tukey's test were used to assess differences between groups and before and after operations. In all cases significant differences were considered when ($P<0.05$).

Results

Cortisol in plasma and mucus

Looking at the cortisol values at the end of the experiment (before cage operation) no significant differences were seen between groups and compared to base line values **(Figure 32)**. However, levels in C1 showed a clear increase in both plasma and mucus cortisol. C2 also showed an increase in plasma, not as evident in this case, but levels in mucus were lower. C3 showed an odd (very low) value in plasma and a similar level than C2 in skin mucus.

Cortisol dynamics across each appropriate cage operation at the end of the experiment showed a different pattern between C1 and C2, C3. In plasma, cortisol levels in C1 clearly increased right after net replacing and remained high 24 hours after the operation. On the other hand, in the on-site cleaning strategy conditions, the reaction right after the maneuver was also seen, but

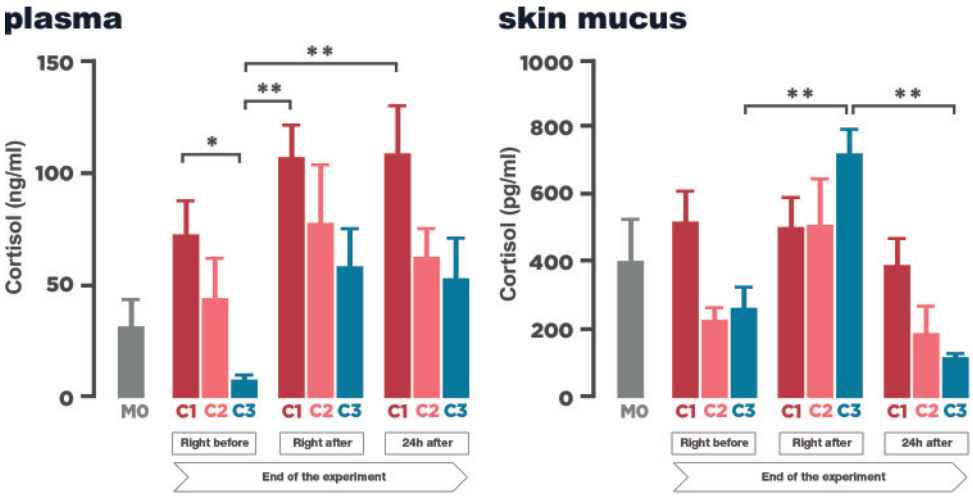


Figure 32. Cortisol release over the experiment in plasma and skin mucus.

Values are means of three replicates (n=9) except base line values (M0) obtained from a pool of 9 animals. Asterisks denote significant differences ($P<0.05$).

24 hours later cortisol levels returned to similar values right before starting the operation. In skin mucus, C2 and C3 behaved very similarly to what they did in plasma. In this case no significant reaction in cortisol values was observed in C1.

Apart from those caused by the odd cortisol value of C3 right before starting the las net cleaning operation, the only significant differences were recorded in skin mucus within the C3 group in skin mucus provoked by the peak right after net cleaning.

hsp70 expression

The fold change of the expression of *hsp70* was used to assess the possible effects of both the chronic factors and the temperature increase between the beginning and the end of the experiment (right before cage operation). To assess gene expression throughout the maneuver at the last stage of the experiment, the results were calculated as normalized (with themselves and the housekeeping genes) relative expression.

After seven months of rearing conditions, the expression of the *hsp70* gene displayed minor changes in organs (liver and spleen). No significant differences between groups were detected, but while in C1 a mild up regulation was registered in liber, in C2 and C3 the gene was slightly down regulated in both tissues **(Figure 33)**.

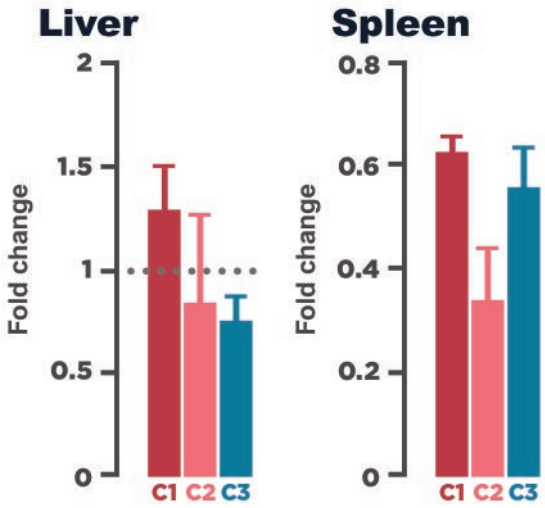


Figure 33. *hsp70* gene expression fold change in liver and spleen.

Changes in expression throughout experiment (before last cage operation).Values are means of three replicates (n=9), (P<0.05).

When looking at what happened during cage operation at the end of the experiment, in liver, the general trend was a slight down regulation 24 hours right after the operation in C2 and C3 and no changes were observed in C1. Only in C2 such down regulation of the gene was significant. In spleen, *hsp70* significantly down regulated in C1 and C3 across the cage operation. On the contrary, the tendency in C2 was a significant up regulation as net cleaning was performed **(Figure 34)**.

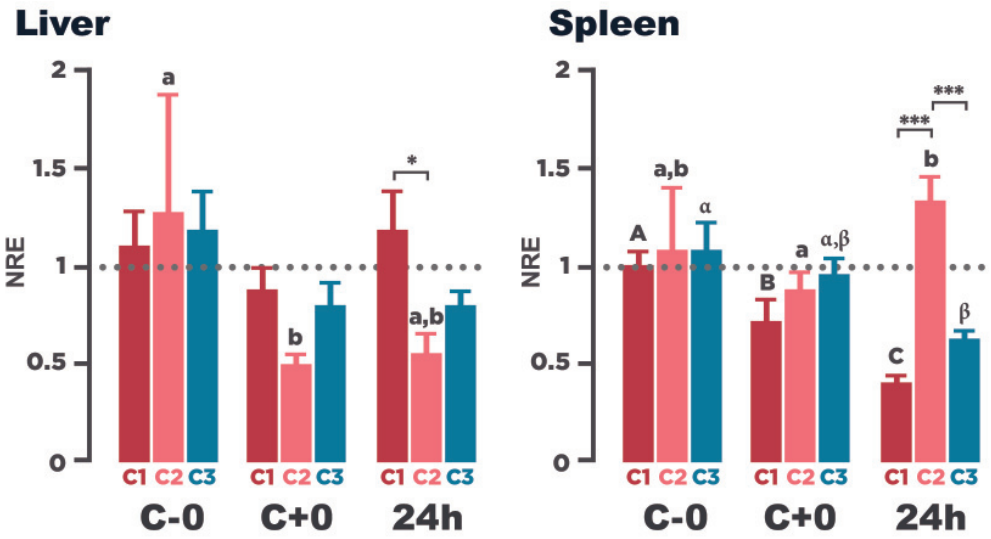


Figure 34. Relative expression of *hsp70* gene in liver and spleen.
Letters (upper-case, lower case and Greek characters) denote differences within groups and asterisks between groups. Values are means of three replicates *(n=9),* (P<0.05).

Discussion

Commonly, fish farming conditions can lead fish to chronic or acute stress situations. In this experiment animals were exposed to simulated real rearing conditions involving cage operations which could have provoked those stress scenarios. For seven months, two of the groups were periodically disturbed as a consequence of on-site cleaning operations (C2, C3) while the third group (C1) remained unaffected by cage operations. At the end of the experiment, C1 cages underwent a net change at the time that C2, C3 nets were for the last time *in situ* cleaned.

Cortisol is released as a consequence of the H-P-I axis activation as a result of a stressful situation that can be acute or chronic, and farming conditions constitute often an appropriate environment to induce those situations in fish. In this study, the experimental rearing period, from December to June, comprised the coldest half of the year in the area. During that time, fish were exposed to an array of unpredictable and uncontrollable events that occurred naturally as low temperatures, substantial daily and seasonal temperature variations, bad weather, predator's presence and an episode of lymphocystis infection during the coldest months.

Although fish were acclimatized in the cages for 15 days prior to the beginning of the experiment, in this work, base line values of cortisol in plasma were higher than those majorly reported in other works (Barton et al., 2005; Rotllant et al., 2001) probably due to the peculiar experimental conditions already cited. However, Guardiola et al., (2016) reported much higher base line values for the same species and a similar size.

According to the results, the seven months rearing period did not seem to generate any peculiar stress situation since no significant differences were detected between final and base line levels of cortisol. It could be understood that fish were under a mild chronic stress scenario to which became acclimated. Basal cortisol values, at least in plasma, were substantially higher in C1 than in the other two groups, though, in a manner similar to that reported by (Rotllant and Tort, 1997) with this species under stress conditions. Under these circumstances both the mild cortisol release response and the occurrence of feedback mechanisms in the HPI axis could explain our results.

Throughout the last maneuver, cortisol levels in C2 and C3 showed the classical peak- recovery dynamics pattern within the posterior 24 hours after the exposure to the stress source both in plasma and mucus, but only a significant increase was recorded in C3, in skin mucus, right after net cleaning. On the other hand, in C1, plasmatic cortisol levels did not recover and remained high 24 hours after net changing while in skin mucus, C1 did not show major changes. The reason why cortisol levels did not recover in C1 plasma could be explained due to the intensity and duration of the operation and even because of the synergistic effect (Flos et al., 1988) of both confinement plus handling plus time of the stressor application. After stressor exposure, cortisol levels were similar to those reported by Barton et al., (2005) in sea bream under a similar nature of stressor but lower in intensity, shorter in time and within a much more controlled experimental environment.

Although cortisol in mucus behaved similarly during the maneuver there was not a clear correlation between levels in plasma and skin. As mentioned above, the peculiar conditions in which the trial was carried out might have had some effect on the results. Cortisol levels in mucus measured in this experiment were well below those reported by Guardiola et al., (2016) under different stress sources. Responses to stress in mucus seemed to happen later compared to changes in plasma. The periodically cleaning operation performed in C2 and C3 could have attenuated the response to the maneuver and promoted a more rapid recovery in those conditions compared to C1 (Laflamme et al., 2000).

Looking at the expression of the *hsp70 gene*, the results suggest that the rearing period as such did not represent any major source of chronic welfare impairment to the animals even considering the difference of temperature between the beginning and the end of the experiment (13ºC to 23ºC) which would confirm the ability of this species to adapt to those temperatures as suggested (Feidantsis et al., 2009). No significant changes were found in the expression of the gene, and in spleen it was even down regulated. Throughout the operation a down regulation pattern was detected. It occurred mainly in liver in C2 and in spleen in C1 and C3. Exposure to copper, mainly in C1 did not promoted any significant change in terms of stress at molecular level.

Conclusions

Animals successfully adapted to the experimental conditions (simulating those in commercial cages) which did not seem to suppose any major stress source to them. The operations to which fish groups were exposed provoked only a mild primary stress response which in the case of C1 was still evident after 24 hours after exposure to the stressor source. The pattern of expression of the *hsp70* gene also indicate a non-special stressful situation in any of the experimental groups, even when those experienced a cage manoeuvre or were exposed to copper. The low intensity in the response could have been the result of the chronical stress conditions suggested above (Laflamme et al., 2000).

A high individual variability and the special characteristics of this work, may specially have potentiated this variation making difficult to detect a clear tendency among the results. In fish, high individual variation is commonly found, a trait that is easy to see when reviewing literature (Kokou et al., 2016), and although sample size in this work was similar to other studies it seems easy to conclude that in real conditions the overall variability

would probably be higher because of the higher number of uncontrolled environmental variables.

Despite the above consideration, looking at the results it is difficult to point out clear differences between both strategies in terms of stress response. Values obtained from the analysis do not follow a very clear pattern and no correlations are found between them.

As a conclusion it could be stated that there was no indication of a major stress source along the growth cycle or throughout the operations performed in the cages. Although, a higher stress intensity after net replacing over net cleaning was suggested by a higher release of cortisol in plasma. Moreover some behavioural changes such as changes in feeding activity were detected after those operations which sometimes were evident even the day after the manoeuvre, in particular the feeding behaviour in all groups. The fact that C2 and C3 were more often perturbed supposed a major degree of affectation.

The secondary stress response: The metabolic response

Blood and skin mucus biochemical parameters as stress and health status indicators

As a consequence of the primary response to an acute stressor the allostatic load imposed, undergoes a series of metabolic adjustments to face the energy demanding situation in which the organism encounters. This arrangement basically consists in modifying the metabolic pathways to provide the necessary energy and oxygen to those vital areas of the organism, mainly brain, gills and muscle, to trigger a fight or flight reaction. Under a chronic exposure to stressful conditions this metabolic adjustment remains at a lower degree of intensity allowing the animal to survive while coping with the cost of the stress situation. The long term adjustments to cope with the chronic stress may have detrimental effects on the biological functions of the organism because some functions are reduced or postponed and energy is diverted because of this allostatic load, therefore compromising the performance adequately.

The strategy through which the goal of redirecting energy is pursued, consists in reducing the consumption of energy in those non-vital tissues and promoting a mobilization of energy substrates (carbohydrates, proteins and lipids) to increase gluconeogenesis. The effect of cortisol in peripheral tissues causes a reduction of protein synthesis and glycolysis and promotes an increase of lipolysis and proteolysis as well as production of lactate. This releases amino acids, fatty acids and lactate into the bloodstream that are transported to the liver to provide a substrate for gluconeogenesis.

All this metabolic reorganization results in blood chemistry alteration that can be measured in plasma giving an indication of the existence and intensity of a secondary stress response. In fact, plasma biochemistry parameters are usually used as indicators of the physiological status of pets and farm animals (Knox et al., 1998) and could also be used in fish farming on a regular basis for the same purpose. Today reference baseline values for aquaculture species, among them sea bream are still scarce, but some works studying the effect of several stressors on blood parameters (Alves et al., 2010; Montero et al., 1999; Pagés et al., 1995; Sala-Rabanal et al., 2003) do exist.

Plasma glucose and lactate concentrations have been used as indicators of stressed states in fish (Barton et al., 2005; Pagés et al., 1995; Rotllant et al., 2001; Rotllant and Tort, 1997), and they are probably the most commonly measured secondary change markers that occur during the stress response in fish. Apart from energy substrates, the detection of some specific metabolic enzymes and metabolites can also indicate significant physiological changes as a result of a stress episode (Peres et al., 2013; Roncarati et al., 2006).

In this section the secondary response corresponding to the situation described in the previous section will be assessed. Plasmatic glucose levels will be analyzed as well as lactate, triglycerides and total proteins as precursors for gluconeogenesis. Alanine aminotransferase and creatinine will be also determined as the enzyme and metabolite that indicate liver and muscle activity after stress (Montero et al., 1995). Additionally the same parameters will also be determined in skin mucus to verify any correlation between both tissues, making the analysis easier.

Materials and methods

Blood serum, skin mucus and tissues were obtained as described in chapter 2 from nine animals at the beginning of the experiment and eighty one animals (3 animals per cage, 9 animals per group per each sampling point) at the end. Baseline values were determined in pooled samples of plasma and mucus from the 9 initial animals. Animals were starved 24 hours before sampling.

Parameters determination

Glucose was determined using the hexokinase procedure, lactate through LOD (lactate oxidase) procedure, total proteins were determined with bovine serum albumin as standard, triglycerides with the GPO (glycerol phosphate oxidase) methodology, and ALT and creatinine using the method suggested by the IFCC (International Federation of Clinical Chemistry). All the analyses were performed in an Olympus AU400 analyzer (Olympus, Germany) using OSR reagents (Beckman Coulter, Ireland).

Statistical analysis

After checking normality using the Anderson-Darling Test, General Linear Model (GLM) was used to see differences between baseline values and the experimental groups at different moments. One-way ANOVA and Tukey's test were used to see differences within groups in each specific moment. In all cases significant differences were considered when ($P<0.05$).

Results

Glucose and lactate

At the end of the trial, the groups reared under the on-site cleaning strategy showed higher values of glucose compared to base line values recorded seven months before. That increase was significant in plasma in C3 and in skin mucus in C2. In C1, values did not significantly change. Throughout the final operation, glucose in plasma did not experience significant changes, and the difference between C3 and the other two groups remained constant. In mucus the difference recorded right before the operation between C2 and C1, C3 was restored and 24 hours later glucose levels in all three conditions were similar to basal values **(Figure 35)**.

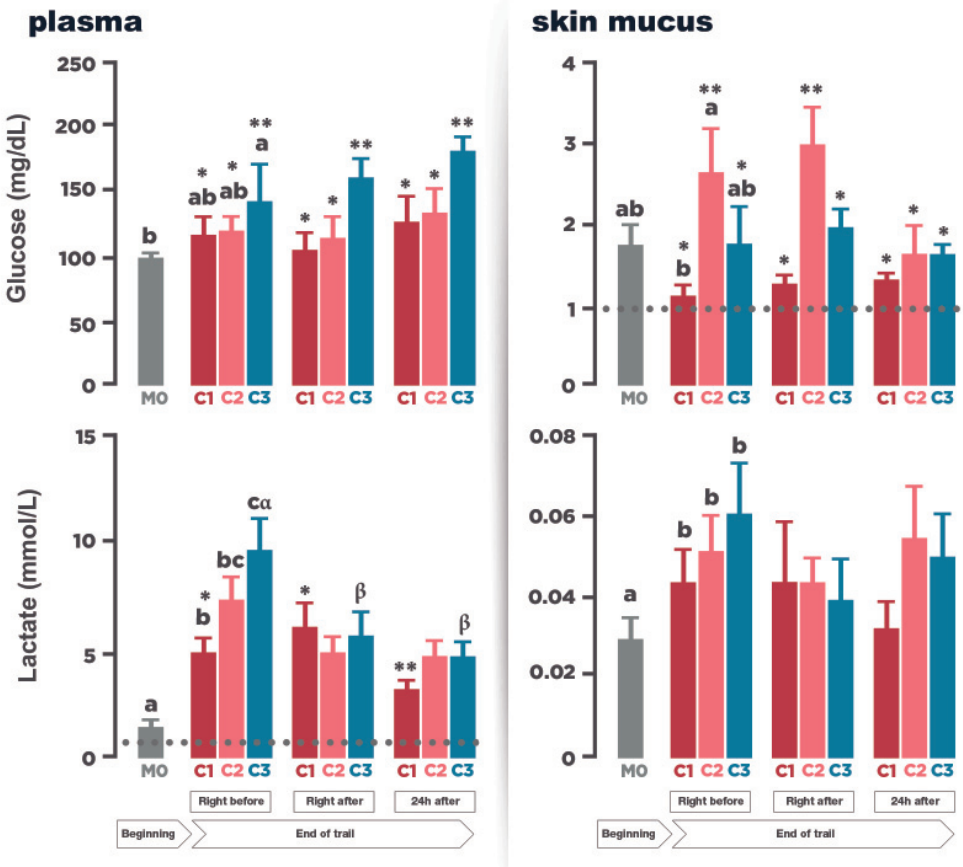


Figure 35. Plasma and skin mucus glucose and lactate levels during the experiment.

Base line values (M0) are the mean of a pooled sample. Values at the end of the trial are means of three replicates (n=9). Letters explain significant differences between baseline values and group values at the end of the experiment before final cage operation. Asterisks and Greek characters denote differences within and between groups at the end of the experiment (P<0.05).

The presence of lactate in plasma was very significantly higher at the end of the trial compared to the beginning, especially in those groups belonging to the strategy of on-site cleaning (C2 and C3). During net cleaning/ changing operation levels of lactate in plasma decrease in all conditions but only significantly in C1. Twenty four hours after cage operation, lactate levels in plasma were higher in C2, C3 compared to C1. Looking at skin mucus, and similarly to plasma, levels of lactate significantly increased in all groups compared to those seven months before. The different experimental conditions did not show differences among them at this stage. Throughout the operation at the end of the experiment lactate in mucus did not experience significant differences **(Figure 35)**. The relationship between lactate concentration in plasma and mucus resulted to be slightly significant. The variability of lactate in plasma could be explained in a 37.8% (R-Sq) by the concentration in mucus.

Total proteins and triglycerides

Small but significant differences due to the homogeneity in the values were found in plasmatic total protein content between C3 right before net cleaning operation at the end of the experiment compared to C1, C2 and also compared to base line values. At the end of the trial, during the cage operation total proteins in plasma continued to be very homogeneous among all sampled fish. The lower content registered in C3 right before net cleaning was restored 24 hours after the cleaning operation.

In skin mucus, the only significant differences were found at the end of the experiment between those cages under the on-site cleaning strategy (C2, C3) compared to those in which nets were replaced by new ones (C1). In the latter, the content of total proteins was lower. Across the maneuver those differences remained significant in the same way **(Figure 36)**.

Triglycerides concentration in plasma dramatically dropped during the experiment but did not experience significant differences throughout cage operations at the end of the experiment. In skin mucus, levels of triglycerides at the end of the experiment also significantly decreased although in a much lower degree compared to what happened n plasma. Throughout the maneuver levels in C3 significantly increased whilst in the other two groups remained equal.

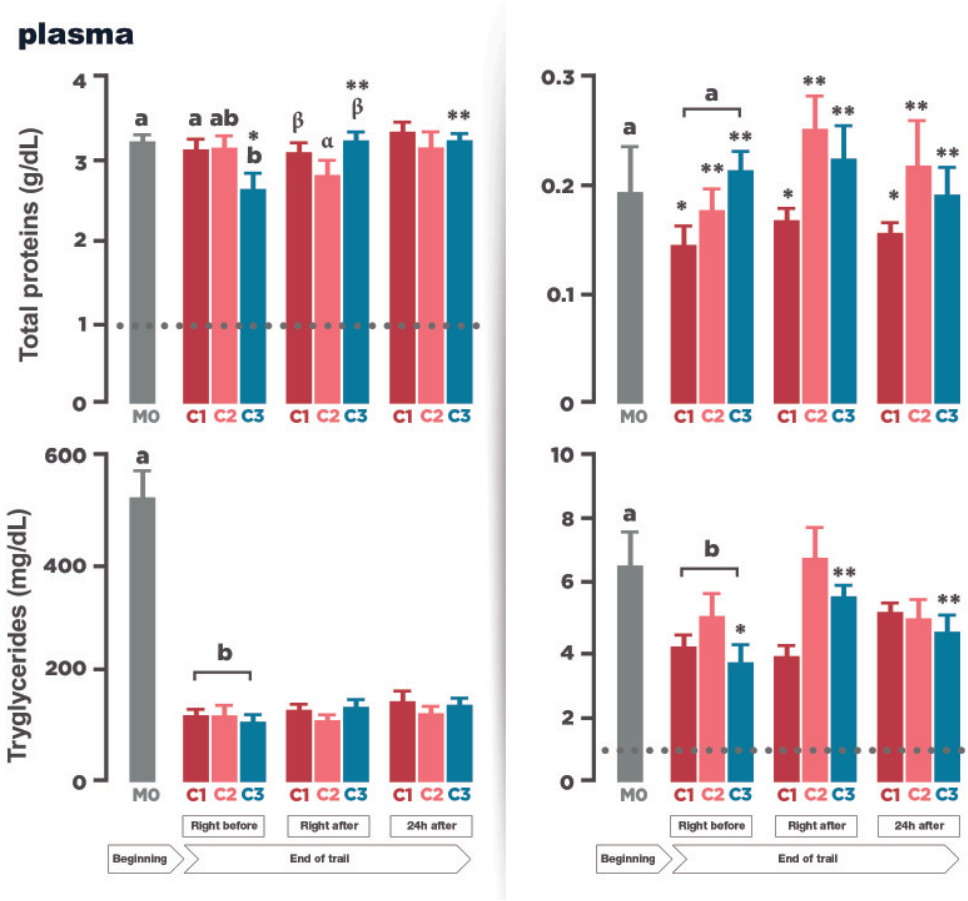


Figure 36. Plasma and mucus proteins and triglycerides.

Base line values (M0) are the mean of pooled samples. Values at the end of the trial are means of three replicates (n=9).Letters explain significant differences between baseline values and group values at the end of the experiment before final cage operation. Asterisks and Greek characters denote differences within and between groups at the end of the experiment (P<0.05).

Creatinine and ALT

Creatinine levels significantly changed only in skin mucus along the experiment. In plasma, right before the last net handling creatinine levels decreased, especially in C1 and C3 although not significantly. Throughout the operation no differences were found within or between groups. On the other hand, in skin mucus levels at the end of the trial values were higher than base line figures registered in the initial pool. Those values clearly increased as cage operations were performed in all three experimental conditions. During the time course of cage operation and the posterior to 24 hours, differences within groups were only detected in C3 but not in the other two groups **(Figure 37)**.

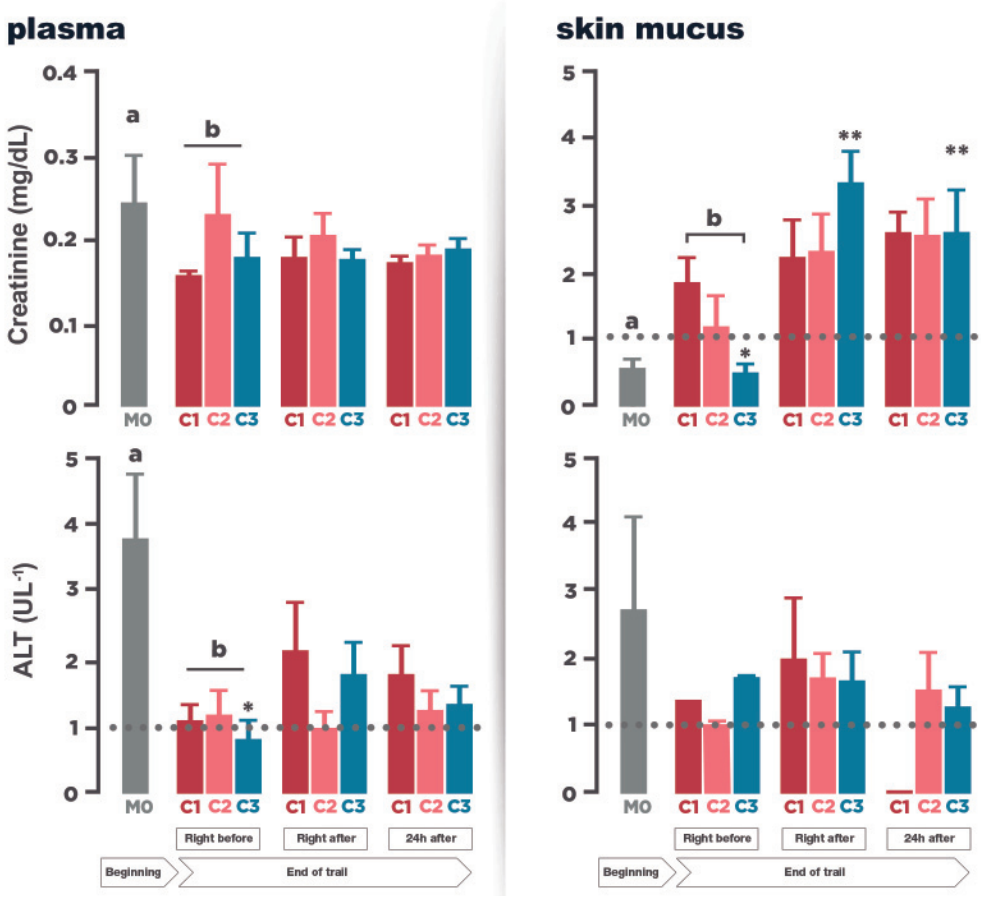


Figure 37. Creatinine and ALT in plasma and skin mucus during the experiment.

Base line values (M0) are the mean of a pooled sample. Values at the end of the trial are means of three replicates (n=9) Letters explain significant differences between baseline values and group values at the end of the experiment before final cage operation. Asterisks denote differences within groups at the end of the experiment ($P<0.05$).

To analyze ALT dynamics across the experiment only a proper statistical analyses was performed in plasma since the values in most skin mucus samples were below the technique detection threshold. During the experimental time the enzyme levels in plasma significantly decreased in all three experimental groups. No significant changes were observed throughout net operation at the end of the experiment although values suggest a light increase right after the maneuver which was still evident 24 hours after. In skin mucus, levels of ALT behaved similarly to those in plasma at the end of the experimental time course. Data obtained right after and 24 hours after cage operation seemed to indicate an increase with a posterior recovery 24 hours after the net cleaning. No data was available from C1 24 hours after net replacing.

Discussion

Primary stress response and the stressor itself cause a metabolic re-arrangement that allows the animal to overtake the stress situation. This pathway reorganization constitutes the main characteristics of a secondary stress response. Thus, the physiological adaptation of the organism can be assessed through analyzing indicators in blood but also in other body fluids like skin mucus (Guardiola et al., 2016). Glucose and lactate are two of the most sensible indicators after a stress situation and have been widely measured in different fish species to assess the effects of stress (Barton et al., 2005; Rotllant and Tort, 1997). In a stress scenario, glucose levels in blood increase due to glycolysis in non-vital tissues and gluconeogenesis increase in liver with the purpose of providing brain and muscles this main substrate to feed the critic acid cycle (CAC) or tricarboxylic acid cycle (TCA) and obtain energy (Lehninger, A. L., Nelson, D. L. 1., & Cox, 2008).

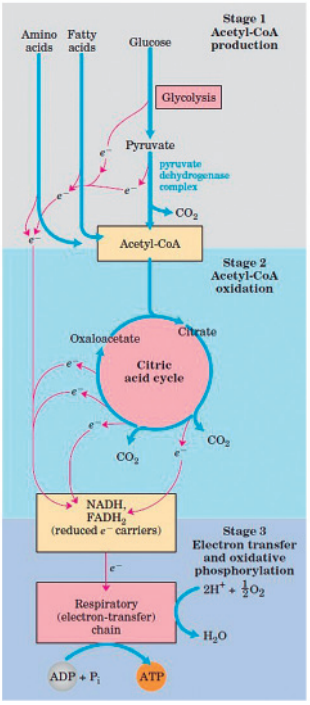


Figure 38. Citric acid cycle or tricarboxylic acid cycle (TCA)..

Principles of Biochemistry, 2008. (Lehninger, A. L., Nelson, D. L. 1., & Cox, 2008)

Lactate from anaerobic metabolism in muscles is released to blood vessels, transported to liver where it can be re-used again as a substrate for gluconeogenesis. Amino acids and lipids are also catabolized with the same purpose of feeding TCA to obtain energy **(Figure 38)**.

Data from the primary stress response in this study suggested that animals underwent a mild chronic stress situation during the time course of the experiment and reacted right after handling operations although without a significant increase of cortisol levels.

Glucose and lactate levels at the end of the experiment were significantly higher than those base line values recorded seven months before. Glucose baseline values were slightly higher compared to those previously reported levels in plasma by other authors (Ballester-Lozano et al., 2015; Barton et al., 2005; Benedito-Palos et al., 2016; Caruso et al., 2005; Maricchiolo et al., 2011; Roncarati et al., 2006) and similar to what Peres et al., (2013) des-

cribed after a short starving period similar to the context in which animals in this trial were sampled. Throughout the maneuver, these parameters behaved similarly to what has been reported (Barton et al., 2005; Rotllant et al., 2001) after a stress episode but in general terms, they did not experience significant changes although some erratic significant increase was recorded, for instance it was difficult to explain the higher levels of lactate in C2 and C3. No significant correlations were found between or within those parameters in plasma and/or mucus. Levels in plasma of glucose and lactate were in the range of those reported for this species Although fish were not fed from 24 hours prior to the sampling right before net operation glucose levels did not decrease most likely as a result of the fast reaction of liver gluconeogenesis (Navarro and Gutiérrez, 1995).

As proteins and lipids can also serve as a source of Acetyl-CoA in the TCA, these molecules are also susceptible to be mobilized in stress episodes. The variability among protein levels in all samples was very low and no major changes were detected during the entire time course of the experiment. Although there was a substantial difference in temperature between the beginning and the end of the trial, and considering this can modulate total protein content in plasma (Montero et al., 1999), levels recorded were very similar to those reported previously by other authors in sea bream (Benedito-Palos et al., 2016; Peres et al., 2013; Roncarati et al., 2006). In skin mucus, total protein content in those groups subjected to on-site cleaning was significantly higher than levels registered in the cages where nets were replaced. A further future step will be to perform a proteomic profile of the skin mucus with samples from this stage of the experiment to see the nature of the protein content. The results of these analysis will not be included in this work, though.

A very clear decrease of the content of triglycerides in plasma was seen at the end of the experiment. This reduction would match with the reduction of HSI at the end of the experiment. In fact, the drop was considerable but values at the end of the experiment were similar to values reported for this species in other works. Peres, H. et al. (2013) reported slightly higher values in fish starved for 7 days. Benedito-Palos, L. et al. (2016) and Roncatari, et al. (2006) reported similar or even lower values. On the other hand, baseline values at the beginning of the experiment were clearly higher than levels reported for *Sparus aurata* in the above works which would indicate the fat status of fish and would reflect the optimal feeding regime to which fish were subjected during the pre-growth in tanks and its preservation during the acclimation period in the experimental pond. As it was mentioned in

a previous chapter, the fact that despite undergone detrimental events such as considerable temperature oscillations and episodic diseases, the growth attained at the end of the experiment, at least in C1 (with similar triglycerides values than other groups), was not dramatically lower than expected would possibly indicate an overfeeding during cold months in this species in commercial conditions that would probably not be cost effective.

Triglycerides in skin mucus did not significantly change at the end of the experiment in general terms. A response right after the maneuver seemed to be detected by looking at values in C2 and C3. In C3 this increase was significant compared to the values right before the net cleaning operation, but it is difficult to extract clear information from these results. No relationship could be established at this stage between values in mucus and plasma.

On the contrary to what happened with the other analyzed parameters, levels of creatinine were higher in skin mucus that in plasma. Creatinine values both at the beginning and the end of the trial were in the upper part of the range for those values reported by Ballester- Lozano et al., (2015); Benedito-Palos et al., (2016). However, at the end of the experiment levels were significantly lower than baseline values. Creatinine in blood denotes kidney malfunction but also amino-acid catabolism in muscle after a stress episode. In this work, the operations to which cages in all groups were subjected did not suppose any significant change of this metabolite in blood. Instead, in skin mucus values at the end of the experiment were significantly higher than basal levels and the effect of cage-net operation caused an even more marked increase. Metabolite profiling in fish skin mucus has been recently generating interest as a non-invasive and fast approach to determine health status of the animals and as a bio-indicator (Ekman et al., 2015):The results obtained in this work do not evidence clear differences between groups.

Another possible biochemical indicator of metabolic adjustments is ALT or ALAT which is a pancreatic enzyme involved in gluconeogenesis and protein catabolism (Moves and West, 1995). Similarly to what creatinine showed, levels of ALT were similar in plasma and mucus differently to what happened with carbohydrates, lipids and proteins. Baseline levels in plasma were in line with what Ballester-Lozano et al., (2015; Peres et al., (2013) measured in their works and slightly higher than what (Benedito-Palos et al., 2016) described for this species. The effect of handling in all groups suggested a mild increase both in plasma and mucus with a subsequent recovery after 24 hours. A substantial part of the skin mucus samples did not show detectable quantities of ALT and were not used for the analysis. No

clear relationship could be established between creatinine and ALT results in this work.

Conclusions

The reliability and use of biochemical parameters in plasma as a diagnostic tool to determine health status of farmed animals has been corroborated in terrestrial productions and pets. However in this work, data did not provide substantial evidence of the differences between the experimental strategies tested. The idea of correlating what happens in skin to what happens in plasma did not work. Possibly, the measurement of welfare and health condition in fish is more difficult than in other animals due to its physiology or the specific conditions in this work. The variability of the different parameters analyzed here that have been also reported in the literature is high, and when measurements are made in the context in which this work was performed, variability becomes even higher due to the existence of many factors that can exert an effect on fish, thus increasing individual variation. This, complicates the job, but reflects the reality. It would be of great interest to explore the possibility of implementing this tool into commercial fish productions despite it might have some limitations at least in offshore conditions. However, it seems clear that sample size in those conditions should be higher than in trials conducted in the laboratory.

The immune response

The physiological, endocrine, metabolic, cellular or molecular arrangements orchestrated by an animal in the context of a stress episode may also affect its immune function. The immune response will be modulated depending on the nature, intensity and duration of the stressor. Often the immune activation is coupled with the stress response which acts as an adjuvant (Dhabhar, 2014). From a practical point of view, several types of stressors may induce an immunosuppressed status that will result in a lack of performance in terms of lower potency, delayed response and an increase of costs which may greatly influence the growth performance, reproductive ability and even the chances of survival.

Fish immunity

The immune system is the set of structures and networks that allow the organism to fight and eliminate external agents and thus keep integrity. In vertebrates, immunity is divided into two branches: the innate immune response (non-specific) and the adaptive or acquired immune response (antigen specific). The innate immune system is phylogenetically very old. It is shared by vertebrates, invertebrates and even plants (Ausubel, 2005) while the adaptive, more recently developed from an evolutionary point of view, is only present in vertebrates. Fish are the first group along the phylogeny presenting both systems (Andersson et al., 1995). Nonetheless, innate immunity plays a primordial role in fish together with the adaptive immunity which is found in a primary stage of development compared to other vertebrates (Plouffe et al., 2005).

The innate immune system

Innate immunity represents the first line of defense to external agents (Magnadottir, 2010) and reacts in a time lapse of minutes or hours to the presence of external agents characteristically manifesting an inflammatory response (Lieschke and Trede, 2009). The organism perception of foreign particles (antigens) relies on the recognition of well- conserved structures common in microorganisms including viruses, bacteria, yeasts and parasites known as pathogen associated molecular patterns (PAMPs) by pattern- recognition receptors (PRRs) which trigger the defense response. The innate immune system is composed of three basic compartments: physical barriers, cellular components (myeloid and lymphoid line cells) and humoral factors (Magnadottir, 2010). By physical barriers it is basically understood that they include those mucosal layers in skin, gills and gut and even nose. These

surfaces are major places for the entrance of foreign organisms as living surfaces in direct contact with the surrounding media. Mucus secreted in this surfaces trap and avoid the entrance of microorganisms and also contain antibacterial substances (Salinas et al., 2011). Non-specific cytotoxic cells and phagocytic cells (granulocytes –neutrophils- and monocytes/macrophages and B-lymphocytes in the case of fish) can detect pathogens by PAMP-PRR interaction. Epithelial and dendritic cells also participate in the innate defense in fish. Lastly, humoral factors can be secreted or membrane-bound molecules that include enzymes like lysozyme, complement proteins, natural antibodies and peptides among others. If a pathogen is able to evade the innate immune system repertoire of weapons and strategies, the acquired immunity represents a second chance for the organism to eliminate it. Innate and acquired immunity work coordinately.

The adaptive immune system

The adaptive immune system relies on the presence and activation of the lymphoid line cells including B, T and natural killer (NK) cells and the generation of antigen receptors. B cells express their antigen receptors on their cell surface as B cell receptors (BCR) and secrete them as immunoglobulins (Ig) or antibodies, whereas T cell receptors (TCR) are always cell-surface bound. T cell receptors only recognize antigens when exposed in the context of an isogenic major histocompatibility complex (MHC) either class I or II present in the cell surface (Beck and Peatman, 2015).

In fish, head kidney is the main hematopoietic tissue (Sunyer, 2013) although thymus and spleen are also important hematopoietic organs. Additionally, mucosal surfaces contain the named mucosal associated lymphoid tissue (MALT) (Salinas et al., 2011) which contain B and T cells. Recently, in the gill associated lymphoid tissue (GIALT) it has been described another immune structure designated as *interbranchial lymphoid tissue* (ILT) (Haugarvoll et al., 2008) which is mainly composed of T cells embedded in a meshwork of epithelial cells (Beck and Peatman, 2015).

In the event of an external intrusion, both innate and adaptive immune systems interact by stimulating each other, thus enhancing the chances to overcome the infection (Iwasakiand Medzhitov, 2010). **Figure 39** shows the general way of action of the fish immune response (Beck and Peatman, 2015).

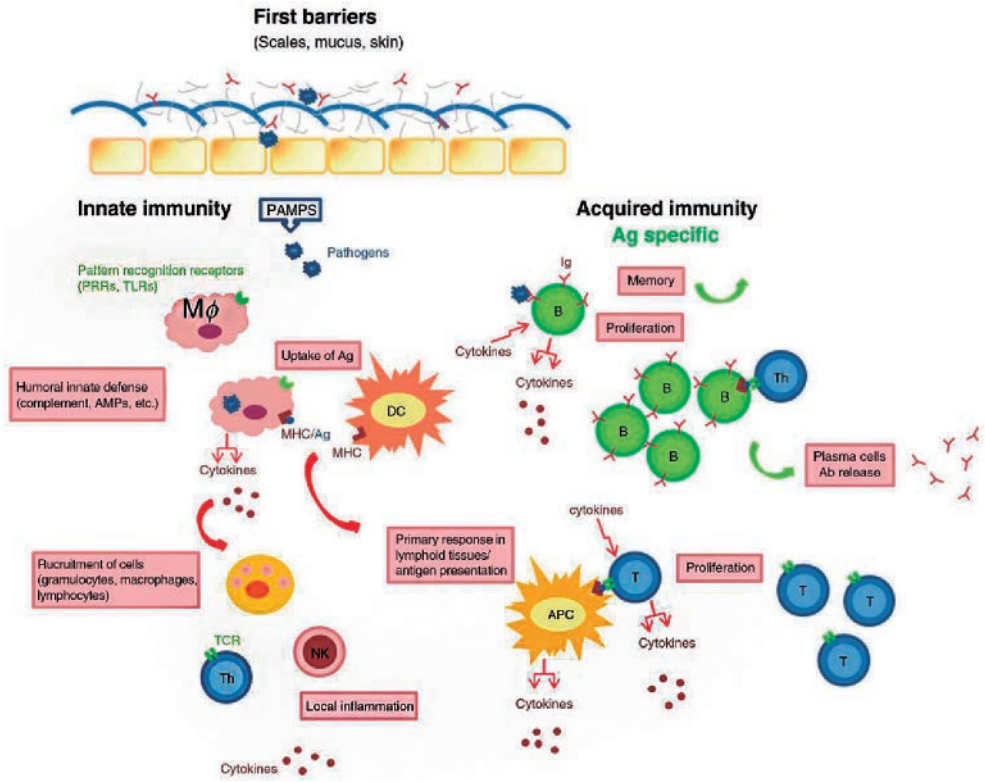


Figure 39. General mechanisms of the fish immune response.

The encounter with a pathogenic organism via mucosal tissues, such as gills, skin, or gut, is primarily blocked or limited by physical barriers such as the mucus, the scales, and the epithelium. The mucus contains different humoral components with antimicrobial activity such as complement factors, lysozyme or Ig's. In case the pathogen succeeds to penetrate the epithelium, it encounters the innate cellular machinery, triggered in a first step by those cell types bearing invariable receptors called pattern recognition receptors (PRRs), able to recognize common conserved molecules (PAMPs) characteristic of many microbial agents. The uptake of the antigen leads, on one hand, to the release of cytokine mediators and attractants for different cell types to unleash the inflammatory process and, on the other hand, to the antigenic presentation through the MHC in the lymphoid tissues for the activation of the primary responses of antigen-specific lymphocytes bearing variable receptors able to specifically recognize molecules distinctive of the pathogen, setting the bases for further secondary responses and memory. From (Beck and Peatman, 2015).

The duality of the immune response to stressors

The endocrine and the immune system interact in many and complex ways (Tort, 2011). Stress has generally been seen as a negative concept, and indeed it has been shown that in many cases disease appears after fish is subjected to a stress situation and the deleterious effects of stress on immune response have been attributed mainly to elevated levels of cortisol. In this sense, Mauri et al., (2011) showed in *Sparus aurata*, that high density increased levels of cortisol and reduced complement activity, Ortuño et al.,

(2001) reported that handling stress affected cellular and humoral non-specific immune response also in sea bream, and Montero et al., (1999a, 1999b, 1998) described immune alterations as a consequence of high stocking densities and dietary deficiencies.

From an evolutionary point of view, though the negative effects on the immune response produced by stress should not be adaptive, they may contribute to the survival of the animal, as the main goal of the stress response. Considering this, Dhabhar and McEwen, (1997) hypothesized that as stress response prepares the organism for a fight or flight reaction it should also prepare the immune system for the challenges imposed by the stressor and the physiological consequences derived from this. After several works, (Dhabhar, 2008, 2002, 2000; Dhabhar et al., 1995) these authors have proposed ***The Stress Spectrum Hypothesis*** **(Figure 40)** which suggests a dual stress response depending on the intensity and duration of the stressor. The hypothesis states that while short term-acute stress would activate immune response and enhance humoral immunity by increasing responses such as lysozyme and complement activity, and mobilizing-activating leukocytes in an adaptive immune cell redistribution, long term stress would negatively affect protective immunity by reducing lymphocyte proliferation and circulation, reducing antibody production, natural killer cytotoxicity, phagocytosis and pro- inflammatory cytokine production, thus increasing susceptibility to infection.

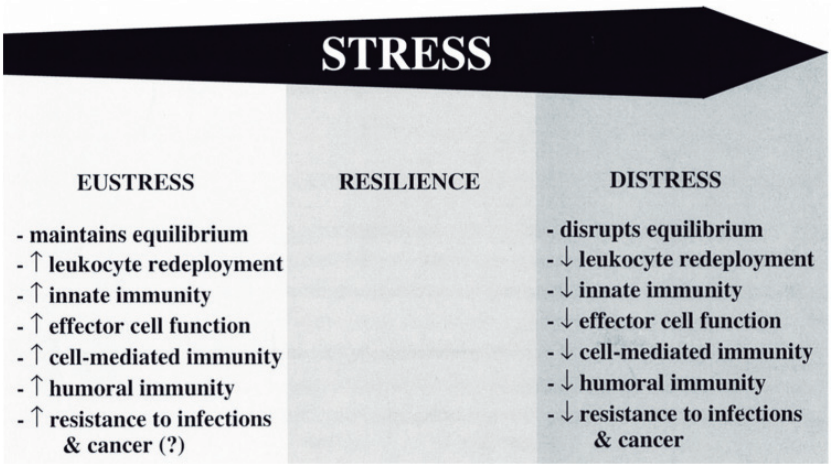


Figure 40. Model representing the stress spectrum.

Eustress represents acute stress which may result in an enhancement of immune response. Distress would represent chronic stress episodes resulting in a depletion of immunity. Right in the middle, the resilience area would represent the homeostatic capacity of individuals (Dhabhar and Mcewen, 1997).

The rationale of this model is based on the assumption that, under alarm situations, some responses require immediate changes and fast energy supply while others that need more time to be implemented, such as some adaptive immune responses, may be reduced or delayed to give energy priority to the first ones.

The fish farming environment

In fish farming, both acute and chronic stress episodes are commonly found. The rearing scenario as such, supposes an unnatural environment for fish which continuously may affect their welfare status by for instance, disabling animals to freely move according to their needs, impairing natural behavior, eliminating hierarchies and also by eventually being exposed to low water quality or contaminants as copper, among others. On the other handling operations as net replacing or on-site cleaning can suppose acute stressful events if not conducted conveniently.

In this work, some variables that have been shown as being indicators of the innate and adaptive immunity status of the animals were assessed at the end of the experiment in the three experimental conditions. Lysozyme and complement activities were analyzed in plasma and the expression of *c3, il1ß* and *il6* genes as innate immunity factors, as well as the expression of *igm* gene as an indicators of the response of adaptive immunity.

Lysozyme activity is one of the most studied immune responses in fish. Lysozyme is a bacteriolytic enzyme secreted by leukocytes and widely distributed throughout the body and is part of the innate defense mechanisms in most animals including fish (Uribe1 et al., 2011). It acts on the peptidoglycan layer of bacterial cell walls resulting in the lysis of the bacteria (Tort et al., 2003). Lysozyme also promotes the activation of the complement system and phagocytosis (Grinde, 1989). The complement system constitutes one of the central immune responses in fish. It has numerous functions but one of the most studied is the capacity to kill pathogens through the membrane attack complex by creating pores in their surface membranes (Holland and Lambris, 2002), being C3 the key complement protein (Sunyer et al., 1997). IL1ß is involved in the stimulation of T cells and in the pro-inflammatory response (Magnadottir, 2010; Williams, 2012) and IL6 is mainly involved in lymphocytes differentiation (Savan and Sakai, 2006; Williams, 2012). IgM is the predominant immunoglobulin in teleosts (Uribe1 et al., 2011) and circulating total IgM levels reflect the immune system status of fish regardless any specific antigen (Yada et al., 1999).

Materials and methods

Blood serum, skin mucus and tissues were obtained as described in chapter 2 from nine animals at the beginning of the experiment and eighty one animals, (3 animals per cage, 9 animals per group per each sampling point) at the end. Baseline values were determined in pooled samples of plasma and mucus from the 9 initial animals. Plasma IgM and lysozyme and complement activities were measured at the end of the experiment right before and 24 hours after the appropriate cage operation in each group. Expression of *c3, il1ß, il6* and *igm* genes was analyzed before, right after and 24 hours after operation at the end of the trial and compared with base line values from the beginning of the experiment.

Lysozyme analysis

Lysozyme activity assays were performed by a turbidimetric method that uses the lysis of *Micrococcus luteus* by the action of lysozyme activity (Ellis, 1990) using human lysozyme as a standard. A volume of 10 µL of plasma were suspended into a 200 µl suspension of *M. luteus* containing 0.2 g of bacteria per liter. Reads were done in a Victor3 spectrophotometer (Perkin Elmer, Massachusetts) at 540 nm, (0.1 s) every 10 minutes for a time lapse of 60 minutes.

Complement analyses

The measurement of the complement activity was performed using sheep red blood cells as antigen reactive or target (Sunyer et al., 1997).A volume of 15 ml of sheep blood were withdrawn and placed in a 50 ml falcon with 0.2 ml of heparin (Deltalab, Spain). Then, blood was washed 3 times with saline solution after centrifugation at 2000 rpm for 5 min, adding 4 volumes of saline solution per volume of blood. The final volume was adjusted to $2.8{\cdot}10^{8}$ cells·ml^{-1}. The fish serum was then placed in an ELISA V bottom plate and subjected to two serial dilutions with the Test Buffer (0.1% gelatin, 5 mM sodium barbiturate, 0.13 M NaCl, 10 mM Mg^{2}+, 10 mM EGTA, pH 7.3) following the series: 1/32, 1/48, 1/64, 1/96, 1/128, 1/192. After this, 10 µl of diluted sheep blood were added and the plates were incubated during 100 min, at room temperature agitating every 20 minutes. After 100 min, 150 µl of Stop Buffer (0.1% gelatin, 5 mM sodium barbiturate,

0.13 M NaCl, 20 mM EDTA, pH 7.3) was added to stop the lytic reaction, and 100 µl of supernatant were collected after centrifugation (1000 rpm, 2.5

min) and put into flat bottom plate to be read in a Victor3 spectrophotometer (Perkin Elmer, Massachusetts) at 414 nm. The results were expressed in ACH50 units, as the titer at which 50% hemolysis is produced.

IgM determination

Plasma total IgM levels were measured by the enzyme-linked immunosorbent assay (ELISA). Serum was 1:100 diluted in a PBS+EDTA 5 mM solution to avoid complement activation. A volume of 100 µl per well of the 1/100 diluted serum were placed in flat-bottomed 96- well MaxiSorp™ plates in duplicate for overnight incubation at 4 ºC. After having rinsed two times with 200 µL per well with PBS, each well was filled with 100 µL of milk powder 5% in PBS, afterwards wells were rinsed 2 times with PBS. Plates were then incubated for 1.5 h with 50 µl per well of mouse anti-gilthead seabream IgM monoclonal antibody (Aquatic Diagnostics Ltd.) 1/1000 diluted in PBS, washed three times with PBS- Tween (0.15%) and incubated again 1 hour with 50µL of the secondary antibody anti- mouse Ig GHRP 1/4000 diluted in PBS. After washing 5 times with PBS-Tween, plates were developed using 60 of 3'3-5'5 TMB and afterwards the reaction was stopped with 60 µL of H_2SO_4. Plates were read in a Victor3 spectrophotometer (Perkin Elmer, Massachusetts) at 450 nm, (0.1 s).

Gene expression analysis

The procedures followed for gene expression have been already detailed in the introductory section of this chapter.

Statistical analysis

The Anderson-Darling Test was used to check data normal distribution. One-way ANOVA and Tukey's test were used to see differences between groups and base line values. General Linear Model (GLM) was used to see differences between groups (conditions) at different moments throughout cage operation at the end of the experiment, and one-way ANOVA and Tukey's test were used to see specific differences within the groups during the operation or between groups in every specific moment.

For gene expression, within differences among groups were analyzed separately with one- way ANOVA and Tukey's test comparing initial values of expression with those seven month later right before cage operation. At the end of the experiment General Linear Model (GLM) and Tukey's test were used to assess differences between groups and before and after operations. In all cases significant differences were considered when ($P<0.05$).

Results

Lysozyme activity

Serum lysozyme activity remained unchangeable at the end of the experiment. No differences were found between groups and base line values. Across cage operation, in C2 and C3, lysozyme activity significantly decreased compared to values prior to operation **(Figure 41)**.

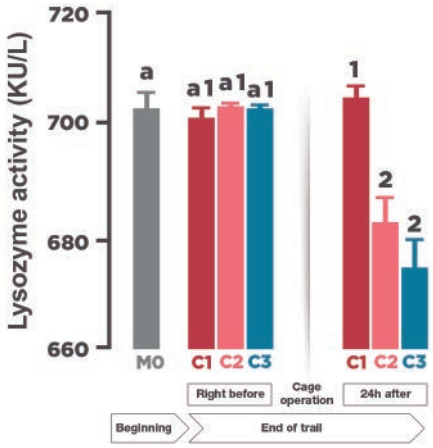


Figure 41. Lysozyme activity throughout the experiment.

Values are means of three replicates (n=9) except base line values (M0) obtained from a pool of 9 animals. Values sharing numbers or letters do not show significant differences (P<0.05).

Complement activity

Complement activity was higher at the end of the experimental period especially in C3 where the increase was statistically significant. Looking specifically right before cage operations there were no significant differences between groups. 24 hours after net cleaning, the activity in C3 significantly decreased compared to the other groups that increased moderately but not significantly **(Figure 42)**.

Total IgM

Looking at the adaptive response, total IgM content in plasma at the end of the experiment was significantly higher in all groups compared to initial values. Those values did not experienced significant changes during the time course of the cage operation **(Figure 43)**.

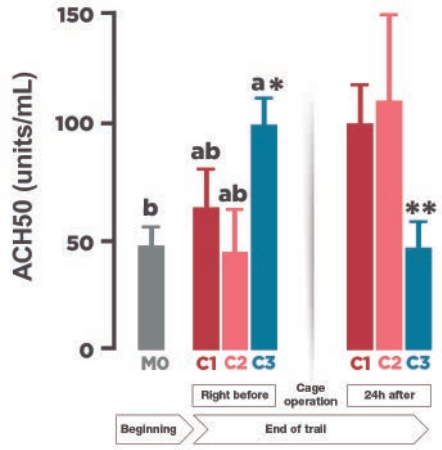


Figure 42. Complement activity throughout the experiment.

Values are means of three replicates (n=9) except base line values (M0) obtained from a pool of 9 animals. Values sharing letters do not show significant differences. Asterisks denote differences throughout cage operation ($P<0.05$).

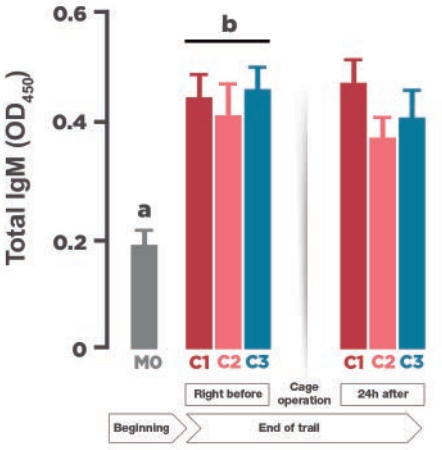


Figure 43. IgM in plasma throughout the experiment.

Values are means of three replicates (n=9) except base line values (M0) obtained from a pool of 9 animals. Values sharing letters do not show significant differences ($P<0.05$).

Gene expression

The expression of immune related genes were analyzed in spleen and gills. After head kidney, spleen is the major organ involved in the immune function, and gills are one of the most exposed organs to net handling and cage operation effects.

Chronic effects

The fold change in the expression of selected genes was used to assess the possible effects of chronic factors and the substantial temperature increase between the beginning and the end of the experiment (right before cage operation).

When looking at innate immunity parameters, the general trend in gills, and spleen along the experiment was a down regulation of *c3* gene and an up regulation of the genes involved in the inflammatory response, *il1ß* and *il6*. However, no significant differences were found between groups with the exception of *il6* that was significantly up regulated in gills in C2 compared to C1 and C3 **(Figure 44)**. Although not shown in the graphs, *c3* gene showed a down regulation in liver in all groups at the end of the experiment without significant differences between them.

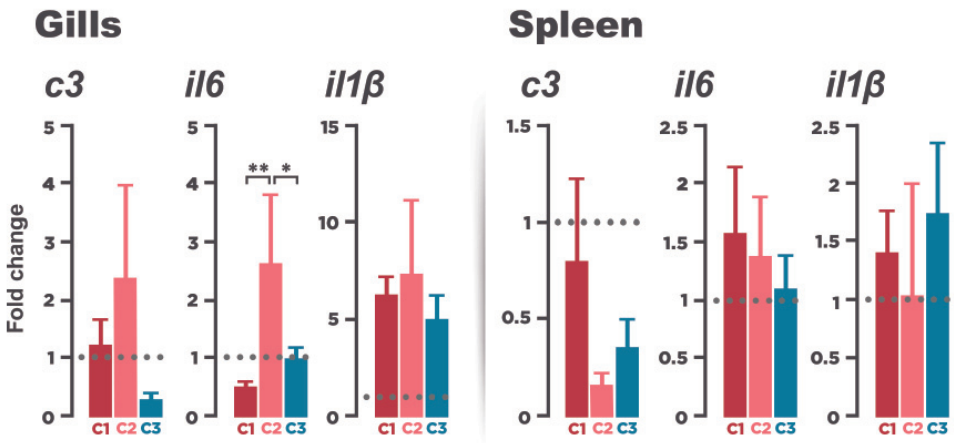


Figure 44. Fold change expression of genes encoding complement protein C3 and interleukins IL6 and IL1ß between the beginning and the end of the experiment (right before cage operation).

Values are means of three replicates (n=9). Asterisks denote significant differences between groups (P<0.05).

Regarding the adaptive parameters, when looking the expression of the gene encoding the IgM protein, a different behaviour can be observed between groups. Whereas in C1 *igm* was up regulated, especially in spleen, in C2 and C3 a clear down regulation, mainly in gill, was observed, **(Figure 45)**.

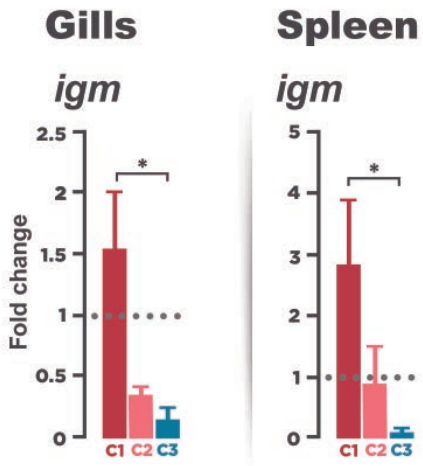


Figure 45. Fold change expression of *igm* gene in gill and liver between the beginning and the end of the experiment (right before cage operation).

Values are means of three replicates (n=9). Asterisks denote significant differences (P<0.05).

Acute effects

To assess gene expression throughout the maneuver at the last stage of the experiment, the results were expressed as normalized relative expression (with themselves and the housekeeping genes).

The modulation of the expression during the operations at the end of the experiment showed a heterogeneous pattern among the innate immunity factors with few significant differences. However a likely up regulation of pro-inflammatory factors is perceived right after net handling with a posterior recovery indistinctly in conditions of both strategies tested in the trial. In gill, *c3* gene significantly up regulated 24 hours after net cleaning in C3 and *il6* significantly up regulated right after net changing in C1 for a posterior level of expression restoration. In spleen, *il1ß* gene, displayed the same dynamics in C2 after net cleaning whereas in C1 significantly up regulated throughout the operation. No significant differences were observed in *il1ß* in gill or in *c3* and *il6* in spleen during cage operations **(Figure 46)**. In liver, only available data are the expression levels of c3, which did not show significant changes and are not graphically represented.

When looking at what happened at adaptive level, a much higher and significant level of expression of the *igm* gene was seen in C3 compared with the other two groups. After 24 hours post manoeuvre, *igm* gene clearly upregulated in C3 in both gill and spleen while the in C1 in spleen *igm* down regulated **(Figure 47)**.

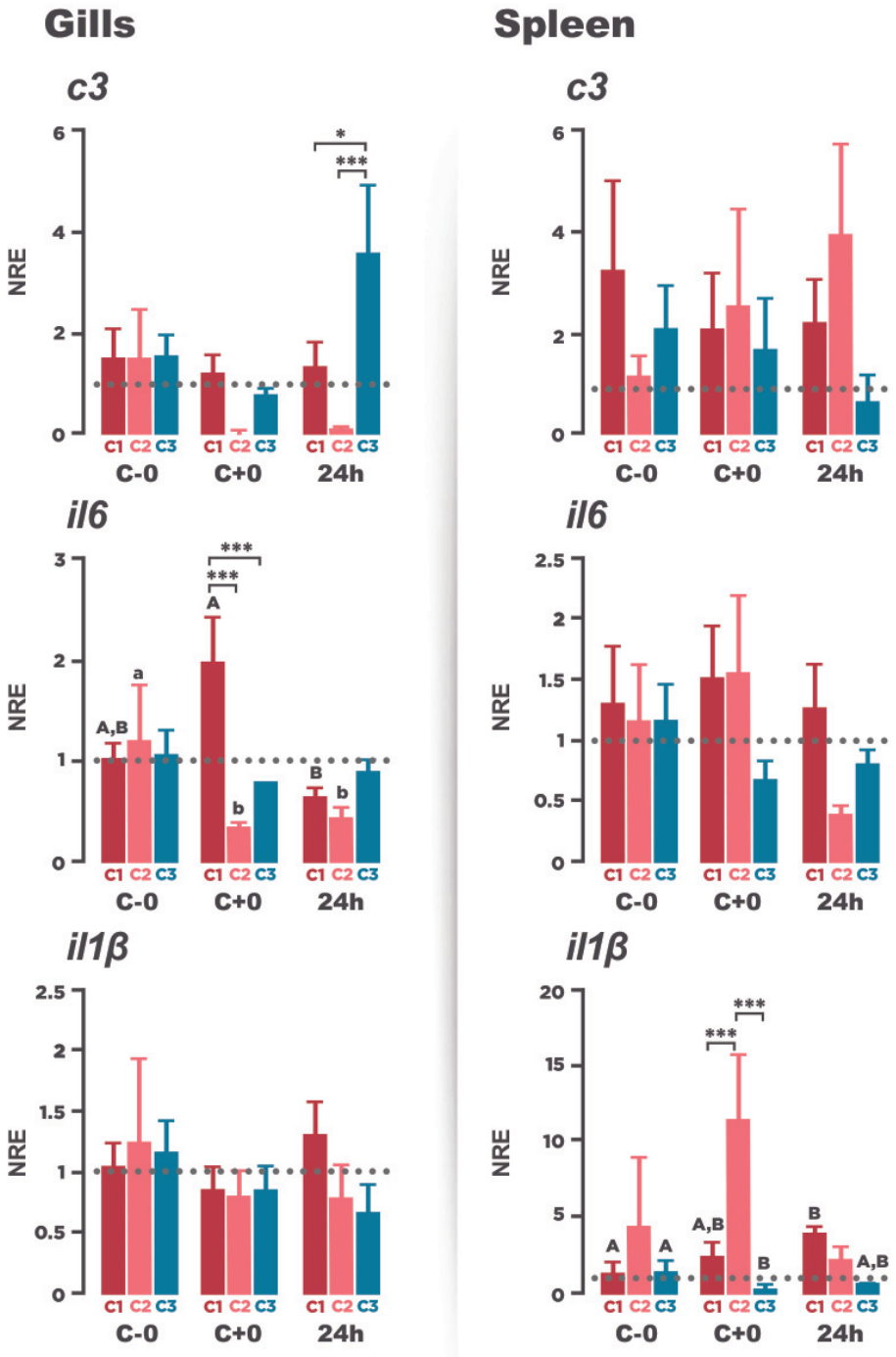


Figure 46. Relative expression efficiency of *c3 il6* and *il1ß* during cage operation at the end of the experiment..

Values are means of three replicates (n=9). Different letters mean significant differences within groups and asterisks denote significant differences between groups (P<0.05).

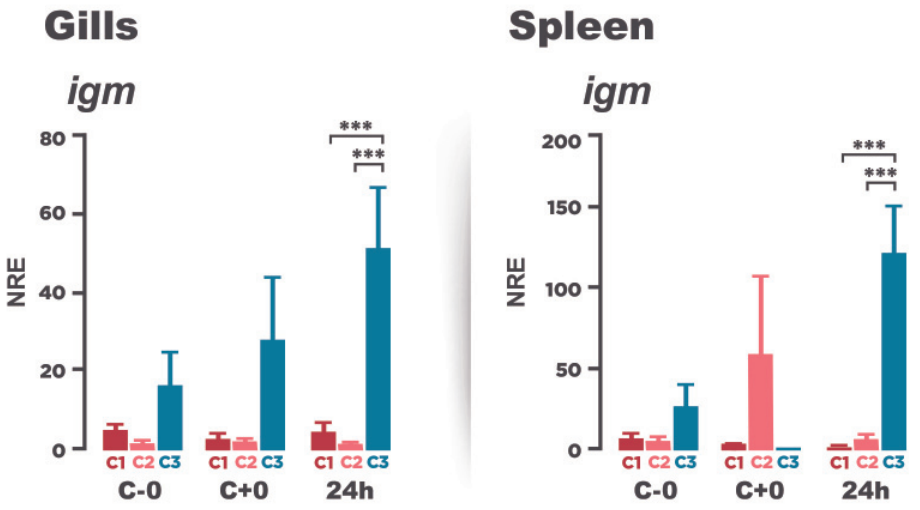


Figure 47. Relative expression efficiency of *igm* gene during cage operation at the end of the experiment. Values are means of three replicates (n=9). Asterisks denote significant differences (P<0.05).

Discussion

Many factors can affect the immunological status of fish. As part of the secondary stress response, all situations away from optimal welfare conditions might be susceptible of provoking changes in the immune system of animals. In a fish farming context, and particularly in the framework in which this study was carried out there were chronic and acute stressors that could have modulated the immune response. Apart from the captivity conditions as such, cage operations, copper and parasites exposure, environmental factors, diseases and predators presence could have exerted effects both in the innate and acquired immunity of the experimental groups.

An extensive effort has been done in studying the immunomodulation in fish and investigating which factors and how they affect the immune response but when looking for data obtained in real commercial aquaculture conditions there is a lack of data and only a very few works are found (Caruso et al., 2005; Contessi et al., 2006; Hernández and Tort, 2003; Tort et al., 1998).

In this work, in general terms the humoral response denoted a mild immune activation along the time course of the experiment. The effect of the net operations at the very last stage of the experiment was not clearly detectable

among the set of data obtained. In addition, a high variability was observed after the analysis, probably due to many factors affecting each individual.

In 2003, Hernández and Tort, suggested a positive correlation between temperature and complement activity in sea bream which would be in accordance with the results obtained in this experiment. Here, complement activity values were within the rage of those reported by other authors (Cuesta et al., 2006; Esteban et al., 2001; Hernández and Tort, 2003; Sitjà-Bobadilla et al., 2003; Tort et al., 2004) but much below those values reported by Contessi et al., (2006) in fish taken from commercial cages. Lysozyme activity values did not change over the experimental time and were in line with values reported previously (Hernández and Tort, 2003), higher than those reported by Sitjà-Bobadilla et al., (2003) and much higher of those reported by Ballester-Lozano et al., (2015);or Benedito-Palos et al., (2016).

Despite the fact that a mild, but not significant immune activation was observed in complement activity after the final operation in C1 and C2, the only significant difference found at this point was a significant reduction of the hemolytic activity in C3. Several works describe a reduction of the complement activity after an acute stress episode and link it with elevated levels of circulating cortisol (Contessi et al., 2006; Cuesta et al., 2006; Esteban et al., 2001; Tort et al., 1996). However (Cuesta et al., 2006) did not find changes in this parameter after cortisol injection. In this work, the relationship between cortisol and complement activity is not very evident. Lysozyme activity did not seem to be affected by rearing conditions. Values observed at the end of the trial were very similar to control values. Here, as described before (Hernández and Tort 2003), temperature did not seem to influence this parameter. Curiously, 24 hours after net cleaning in both C2 and C3 lysozyme activity very significantly decreased compared to values right before the operation. That would be in line of what occurred with complement in C3 but a clear cause for that was not found.

Elevated levels of circulating cortisol are related with higher presence of IgM in plasma (Cuesta et al., 2006). In this work, the higher levels of cortisol in plasma displayed at the end of the experiment would have rather a chronic connotation and therefore could explain the significantly higher content of IgM in all groups at the end of the trial before exposing the animals to the final cage operation. At this stage the higher exposure to parasites or copper would have not caused changes between C1 and the groups belonging to the other net management strategy. Throughout the last cage operation in the experiment IgM content did not significantly change likely to what repor-

ted also in sea bream (Cuesta et al., 2004; Guardiola et al., 2016) after acute exposure to stress conditions.

Moving to the gene expression level, at first glance, the fold change expression along the experiment (from the beginning to right before the last cage maneuvers) suggest slight differences between the two strategies contrasted in this study. Broadly, the set of genes encoding for the innate immune system proteins were slightly up regulated in C2 and C3 compared to C1, in particular in the inflammatory factors. This was more evident in gills rather than spleen, where a mild up and down regulation was observed indistinctly in all three groups without significant differences between them. This results would match with the damage produced in gills during *in situ* cleaning episodes in those groups. Looking at the adaptive immunity (*igm*), both in gill and spleen a significant up regulation of the gene was found in C1 compared to the down regulation in the other two groups. The intensity of the up regulation was higher in spleen, where phagocytosis of antigen- antibody complexes is conducted (Uribe1 et al., 2011). This fact could be related to a higher chronic exposure to water borne copper (Geist et al., 2007) and subsequent accumulation in C1 compared to C2 and C3. The higher level of expression in C1 would be in accordance with higher circulating levels of the immunoglobulin. The odd fact is that in C2 and C3 those elevated levels in blood were also found. Interestingly, looking at what happened at the end of the trial, *igm* gene was very much up regulated in spleen and gills compared to C2, and C1where the gen was in a minor but significant way down regulated. This acute up regulation would be in line with the results described by Mauri et al., (2011).

The innate immune parameters across the last net-cage operation showed an erratic pattern of expression. The most remarkable outcome of the data analysis was a rapid (right after operation) but not very significant reaction detected among inflammatory genes in both tested strategies.

Conclusions

Probably both the fact that no huge differences among groups and again the context in which the experiment was performed, makes difficult to extract a clear conclusion from the outcome data set of immune results. As in the metabolic responses, high individual variation was found when analyzing the results of the immune parameters.

Reviewing the literature, the humoral values found in this study for both innate and adaptive factors were inside the normal range for this species

and the responses, despite not very uniformly among the groups followed the general trends described *in vitro* or *in vitro* laboratory conditions. In general terms the rearing conditions to which the experimental groups were subjected, caused a mild chronic immune activation which was not very much altered during the operations conducted to every cage at the end of the experiment according to changes in lysozyme and complement activity. According to the circulating immunoglobulins levels we could state the immunological status of the animals was not affected. The expression of genes involved in the inflammatory response was slightly higher from both a chronic and an acute perspective in the gills of those groups subjected to the on-site cleaning strategy which would confirm the damage caused by this management methodology in fish.

Overall, from the present results it is difficult to see substantial difference on the immunity status of the animals between different groups. However, it seems clear that on site cleaning promotes an acute inflammatory response at gill level.

The antioxidant response

As aerobic organisms, fishes experience another consequence after stress, i.e. the exposure to reactive oxygen species (ROS) causing oxidative stress. Oxidative stress can be originated endogenously as a by-product of metabolism (Costantini, 2008) and the immune response (Marri and Richner, 2015) or exogenously as a result of exposure to challenging environments, such as contaminated areas (Bacanskas et al., 2004). ROS species can impair many physiological functions by producing damage to lipids, proteins and DNA (Finkel and Holbrook, 2000) **(Figure 48)**.

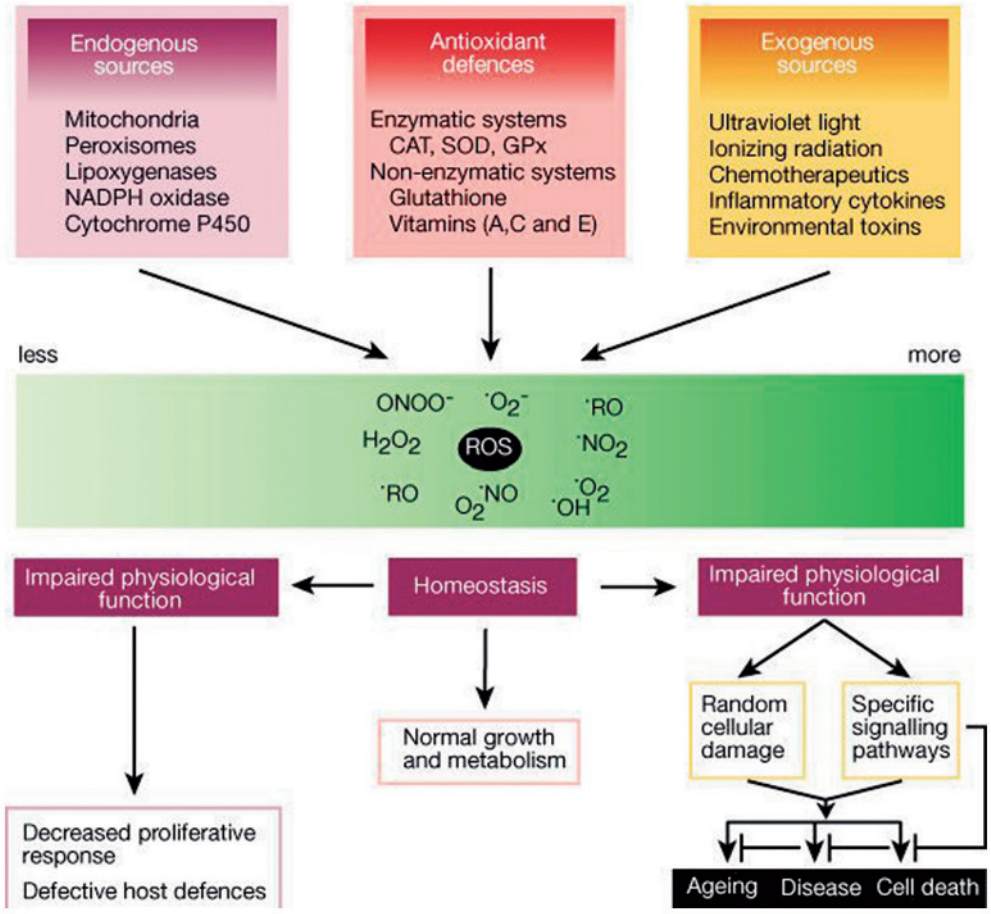


Figure 48. The sources and cellular responses to reactive oxygen species (ROS).

A sophisticated enzymatic and non-enzymatic antioxidant defense system including catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) counteracts and regulates overall ROS levels to maintain physiological homeostasis. From (Finkel and Holbrook, 2000).

Organisms have developed antioxidant defenses such as specific enzymatic proteins including superoxide dismutase and catalase, glutathione peroxidase and metallothionein. Those defenses are modulated by age, infection, xenobiotic exposure, nutritional condition, and environmental and behavioral factors (Martínez-Álvarez et al., 2005). The scenario of oxidative stress appears when the presence of these antioxidant defenses and ROS production is not balanced.

In aquaculture, apart from the above mentioned metabolic and immunological endogenous sources, animals are frequently exposed to external factors potentially promoting ROS production. One of the most evident is exposure to heavy metals in a context of environmental pollution or due to their use in several practices within the aquaculture industry. As it is known, copper is widely used in the antifouling treatment of aquaculture nets. We saw in chapter 4 in this thesis that copper redox activity ($Cu^{1}+ \rightarrow Cu^{2}+$) promotes formation of ROS species and as copper is freely soluble it can pose animals at risk (Minghetti, 2009). Chronic exposure leads to accumulation in tissues, especially liver it is the major detoxifying organ, but at the cellular level, copper can interferes with several metabolic pathways and thereby induce different cellular responses.

Several biomarkers are used to measure oxidative stress caused by copper in fish, most of them enzymes involved in copper homeostasis, among them, Cu/Zn superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), which constitute the first line of defense against ROS (Kammer et al., 2011). Their great sensitivity to wide- ranging stress conditions makes the gene expression of these antioxidant enzymes genes suitable for use as fish biomarkers. Apart from those, metallothioneins (MT) and cytochrome P450 are also molecules often used as bio-indicators of oxidative stress. MT, which are found ubiquitously in body tissues, are also involved in protection against oxidative stress (Evans and Halliwell, 2001) and are also frequently used as a biomarker both under laboratory and field conditions (Bervoets et al., 2013). MT are highly involved in the metabolism of copper with functions of detoxification and storage (Templeton, D.M., Cherian, 1991) protection against ROS and Cu recruitment in case of copper deficit (Suzuki et al., 2002). Cytochrome P4501 family is a major family of drug metabolizing enzymes (DME) and CYP1A is especially involved in metabolizing environmental pollutants (Shimada et al., 2001) towards which responds in a dose-dependent manner and is commonly used also in field and laboratory studies as a marker (Chipman, 2006).

Due to the major detoxifying role of liver and the direct contact of gills with the surrounding water, these are often considered target organs to study the modulation of the expression of genes encoding proteins involved in xenobiotic metabolism and oxidative stress protection. However, as stated above, immune activation also releases highly reactive species. Despite this fact, spleen is not commonly considered as a target organ when studying the antioxidant response of exposed animals.

This section will assess the expression of those genes encoding for the four enzymes and proteins above described in gills, liver and spleen.

Materials and methods

The procedures followed for gene expression have been already detailed in the introductory section of this chapter.

Statistical analysis

After checking normality with the Anderson-Darling test, within differences among groups were analyzed separately with one-way ANOVA and Tukey's test comparing initial values of expression with those seven months later, right before cage operation. At the end of the experiment General Linear Model (GLM) and Tukey's test were used to assess differences between groups and before and after operations. In all cases significant differences were considered when ($P<0.05$).

Results

Chronic effects

The fold change in the expression of selected genes was used to assess the possible effects of chronic factors and the substantial temperature increase between the beginning and the end of the experiment (right before cage operation).

In gills, all genes encoding antioxidant enzymes (SOD, CAT and GPX) were down regulated during the experiment in all conditions except gpx, which expression did not change compared with expression intensity at the beginning of the experiment. Significant down regulation was observed specially in C1. *cyp1a1* gene expression was higher at the end of the experiment in C3, but in contrast in C1 and C2 they were clearly downregulated. However, due to individual variation the difference observed between groups was not significant **(Figure 49)**. There were no available data regarding *mt* expression in gill.

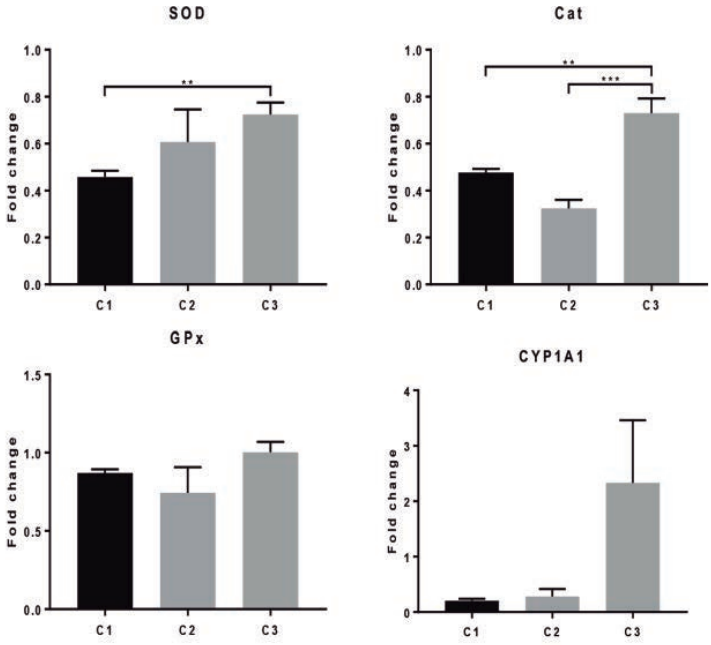


Figure 49. Fold change expression of genes encoding SOD, CAT, GPX and CYP1A1 in gill between the beginning and the end of the experiment (right before cage operation).

Values are means of three replicates (n=9). Asterisks denote significant differences between groups (P<0.05).

In liver, the genes displayed almost the same expression pattern than in gills. In this organ, though, *sod* and *cat* weredown regulated in C1 and C3 but slightly up regulated in C3. The difference tendency between C1 and C3 was significant. *gpx* expression did not show changes in any condition. *cyp1a1* up regulated only in C3, but in a non-significant way and *mt* was the only gene which up regulated in all three conditions. The increase in the expression pattern was similar in all groups **(Figure 50)**.

In spleen, a slight up regulation of *gpx* was observed in C3. *sod* and *cat* showed significant down regulation in C1 and C2, whereas *cyp1a1* were up regulated in C3. Looking at the data, this up regulation in C3 was very evident, but again not significant due to high variation. *mt* was down regulated in all groups during the experimental period **(Figure 51)**.

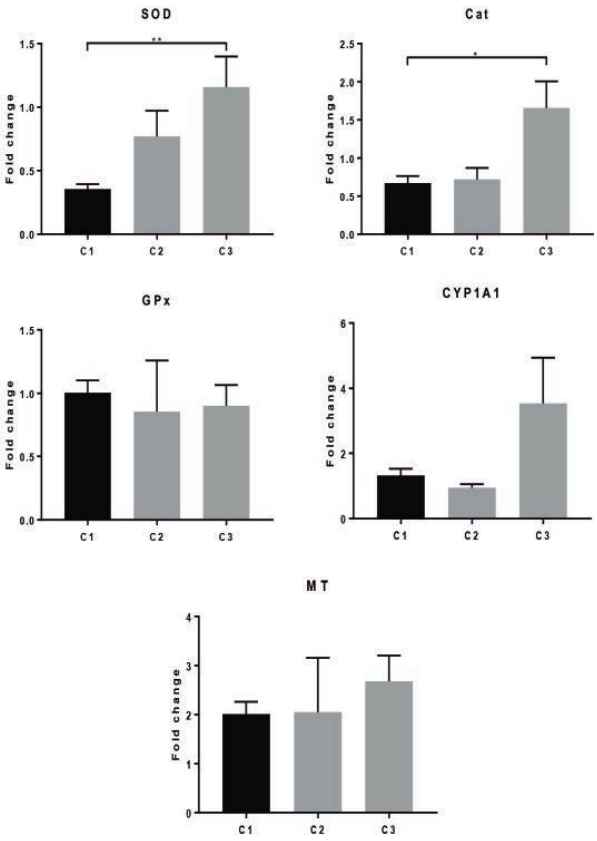


Figure 50. Values are means of three replicates (n=9). Asterisks denote significant differences between groups (P<0.05).

Values are means of three replicates (n=9). Asterisks denote significant differences between groups (P<0.05).

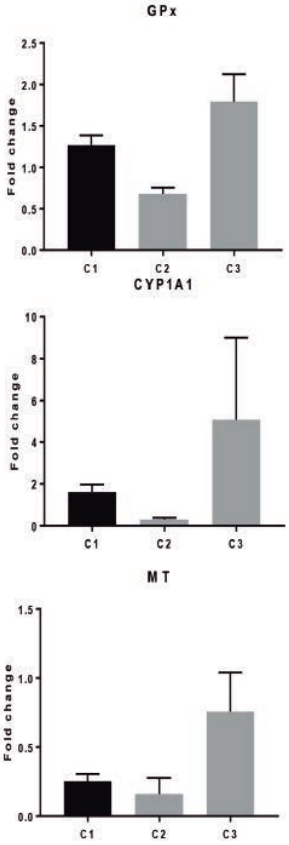


Figure 51. Fold change expression of genes encoding SOD, CAT, GPX, CYP1A1 and MT in spleen between the beginning and the end of the experiment (right before cage operation).

Values are means of three replicates (n=9). Asterisks denote significant differences between groups (P<0.05).

Acute effects

To assess gene expression throughout the maneuver at the last stage of the experiment, the results were expressed as normalized relative expression (with themselves and the housekeeping genes).

The general reaction to the operations observed in gills was a slight up regulation of the antioxidant enzyme genes and *cyp1a1* in C2 and C3, *sod* expression significantly increased in C1 right after the operation, *cat* did the same in C1 and C2 24 hours later as well as *cyp1a1* in C1. 24 hours after cage operation the level of expression of *cat, gpx* and *cyp1a1* was higher in

C1 and C2 compared to C3 **(Figure 52)**. As samples showed unspecified bands for *mt,* the analysis for this gene was not performed in gills.

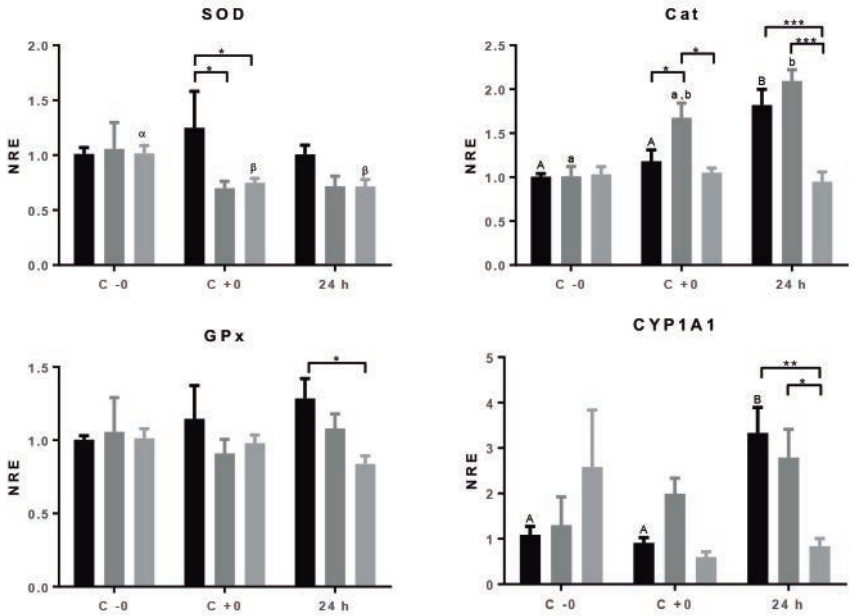


Figure 52. Relative expression efficiency of *c3*, *il6* and *il1ß* during cage operation at the end of the experiment. Values are means of three replicates (n=9). Different letters mean significant differences within groups and asterisks denote significant differences between groups (P<0.05).

In liver, C1 showed a clear and significant up regulation pattern for all the analysed gens except *cyp1a1* compared to the conditions under the on-site cleaning strategy. *cyp1a1* significantly up regulated 24 hours after net cleaning in C2 **(Figure 53)**.

In spleen a general down regulation trend was found among the antioxidant enzyme genes 24 hours after the appropriate operations in C1 and C3, while in C2 those values did not change. *cyp1a1* and *mt* expression values showed a mild up regulation, which in the case of *mt* caused a significant difference right after net changing in C2 compared to the other groups **(Figure 54)**.

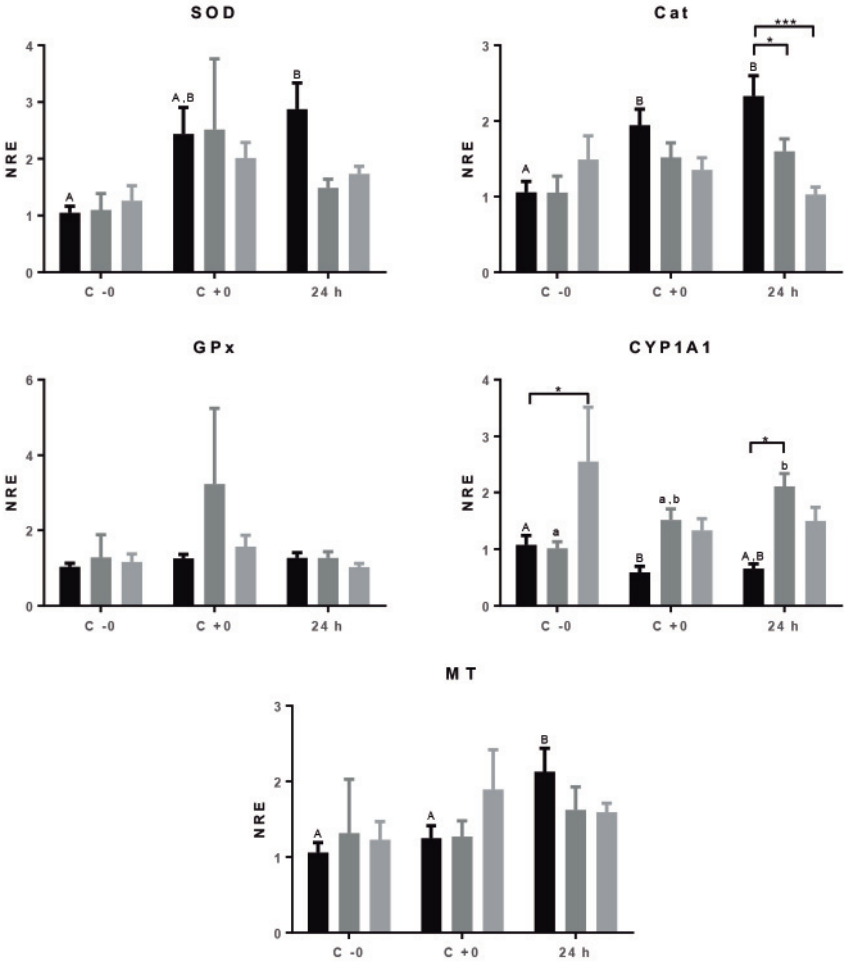


Figure 52. Relative expression efficiency of *c3 il6* and *il1ß* during cage operation at the end of the experiment.

Values are means of three replicates (n=9). Different letters mean significant differences within groups and asterisks denote significant differences between groups (P<0.05).

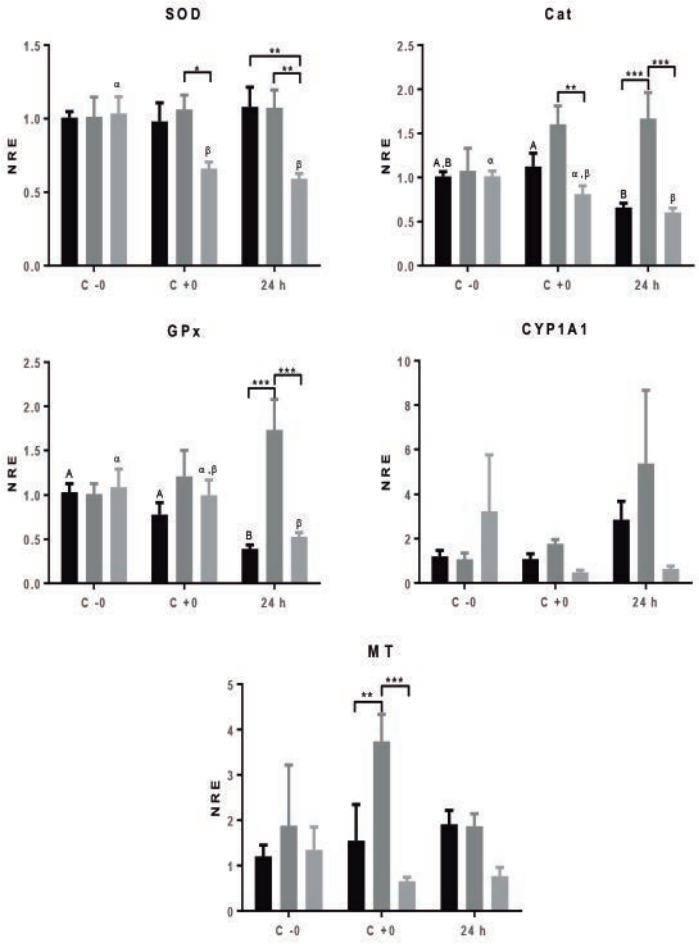


Figure 54. Relative expression efficiency of *c3 il6* and *il1ß* during cage operation at the end of the experiment..
Values are means of three replicates (n=9). Different letters mean significant differences within groups and asterisks denote significant differences between groups (P<0.05).

Discussion

As in real aquaculture practices, copper exposure, predator presence or the periodically repeated cleaning operations such as those conducted in C2 and C3, are some of the factors that most clearly could have originated oxidative stress in the experimental work in this study. Apart from those, temperature oscillations registered along the time course of the trial, could involve a source of potential oxidative stress, and in this case in a much higher intensity than what happens in actual commercial conditions in off shore cages.

The most generalized outcome of the effect of the experimental rearing conditions among the groups was the mild, although not significant up regulation pattern on *mt* gene in the liver. Apart from that, the general observed trend was a down regulation of all analyzed genes in C1 and C2. Interestingly, C3 showed a smooth up regulation pattern of *cyp1a1* in all tissues as well as an up regulation of the inflammatory factors in liver. The only differences between them was the higher parasites prevalence and the larger amount of fouling generated in C3 which could have propitiated a longer cleaning time, and that indeed caused a more considerable amount of suspended debris in the cage during cleaning operations. Unfortunately, the exact time dedicated to clean each net was not recorded, and could have probably been a worthy data.

By looking at the results, the general impression of the effect of the cage operation on antioxidant gene expression at the end of the experiment was an up regulation trend in C1 and C2 while C3 showed a general down regulation pattern. More significant changes occurred in gill and liver rather than in spleen as those organs are more exposed to oxidative stress. Gills are in direct contact with the surrounding medium, and in this experiment they were specially exposed to the effects of on-site cleaning. Previously in this work, the physical damage caused by net cleaning operations on to the gills was observed and also the mild but significant accumulation of copper in this tissue. This probably originated an oxidative response in this organ. Due to the detoxifying role of liver and the higher concentration of copper found in this organ (Grosell, 2011), the antioxidant response shown in the results in this organ is also coherent.

Another evident outcome from the results in the very last stage of the experiment is the significant increase in the intensity of the expression of the *mt* gene at the end of the experiment in liver in C1 24 hours after replacing the nets by new ones. This could be explained by the fact that when installing a new net onto a cage, often some antifouling paint leaches out rapidly, even if the net seems to be dry. This tendency would agree with the function of metallothionein, the detoxifying function of the organ and the accumulation of copper observed at the same stage of the experiment in all conditions.

Conclusions

As a conclusion we could state that according to the magnitude of the antioxidant response detected through the changes in the expression pattern of the analyzed biomarkers, neither the rearing conditions nor the operations at the end of the experiment or any other event occurred during the

experimental time became a substantial source of oxidative stress at gene expression level. As it was expected, liver showed most of the changes recorded, especially in the expression of *mt* gene. Gene expression in spleen did not appear to be an organ specially affected by oxidative stress.

Chapter conclusions

The overall conclusion which arise from the results in this chapter, could be reduced to the following statement: In terms of primary and secondary stress responses, the two fouling strategies tested in this trial did not seem to cause major differences among the experimental groups.

The observed primary stress response suggests a moderate chronic stress scenario and an expected response to the acute perturbations caused by net-cage operations. In terms of levels of cortisol released into the blood stream it seems that net changing produced a longer effect compared to on-site cleaning. However, those groups subjected to the latter did also react to the operation and this is important considering the higher frequency in which it is conducted in cages at commercial scale. The mild reaction to the operations could have been conditioned by the relatively high levels of cortisol chronically registered along the trial.

It was difficult to distinguish clear metabolic readjustment pattern following the stress response. In general terms all blood biochemistry parameters were within the range of what has been described previously for *Sparus aurata*. No clear tendencies were detected and no correlations could be established among different factors. A general and significant reduction of circulating triglycerides was seen in all groups when comparing final with initial values. Circulating triglycerides at the beginning of the experiment were considerably higher than those reported for sea bream, possibly as a result of an over feeding during the pre-growing period prior to cage stocking. This fact made even more evident the reduction across the experiment. The drop in blood triglycerides more probably correlated with the low feeding rates during the coldest months and lipid mobilization rather than a demand originated as a consequence of a stress situation. This will be further discussed in the general conclusion of this work.

Although the immune status of the animals appeared to be good during the whole time course of the experiment, a moderate chronic activation of the immune system was observed in spleen and gills accordingly with the sub optimal conditions caused by the rearing environment.

Those innate parameters were chronically activated in the groups exposed to the on-site cleaning strategy, while *igm* gene was chronically up regulated in C1. The higher parasites and bacteria presence could have promoted this tendency in C2 and C3 (Alvarez-Pellitero, 2008).The reaction to the last cage operation provoked an erratic and weak inflammatory response, but in contrast a marked adaptive reaction in C3 was detected possibly as a result of the higher level of disturbance provoked by this operation. Net cleaning was shorter in time than net replacing, but C3 cages were those with a larger amount of fouling and thus the time of the operation was longer than in C2. Also, the amount of organic matter washed of the nets was higher and the cloud of debris produced after net cleaning was substantially more important. So, finally the total time during which C2 and C3 cages were under the effects of the maneuver was even higher that the time required for net replacing in C1.

As expected the oxidative response was mainly observed in liver and gills. Although non deleterious for the fish, the presence of copper in the medium was recorded especially in liver showing a higher degree of *mt* gene expression. Both gills and liver reacted to the cage operations, as far as the oxidative response is concerned, in a similar way in all the experimental groups. Net replacing in C1 clearly promoted a response to the presence of copper in the medium directly related to the operation.

Economic impact of the on-site cleaning strategy. A basic approach

One of the major reasons for which the aquaculture industry has largely approached alternatives to the use of copper in aquaculture nets is to reduce operational costs derived from changing the nets of the cages. Despite having been implanted at commercial scale for some years, hardly any data exist on the economic impact of on-site cleaning compared to the use of the antifouling treated nets.

Fish farming, as a primary sector industry has narrow profit margins (EY, 2017) which largely depend on the price fluctuation of the final product. Large salmon producers reported EBITDA margins ranging 10%-20% in 2016 (Marine Harvest, 2017; Grieg Seafood, 2017; Lerøy Seafood, 2017), while in the Mediterranean industry those were a bit lower, around 12% (Andromeda Group, 2017). The latter includes the hatchery production, which is often a more profitable area of the business compared to growth in cages. In both cases, the salmon and the Mediterranean industries, obtained in 2016 the best results for the last ten years.

After having seen the biological implications of on-site cleaning, in this chapter a brief and basic approach to the economic impact of this newly arrived fouling control strategy will be done in this chapter to confirm whether or not it might be considered an alternative to reduce costs and enhance margins, as it was firstly supposed. This analysis uses real published or personal experience data from the aquaculture industry.

Sea bream production cost structure in Spain

Very roughly, the costs structure and margin in the sea bream industry considering realistic feed and fry conversion ratios (stocking fries at 10 g. and harvesting at 420 g.) would be the following **(Table 6)**:

	Fry/feed CR Kg^{-1}	Price (€)	Cost (€ kg^{-1})
Fry cost	2,850	0,295[a]	0,841
Feed cost	2,250	0,990[b]	2,228
			3,068
Overheads*			**0,850**
Cost of goods sold (COGS)			**3,918**
Operating expenses*			**0,950**
Total Cost			**4,918**
Price (EXW)**			**5,460**
Gross profit margin before taxes			**10,8%**

[a,b]Authors' personal knowledge from the industry 12/2017
*Overheads and operating expenses from the author's personal experience (2002-2016)
*Latest price update in the Spanish market 11/12/2017. Source: www.mispeces.com

Table 6. Costs structures in sea bream farming in Spain.

Globally, the cost of fouling management in the aquaculture industry (mainly nets and operations) has been reported to be 5%-10% of the total production cost (Lane and Willemsen, 2004). According to this, and considering an average value of 7.5%, the cost of fouling control in the sea bream industry can be assumed as:

$$3.98€\ Kg^{-1}(COGS)\ x\ 7.5\% = \mathbf{0.294}€\ Kg^{-1}$$

To contrast both strategies tested in this study: *The use of several antifouling treated nets per growth cycle strategy* (S1) and *The on-site cleaning strategy* (S2), the amount and costs of the resources involved in each operation have been pondered. The initial equipment investment, ranging from 8000 € to 350000 € (Riska, K. E., Akvagroup, personal communication) to perform on-site cleaning operations has not been considered. Tables below, show the effect of net and operational factors involved into the operations in terms of cost.

Net and treatment effect

	Strategy 1	*Strategy 2*	*S2 vs S1*
a, Cost of the nets	1	0,985	-2%
b, Antifouling/coating price	1	4	300%
c, Antifouling/coating uptake	1	0,9	-10%
d, Working dillution	0,9	0,5	-44%
e, # nets/growth cycle	3	1	-67%
f, Net propertise loss (BS)	1	1,3	30%
	2,7	**2,30**	**-15%**

Table 7. Relatives weights and costs of net and treatment factors in S1 and S2.

a. The cost of manufacturing the nets is slightly lower in S2 compared to S1 due to the less slack, so the less amount of netting needed. That means a reduction of 2% in the total cost of the net. *Source: Mørenot AS.*

b. The cost per liter of the polyurethane coating is four times that of a regular antifouling. Source: *Netkem AS, Taytech.*

c. The product uptake when treating the netting is slightly higher in the case of the antifouling compared to the coating. The AF penetrates the netting, while the coating does not. *Source: Mørenot AS.*

d. In this study, the experimental products were diluted prior to treat the nets according to current manufacturing procedures.

e. One of the purposes of the on-site cleaning is to reduce the number of nets used per growth cycle, thus the operational cost of fouling management. Normally in the sea bream industry a total of three nets are used to rise a batch of fish to commercial size. Cleaning *in situ* the nets, a unique net (intermediate mesh size) should be used from stocking to harvesting the fish.

f. According to the results obtained in this work (see chapter one), after seven months of a simulated rearing cycle, the effect of on-site cleaning over the netting tensile strength was a reduction of 16% in the breaking strength; i.e., it was needed 16% less strength to break the netting. Considering a growth cycle of 18 months the loss of strength would probably be much higher. Assuming linearity, the loss would be around 30% considering also the effect of cleaning the antifouled three nets per cycle in strategy 1. That would mean that 30% more nets would be needed in the case of using the on-site cleaning strategy.

Bearing all this in mind, in terms of nets and treatments costs, the on-site cleaning strategy would be 15% cheaper than the widely spread strategy of using several nets treated with antifouling.

Operational effect

	Strategy 1	Strategy 2	S2 vs S1
g, Operation manpower	2,5	1	-60%
h, Operation boat	1	1	0%
i, Operation time	1	0,5	-50%
j, # operations /growth cycle	3	36,5	1133%
	7,5	**18,25**	**143%**

Table 8. Relatives weights and costs of the operational factors in S1 and S2.

g. The on-site cleaning operation can be performed by a team of two people, whereas 5 people are usually required to replace a dirty net by a new one, both in the Mediterranean and salmon industry.

h. The same number and characteristics of boats are usually used to execute both operations.

i. In terms of time, the on-site cleaning operation is faster. Approximately a net is cleaned in half of the time required for net replacing.

j. In sea bream production, the strategy of using antifouling treated nets, usually needs a total of three nets to complete a growth cycle. This means two complete operations of net replacing, an initial operation when installing the first new net onto the cage, and a final operation when removing the last net after having harvested the fish. This makes a total of three complete operations. In comparison, for on-site cleaning, the number of cleaning episodes per growth cycle is much higher. According to previous works (Bloecher et al., 2013) and the author's personal experience, on site cleaning is performed every eight weeks (in winter) to two weeks or as often as weekly (in summer) in the salmon industry. This practice has just been introduced in the Mediterranean, with the first tests in 2015. Currently, companies cleaning the nets on-site, conduct these operations every three weeks, although a higher intensity would be necessary. Considering all this, for a 18 months growth cycle (stocking in March), which is the necessary time in Spain to raise fish from 10 g. to 420 g. the following cleaning operations schedule has been proposed (Table 9). It considers temperature and daylight as major factors influencing fouling growth and attachment (Hole, 1952) in an intermediate latitude of the Mediterranean coast of Spain.

	MAR	APR	MAY	JUN	JUL	AUG	SEP	OCT	NOV	DIC	JAN	FEB	MAR	APR	MAY	JUN	JUL	AUG
36,5	0,5	1	1,5	2	4	4	4	3	2	0,5	0,5	0,5	0,5	1	1,5	2	4	4
Water temp. ºC	12,7	15,0	18,3	21,4	25,1	26,2	25,1	21,8	18,0	14,8	13,3	12,3	12,7	15,0	18,3	21,4	25,1	26,2
Daylight (hrs)	12,0	13,3	14,3	15,0	14,5	13,8	12,5	11,0	10,0	9,3	9,8	10,8	12,0	13,3	14,3	15,0	14,5	13,8

*Considering a growth cycle of18 months and stocking fries in March.

Table 9. Required on site cleaning operations for a growth cycle of sea bream in Spain*.

A total of 36.5 cleaning episodes would be necessary with the existing technology and net design to control fouling production in aquaculture nets in Spain. Depending on the latitude this number could change.

According to this, the operational effect on the cost will be much higher in the on-site cleaning strategy rather than in the use of antifouling treatment nets basically due to the high cleaning frequency required and the consequent manpower required.

Conclusion

The overall effect taking into account both factors above analyzed would be an increase of 108% in the cost of fouling management when using the on-site cleaning strategy **(Table 10)**.

	Strategy 1	*Strategy 2*	*S2 vs S1*
Net & treatment effect	**2,70**	**2,30**	**-15%**
Operational effect	**7,50**	**18,25**	**143%**
Overall effect	**20,25**	**42,06**	**108%**

Table 10. Overall effect of on-site cleaning on the cost compared to the use of AF treated nets.

As indicated earlier, the cost of manage fouling production in the Spanish aquaculture industry is approximately 0,294 € (per kg of fish), and an increase of 108% would result in an extra cost of 0.317€ Kg^{-1}.

$$0.294€\ Kg^{-1}\ x\ 108\% = \mathbf{0.317€\ Kg^{-1}}$$

Therefore, an increase in Cogs (cost of goods sold) and thus in the Total Cost of 0.317€ Kg-1 impacts directly in the profit margin, reducing it to only a small 5% before taxes (therefore a dramatic decrease of 53%) **(Table 11)**.

	Fry/feed CR Kg^{-1}	Price (€)	Cost (€ kg^{-1})	
Fry cost	2,850	0,295	0,841	
Feed cost	2,250	0,990	2,228	
			3,068	
Overheads			**0,850**	
Cost of goods sold COGS			**3,918**	**4,235**
Operating expenses			**0,950**	**0,950**
Total Cost			**4,868**	**5,185**
Price (EXW)*			**5,460**	**5,460**
Gross profit margin before taxes			**10,8%**	**5,0%**

Table 11. Effect of on-site cleaning on production cost and business margin.

In this analysis the potential negative effects of using the on-site cleaning strategy on the fish performance have not been taken into account, although it is likely that they occur. Going back into the second chapter of this work it results quite clear that cleaning operations affect the feeding pattern, and this can very easily involve detrimental effects on the feed conversion ratio (FCR). Moreover, it seems also evident that cleaning episodes negatively affect gills health status which can eventually involve disease and a lack of performance. Simulating a reduction of performance of only 2% in both survival- growth and feeding performance the picture results into an even worse situation **(Table 12)**.

	Fry/feed CR Kg^{-1}	Price (€)	Cost (€ kg^{-1})	
Fry cost	2,907	0,295	0,858	
Feed cost	2,295	0,990	2,272	
			3,130	
Overheads			**0,850**	
Cost of goods sold COGS			**3,980**	**4,301**
Operating expenses			**0,950**	**0,950**
Total Cost			**4,930**	**5,251**
Price (EXW)*			**5,460**	**5,460**
Gross profit margin before taxes			**9,7%**	**3,8%**

Table 12. Effect of a 2% lack of performance on cost and gross profit margin.

Though it may seem that on-site cleaning strategy margins are insufficient to cover costs, the strengths of this strategy are clear: the lower quantity of nets and manpower needed to perform the operations, as well as their dedication involved in those operations. Therefore, cost reduction would be achieved if basically the cleaning frequency could be reduced, in turn reducing the damage to the nets and enlarging their lifespan.

Moreover, nets could be better protected by reducing the dilution of the coating. This would increase the treatment cost, but would also reduce the damage caused on the nets. Cleaning efficiency should improve, thus reducing cleaning frequency. This would help in the sense that the impact on fish performance would be lower, and very important, would reduce the re-growth rate of the fouling. In the above exercise, by reducing in 50% the total cleaning episodes (18 instead of 36), practically speaking a monthly cleaning frequency, it would represent a neutral effect on the cost and margin, thus automatically representing an improvement of the results. Currently, this appears to be the major bottle neck of onsite cleaning in terms of profitability.

General discussion

The original aim of this work was to investigate the effects on fish, if any, of a newly implemented technology in fouling management, *on-site cleaning,* in aquaculture cages compared to the largely spread strategy consisting in treating nets with an antifouling paint and replacing them as they become occluded. On-site cleaning implantation had been motivated as a consequence of the need of the aquaculture industry to reduce the complexity and high operational costs involved in fouling management and to minimise the use of copper in aquaculture nets to reduce eventual metal interactions in edible fish tissues and therefore improve social perception among consumers. As the origin of the project was an industrial motivation and need, some company-based considerations were taken into account when planning the work.

It was very rapidly detected that the study needed to cover a wide scope of aspects and not only focus into one specific consequence of the fouling management activity like the number of nets used in a growth cycle as companies profitability may be affected in many ways. Chronic and acute effects were investigated in terms of both fish welfare and stress response to the rearing conditions and cage operations as major factors that might have an effect on fish performance. Apart from those, other elements involved in one or the other strategy/alternative were identified as susceptible to affect health features and fish performance and so they were also included into the experimental design. Apart from analysing the biological consequences in the stock, the effects on the nets was also approached to cover a lack of data in actual working conditions in this field.

As stated right above, the second major consideration was that the experimental work had to be conducted in natural conditions, as similar as possible to a real commercial aquaculture environment. To do so, a long term trial was designed which was carried out in a sea water pond simulating real cage aquaculture conditions. This, on one hand presented some constrains such us high individual variations observed during data analysis probably caused by the many factors influencing the stock, but on the other hand more clearly reflected what happens in the field compared to the results of those trials conducted *in vitro* or under laboratory conditions.

The third important element, and the main goal of the work was to provide clear and reliable information to the industry to facilitate decision making when designing the fouling management strategy. Therefore the experimental design and setting of the work were oriented towards this goal.

Although the work was mainly approached from a biological point of view, the economic implications of one or another alternative were another aspect to be, reviewed to give a more global picture of the on-site cleaning strategy effects compared to the lengthy use of antifouling. This multi-purpose approach was adopted as the planned applied research was promoted and developed in an industrial context.

In general terms, some of the reasons why the aquaculture industry focussed in the on- site cleaning strategy as an alternative to the use of copper indeed exist. Apart from the avoidance of use of copper these reasons are mainly the less quantity of nets needed to rise a batch of fish and the less resources required to perform the cleaning operations compared to net replacing. These two factors could very much reduce the cost of fouling management. However, this strategy presents some limitations that must be tackled and solved in order make on-site cleaning a reliable future option.

The most relevant constrain of on-site cleaning is the high cleaning frequency currently required to keep nets in a constant clean condition. A high cleaning frequency negatively impacts the activity from two points of view. First, it reduces nets and fish performance through several detrimental effects, and second, it supposes a very high operational cost that counters the initial advantages of the on-site cleaning strategy. The reason for this high frequency is that the existing technology and nets design do not allow a complete cleaning of the nets. In many cases, fouling organisms are cut off instead of completely removed from the nets, especially in those areas next to ropes and net folds. As a result of this, there is a high re-growth rate of fouling.

One of the experimental conditions tested in this work (C2), has appeared as a promising option to reduce cleaning frequency. The experimental treatment in this group was a midway solution between the use of copper based antifouling and the plastic coating. In fact, the netting was polyurethane coated and some cuprous oxide, hardly a 3% v/v, was added in the coating dilution. Despite this low quantity of copper, the efficiency in terms of fouling prevention was substantial, right in the middle of the other two groups, the standard antifouling product used in C1 and the coating in C3. This solution would on one hand, considerably reduce the amount of copper necessary for antifouling control and this way accomplishing one of the initial motivations of the industry, and on the other hand would also reduce the cleaning frequency required to prevent fouling accumulation on net panels avoiding all their detrimental effects.

In this study, on-site cleaning operations were performed according to current (or desired) practices in the industry and suggestions from previous works (Bloecher et al., 2013) resulting in a monthly cleaning frequency. However, the results of PNA/PNO levels suggested that a higher cleaning rate would have been necessary, due to the fast re- growing speed of the fouling organisms, which supposed a relatively high presence of fouling along the experiment. A higher cleaning frequency, in the real industry, would then mean an increase of the operational costs.

Like the antifouling paint, the polyurethane coating tested in this work protected the nets against external agents such as temperature variations and UV light, but the actual function of the coating was to protect the netting from the abrasion caused by the high water pressure cleaner disks used to clean the nets *in situ*. In real working conditions, the action of the cleaner disks have detrimental effects on the integrity of the netting. In addition the concentration of the polyurethane dilution used in this work, the one usually applied in the industry at the moment of conducting the experiment, did not seem to provide enough degree of protection. Consequently, under these conditions, the higher cleaning frequency the more damage caused to the nets. To solve this, several alternatives are currently being explored. Polyurethane coatings are being used in a less diluted or even non-diluted manner and in occasions several layers of the product are applied onto the netting instead of the single layer in this work. This increases the cost of the nets but enlarges their lifespan. Furthermore other cleaning alternatives to perform on-site cleaning such as cavitation, low pressure cleaning and the use of polycations enzymes, nanomaterials and photoactive agents (Banerjee et al., 2011) are being investigated.

According to the obtained results, the hygienic status of the nets can be significantly associated to the presence of parasites and bacteria in the surrounding medium and thus compromising animals' health. Apart from preventing fouling formation the action of copper as a biocide can also play a role in preventing the proliferation of such organisms. In this work, fish in those experimental groups supporting a higher amount of fouling in the net panels presented gill parasites and a higher skin microbiota richness which is not bad as such, but suggests a relationship between fouling and bacteria as already suggested for parasites. A more suitable environment for bacteria growth could allow some pathogenic or opportunistic bacteria to reach injured or immunosuppressed fish increasing disease risk. As mentioned right above the suggestion of several authors stating the relationship between fouling and fish parasites was confirmed in this study by the observation of

the presence of the blood sucker *Sparicotyle chrysophrii* only in the gills of C2 and C3 fish. This finding was especially noticeable as it has been reported that this parasite is scarcely found in pond culture (Sitjà-Bobadilla et al., 2010; Sitjà-Bobadilla and Alvarez-Pellitero, 2009).

To keep a relatively low load of fouling, cleaning operations need to be performed today as often as twice a month in average, but this rate is not always achieved due to resources availability and high costs. As a consequence fish gill health is negatively affected. The results of this work directly correlate on-site cleaning practices with gill damage. Significant differences were established between groups when analysing gill status at the end of the experiment. Very likely the damage was not caused by the action of cleaning itself, but by the large amount of organic matter washed off the nets during cleaning operations. Again, rapid re-growth rate of fouling requires a very high cleaning frequency which is not always achieved due to physic limitations. For instance, during seasons with high fouling pressure nets should probably had to be cleaned weekly which in some cases the available resources make it impossible. If the necessary frequency is not reached, fouling accumulates and when it is eventually removed from net panels a thick cloud of debris is generated in and around the cages which most of the times passes through the gills of the animals causing severe gill alterations. This cleaning action had effects at different levels, from tissue damage and cellular response to gene expression. In those groups exposed to on-site cleaning conditions, gills presented physical lesions, inflammation, immune cells mobilization and additionally the expression of inflammatory genes in gills and spleen was slightly upregulated as well as for the *igm* expression (in C3).

In fish farming, a good feeding management is a crucial factor for production efficiency. In the case of sea bream, feed cost represents more than 55% of COGS. A regular feeding pattern is important to ensure a good growth rate, and often regularity is something not easy to achieve and difficult to maintain as many farm operations affect feeding behaviour. Thus, cage operations, often imply the modification of feeding pattern in cages. In most cases fish do not eat the day that the net, in which they are contained, is changed on cleaned. According to the cleaning frequency required, nowadays a batch of fish should be disturbed more than 30 along the growth cycle as a consequence of on-site cleaning, most of them in summer under high temperatures and good growth rates (when fouling production more rapidly occurs). This constitutes another limiting factor for this alternative.

In this experiment, in terms of growth the groups exposed to on-site cleaning showed a poorer growing performance compared to the group reared under the use of antifouled nets, suggesting a possible impact of the cleaning operation in growth. Probably the main part of this lack of performance occurred as a consequence of the disturbance in the regular feeding pattern. Animals were not fed the day in which nets were cleaned and the feeding response after the on-site cleaning episodes was very poor, even when the meal was delivered the next day. The growth achieved in the experiment was contrasted with a model developed from actual data coming from batches reared and harvested in commercial cages in the area. Even in C1, where fish were not exposed to any cage operation until the end of the experiment, growth was below (-6% in weight, -11% in SGR) than expected according to the model. Several reasons could explain that. On one hand, model results are not always achieved as they are rather optimistic since they are conceived as an objective to pursue, which also happens in fish farming. On the other hand, the fact that the trial was conducted in a shallow pond, less than 2 meters deep, originated a high daily and seasonal temperature variations profile which negatively affected fish intake. Moreover, during a substantial part of the experiment, the animals underwent temperatures below 14ºC **(Figure 55)** which is a temperature where sea bream displays very poor growth rates and feeding appetite (see annex VII). Occasionally temperatures below 12ºC were attained. The latter is the threshold below which winter disease syndrome appears in sea bream (Ibarz et al., 2010).

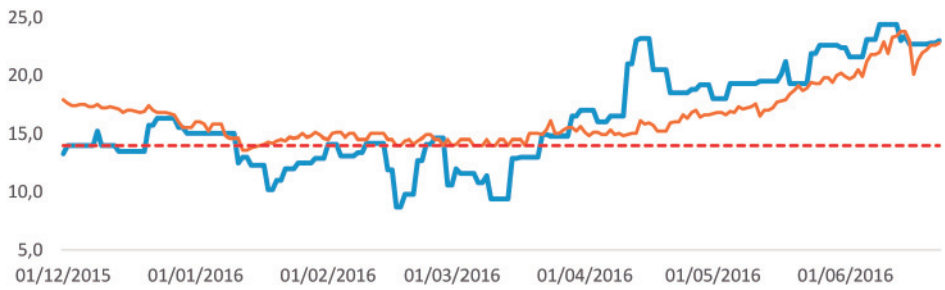


Figure 55. Temperature profile of the experimental pond compared to seawater temperature in the experimental area.

Stapled line represents 14ºC, temperature below which growth rates in sea bream are very poor according to authors' experience. Temperature of 12ºC is reported as the threshold below which winter disease appears in sea bream (Ibarz et al., 2010).

From the welfare point of view the outcome data from the experiment did not show major differences between the two strategies. The general impression was that in both cases fish were exposed to likely suboptimal conditions that moderately activated primary and the subsequent secondary responses. In the group exposed to the use of antifouling, chronic stress seemed to induce more significant responses, although fish apparently acclimated and performed well, whereas in the groups exposed to on-site cleaning the intensity of the chronic stress situation was lower, but the high degree of disturbance caused by cage operations impacted on fish welfare in several ways. The mild chronic stress could have been produced by several factors, including the above mentioned high temperature variation, but many other factors such as predator's presence (bigger fish and birds) or pathogens. Probably the high individual variations and the small differences between treatments did not help in making evident little differences or correlations among the parameters analysed either in plasma or skin mucus. The stress reaction to the cage operation was moderate in both cases, probably modulated by the fact that cortisol levels were chronically elevated.

Despite the fact that blood biochemistry parameters did not show any abnormality, the significant decrease in the circulating triglycerides in all conditions was relevant. Base line values at the beginning of the experiment were exceptionally elevated, and this made even more evident the decrease. As mentioned above, the low average temperature during the experiment, 16.21ºC, (commercial sea bream batches in Spain are reared in an average temperature around 19ºC) and the high temperature variation conditioned a kind of irregular feeding pattern of fish with a subsequent low feeding rate compared to farming conditions. This very probably promoted reserves mobilization which would agree with the reduction of the HSI during the experiment. However, the fact that the growth obtained in C1 was only 6% below than expected opens the door to the possibility of an existing over feeding during winter months in seabream farms. In that case, the animals would accumulate fat instead of dedicating energy to grow.

Apart from the cost, another reason for the industry to move to a new fouling management strategy is the use of copper. The results of this trial showed that after seven months of rearing, although copper accumulation significantly increased in tissues, especially but not exclusively in liver in C1, the recorded amounts of the mineral were below not only the safety recommendations but also the data reported for wild fish, thus not threatening neither fish nor consumers' health.

After having seen that in terms of welfare and stress it can be concluded that there are no major significant differences between both strategies, a first conclusion from the biological point of view would be that under the current state of the art, on-site cleaning not only do not offer any substantial improvement of the rearing conditions but also have several negative effects. The existing technology requires nowadays an undesirable high cleaning frequency from all points of view, to avoid fouling accumulation. If this frequency is achieved, cages are exposed to a tremendously elevated number of disturbances that can easily affect fish feeding pattern and in addition, due to the poor protection provided by the current applied coating procedures, nets' tensile properties become severely compromised as a consequence of the abrasion produced by high water pressure, thus reducing their lifespan and putting stock in risk. If on the contrary, as usually happens, the high cleaning frequency required is not performed, cages hold substantial amounts of fouling which promote bacteria and parasites proliferation. Moreover, when eventually this fouling is washed off the nets the thick cloud of debris severely damages fish gills when passes through them.

On top of that, when approaching the situation from a cost-benefit point of view, and excluding the negative impacts that on-site cleaning might have of fish and nets performance, the obvious advantages of on-site cleaning become countered as a consequence of the repeatedly mentioned high cleaning frequency necessary to keep nets clean.

As a concluding remark it could be stated that a technological improvement is necessary a technology improvement to ensure a better cleaning efficiency, allowing a reduction of the cleaning frequency necessary to maintain nets free of fouling. This would, on one hand reduce the impact on net integrity, thus enlarging the life span of the nets and on the other hand minimize negative effects on fish performance and health. But more important, a reduction of cleaning frequency would mean a reduction of the operation costs which together with the less amount of nets and time required to perform cleaning, would result in a much more profitable solution to keep fouling under control. The intermediate alternative tested in this work appears to be the most promising alternative as it conceals both major objectives of the industry when looking for an alternative to the current practices of fouling control, therefore, under our point of view it clearly deserves further research. A step beyond to this thesis would be to explore which are the best procedures to apply this intermediate solution and challenge it in real framework against the traditional strategy of use of copper in a full growth cycle to better contrast the effects of both. It would be also very interesting

to perform the analysis already done in this work in samples from fish reared in actual commercial cages under each one of the strategies and this is pointed as a future direction in this investigation to conduct both in sea bream and also in salmon as flagship species for the aquaculture industry worldwide.

There are scenarios in the aquaculture industry in which, if economically sustainable, on- site cleaning supposes a clear advantage compared to net changing due to the complexity involved into the operations of net replacing. Today, the tendency in the salmon industry is to concentrate production in massive offshore cages to reduce production costs. These are fully automatized systems run by a few people and are a clear example of this tendency, since replacing such big net panels in offshore conditions, supposes a very difficult task.

Very likely both net management strategies will co-exist in the near future while efforts are put into developing new designs and technologies capable to provide a reliable and sustainable fouling control solution from all points of view.

Conclusions

1. In real working conditions, as in the present work, the antifouling paint and net coating tested in this experiment conferred netting protection.
2. In this work, the net coatings used to avoid abrasion caused by the existing *in situ* cleaning technology did not provide enough degree of protection. Less diluted anti-fouling solutions or multiple layers should be used to protect the nets.
3. The intermediate alternative presented in this work (C2) emerged as an interesting option and deserves further research. It reduces the amount of copper used in antifouling control, and if adequately applied on nets (less diluted, more layers) can confer an acceptable degree of protection against high water pressure abrasion. The use of this alternative would suppose a reduction in the net cleaning frequency without compromising gills integrity and fish health, would reduce fish disturbance, would enlarge the use of nets and limit the damage caused in nets. All this would be translated in better profit margins for aquaculture companies.
4. Although the rearing conditions and different cage operations

did not suppose major differences among fish reared under one or another strategy in terms of welfare impairment and stress primary and secondary responses, the current exposure to the on-site cleaning strategy negatively affected fish performance and health.

5. The current state of the art do not offers a complete efficiency of the on-site cleaning strategy as it allows a fast re-growth rate of the fouling, thus making necessary a high cleaning frequency.
6. On-site cleaning operation disrupts animals feeding pattern. This is an especially negative consequence considering the high cleaning frequency needed nowadays.
7. It has been observed in this work that if not performed as often as necessary, the on-site cleaning strategy involves fouling accumulation in nets. When it is washed off, often debris passes through the gills producing severe damage.
8. Fouling accumulation can be significantly correlated with the presence of bacteria and gill parasites, the latter affecting fish growth.
9. Accumulated levels of copper in tissues after seven months of rearing cycle existed, but were very low and not relevant in terms of fish health and consumers safety. Thus, this should not be a constrain for consumers when choosing among aquaculture or wild caught fish.
10. The current cleaning frequency required to maintain a good fouling control through the on-site cleaning strategy supposes the major drawback of this alternative. It means a huge cost increase making insufficient the resources needed that are limited, (net quantity, manpower and operation time) compared to the traditional strategy of net replacing.
11. According to the results of this work, sample size is very important when conducting research on fish in field conditions. Fish individual variability and multifactorial effects occurring naturally make necessary sample size as big as possible in order to identify significant differences among experimental groups.

Future research should be oriented towards the reduction of the cleaning frequency to effectively reduce cost operations and minimize the negative effects on fish performance and nets integrity.

References

Abolofia, J., Asche, F., Wilen, J.E., 2017. The Cost of Lice: Quantifying the Impacts of Parasitic Sea Lice on Farmed Salmon. Mar. Resour. Econ. 32, 000–000. doi:10.1086/691981

Ahmad, K., Azizullah, A., Shama, S., Khattak, M.N.K., 2014. Determination of heavy metal contents in water, sediments, and fish tissues of *Shizothorax plagiostomus* in river Panjkora at Lower Dir, Khyber Pakhtunkhwa, Pakistan. Environ. Monit. Assess. 186, 7357–7366. doi:10.1007/s10661-014-3932-1

Alsafy, M.A.M., 2013. Gill morphology in two Mediterranean Sea fishes of similar feeding preferences: Sea bream (*Sparus aurata* L) and sea bass (*Dicentrarchus labrax*). Vet. Res. Commun. 37, 163–170. doi:10.1007/s11259-013-9561-2

Alvarez-Pellitero, P., 2008. Fish immunity and parasite infections: from innate immunity to immunoprophylactic prospects. Vet. Immunol. Immunopathol. 126, 171–198. doi:10.1016/j.vetimm.2008.07.013

Alves, R.N., Cordeiro, O., Silva, T.S., Richard, N., de Vareilles, M., Marino, G., Di Marco, P., Rodrigues, P.M., Conceiçao, L.E.C., 2010. Metabolic molecular indicators of chronic stress in gilthead seabream (*Sparus aurata*) using comparative proteomics. Aquaculture 299, 57– 66. doi:10.1016/j.aquaculture.2009.11.014

Andersen, R.J., Luu, H.A., Chen, D.Z.X., Holmes, C.F.B., Kent, M.L., Le Blanc, M., Taylor, F.J.R.M., Williams, D.E., 1993. Chemical and biological evidence links microcystins to salmon "netpen liver disease." Toxicon 31, 1315–1323. doi:10.1016/0041-0101(93)90404- 7

Andersson, E., Peixoto, B., Tormenan, V., Takeshi, M., 1995. Evolution of the immunoglobuUn M constant region genes of salmonid fish , rainbow trout (*Oncorhynchus mykiss*) and Arctic charr (*Salvelinus alpinus*): implications concerning divergence time of. Immunogenetics 83373, 312–315. doi:10.1007/BF00172156

Andromeda Group, 2017. Annual IFRS Financial Statment 2016

Ausubel, F.M., 2005. Are innate immune signaling pathways in plants and animals conserved? Nat. Immunol. 6, 973–979. doi:10.1038/ni1253

Bacanskas, L.R., Whitaker, J., Di Giulio, R.T., 2004. Oxidative stress in two populations of killifish (*Fundulus heteroclitus*) with differing contaminant exposure histories. Mar. Environ. Res. 58, 597–601. doi:10.1016/j.marenvres.2004.03.048

Balm, P.H., Pepels, P., Helfrich, S., Hovens, M.L., Bonga, S.E., 1994. Adrenocorticotropic hormone in relation to interrenal function during stress in tilapia (*Oreochromis mossambicus*). Gen. Comp. Endocrinol. doi:10.1006/gcen.1994.1190

Ballester-Lozano, G.F., Benedito-Palos, L., Estensoro, I., Sitjà-Bobadilla, A., Kaushik, S., Pérez- Sánchez, J., 2015. Comprehensive biometric, biochemical and histopathological assessment of nutrient deficiencies in gilthead sea bream fed semi-purified diets. Br. J. Nutr. 114, 713– 726. doi:10.1017/S0007114515002354

Banerjee, I., Pangule, R.C., Kane, R.S., 2011. Antifouling coatings: Recent developments in the design of surfaces that prevent fouling by proteins, bacteria, and marine organisms. Adv. Mater. 23, 690–718. doi:10.1002/adma.201001215

Barton, B.A., 1997. Stress in finfish: Past, present, and future—a historical perspective., in: G. K. Iwama, A. D. Pickering, J.P.S., Schreck, & C.B. (Eds.), Fish Stress and Health in Aquaculture. Cambridge University Press, Cambridge, U.K., pp. 1–34.

Barton, B.A., Ribas, L., Acerete, L., Tort, L., 2005. Effects of chronic confinement on physiological responses of juvenile gilthead sea bream, *Sparus aurata* L., to acute handling. Aquac. Res. 36, 172–179. doi:10.1111/j.1365-2109.2004.01202.x

Basu, N., Todgham, A.E., Ackerman, P.A., Bibeau, M.R., Nakano, K., Schulte, P.M., Iwama, G.K., 2002. Heat shock protein genes and their functional significance in fish. Gene 295, 173–183. doi:10.1016/S0378-1119(02)00687-X

Baxter, E.J., Sturt, M.M., Ruane, N.M., Doyle, T.K., McAllen, R., Harman, L., Rodger, H.D., 2011. Gill damage to Atlantic Salmon (*Salmo salar*) caused by the common jellyfish (*Aurelia aurita*) under experimental challenge. PLoS One 6, 4–9. doi:10.1371/journal.pone.0018529

Beck, B.H., Peatman, E., 2015. Mucosal Health in Aquaculture Edited by. doi:10.1016/B978-0- 12-417186-2/00014-5

Benedito-Palos, L., Ballester-Lozano, G.F., Simó, P., Karalazos, V., Ortiz, Á., Calduch-Giner, J., Pérez-Sánchez, J., 2016. Lasting effects of butyrate and low FM/FO diets on growth performance, blood haematology/biochemistry and molecular growth-related markers in gilthead sea bream (*Sparus aurata*). Aquaculture 454, 8–18. doi:10.1016/j.aquaculture.2015.12.008

Bervoets, L., Knapen, D., De Jonge, M., Van Campenhout, K., Blust, R., 2013. Differential Hepatic Metal and Metallothionein Levels in Three Feral Fish Species along a Metal Pollution Gradient. PLoS One 8, 1–11. doi:10.1371/journal.pone.0060805

Beveridge, M.C.M., 2004. Cage Aquaculture, Third Edition 361–368.

Bi, C.W., Zhao, Y.P., Dong, G.H., 2015. Numerical study on the hydrodynamic characteristics of biofouled full-scale net cage. China Ocean Eng. 29, 401–414. doi:10.1007/s13344-015- 0028-9

Bi, C.W., Zhao, Y.P., Dong, G.H., Zheng, Y.N., Gui, F.K., 2014. A numerical analysis on the hydrodynamic characteristics of net cages using coupled fluid-structure interaction model. Aquac. Eng. 59, 1–12. doi:10.1016/j.aquaeng.2014.01.002

Blanchard, J., Grosell, M., 2006. Copper toxicity across salinities from freshwater to seawater in the euryhaline fish *Fundulus heteroclitus*: Is copper an ionoregulatory toxicant in high salinities? Aquat. Toxicol. 80, 131–139. doi:10.1016/j.aquatox.2006.08.001

Blancheton, J.P., 2000. Developments in recirculation systems for Mediterranean fish species. Aquac. Eng. 22, 17–31. doi:10.1016/S0144-8609(00)00030-3

Bloecher, N., Olsen, Y., Guenther, J., 2013. Variability of biofouling communities on fish cage nets: A 1-year field study at a Norwegian salmon farm. Aquaculture 416–417, 302–309. doi:10.1016/j.aquaculture.2013.09.025

Bøgwald, J., Dalmo, R.A., 2014. Gastrointestinal Pathogenesis in Aquatic Animals. Aquac. Nutr. 53–74. doi:10.1002/9781118897263.ch3

Bosch-Belmar, M., Giomi, F., Rinaldi, A., Mandich, A., Fuentes, V., Mirto, S., Sarà, G., Piraino, S., 2016a. Concurrent environmental stressors and jellyfish stings impair caged European sea bass (*Dicentrarchus labrax*) physiological performances. Sci. Rep. 6, 1–9. doi:10.1038/srep27929

Bosch-Belmar, M., M'Rabet, C., Dhaouadi, R., Chalghaf, M., Yahia, M.N.D., Fuentes, V., Piraino, S., Yahia, O.K.D., 2016b. Jellyfish stings trigger gill disorders and increased mortality in farmed *Sparus aurata* (linnaeus, 1758) in the mediterranean sea. PLoS One 11, 1–12. doi:10.1371/journal.pone.0154239

Braithwaite, R.A., Carrascosa, M.C.C., McEvoy, L.A., 2007. Biofouling of salmon cage netting and the efficacy of a typical copper-based antifoulant. Aquaculture 262, 219–226. doi:10.1016/j.aquaculture.2006.11.027

Buiakova, O.I., Xu, J., Lutsenko, S., Zeitlin, S., Das, K., Das, S., Ross, B.M., Mekios, C., Scheinberg, I.H., Gilliam, T.C., 1999. Null mutation of the murine ATP7B (Wilson disease) gene results in intracellular copper accumulation and late-onset hepatic nodular transformation. Hum. Mol. Genet. 8, 1665–1671. doi:10.1093/hmg/8.9.1665

Bush, A.O., Lafferty, K.D., Lotz, J.M., Shostak, A.W., 1997. Parasitology Meets Ecology on Its Own Terms: Margolis et al. Revisited. J. Parasitol. 83, 575. doi:10.2307/3284227

Cannon, W.B., 1915. Bodily Changes in Pain, Hunger, Fear and Rage. D. Appleton & Company, New York.

Carl B. Schreck, Lluís Tort, Anthony P. Farrell, C.J.B., 2016. Biology of Stress in Fish, in: Brauner, E. by A.P.F. and C.J., Randall, H.E.W.S.H. and D.J. (Eds.), . Academic Press is an imprint of Elsevier, Oxford, U.K., p. iii. doi:10.1016/B978-0-12-802728-8.00016-3

Caruso, G., Genovese, L., Maricchiolo, G., Modica, a, 2005. Haematological, biochemical and immunological parameters as stress indicators in *Dicentrarchus labrax* and *Sparus aurata* farmed in off-shore cages. Aquac. Int. 13, 67–73. doi:10.1007/s10499-004-9031-5

Casado-Martinez, M.C., Smith, B.D., Rainbow, P.S., 2013. Assessing metal bioaccumulation from estuarine sediments: Comparative experimental results for the polychaete Arenicola marina. J. Soils Sediments 13, 429–440. doi:10.1007/s11368-012-0611-0

Chalmers, L., Taylor, J.F., Roy, W., Preston, A.C., Migaud, H., Adams, A., 2017. A comparison of disease susceptibility and innate immune response between diploid and triploid Atlantic salmon (*Salmo salar*) siblings following experimental infection with *Neoparamoeba perurans*, causative agent of amoebic gill disease. Parasitology 144, 1229–1242. doi:10.1017/S0031182017000622

Chandroo, K.P., Yue, S., Moccia, R.D., 2004. An evaluation of current perspectives on consciousness and pain in fishes. Fish Fish. 5, 281–295. doi:10.1111/j.1467-2679.2004.00163.x

Chatzifotis, S., Kokou, F., Ampatzis, K., Papadakis, I.E., Divanach, P., Dermon, C.R., 2008.

Effects of dietary caffeine on growth, body composition, somatic indexes, and cerebral distribution of acetyl-cholinesterase and nitric oxide synthase in gilthead sea bream (*Sparus aurata*), reared in winter temperature. Aquac. Nutr. 14, 405–415. doi:10.1111/j.1365-2095.2007.00541.x

Chipman, N.A.L.T.D.W.J.K., 2006. Functional Analysis of a Metal Response Element in the Regulatory Region of Flounder Cytochrome P450 1A and Implications for Environmental Monitoring of Pollutants. Toxicol. Sci. 92, 387–393.

Cockell, K.A., Bertinato, J., L'Abbé, M.R., 2008. Regulatory frameworks for copper considering chronic exposures of the population. Am. J. Clin. Nutr. 88, 863–866.

Costantini, D., 2008. Oxidative stress in ecology and evolution: Lessons from avian studies. Ecol. Lett. 11, 1238–1251. doi:10.1111/j.1461-0248.2008.01246.x

Conte, F.S., 2004. Stress and the welfare of cultured fish, in: Applied Animal Behaviour Science. Elsevier, pp. 205–223. doi:10.1016/j.applanim.2004.02.003

Contessi, B., Volpatti, D., Gusmani, L., Galeotti, M., 2006. Evaluation of immunological parameters in farmed gilthead sea bream, *Sparus aurata* L., before and during outbreaks of "winter syndrome." J. Fish Dis. 29, 683–690. doi:10.1111/j.1365-2761.2006.00765.x

Cordeiro, O.D., Silva, T.S., Alves, R.N., Costas, B., Wulff, T., Richard, N., de Vareilles, M., Conceição, L.E.C., Rodrigues, P.M., 2012. Changes in Liver Proteome Expression of Senegalese Sole (*Solea senegalensis*) in Response to Repeated Handling Stress. Mar. Biotechnol. 14, 714–729. doi:10.1007/s10126-012-9437-4

Council of European Union, 2016. Commission Implementing Regulation (Eu) 2016/1095 2016.

Council of European Union, 1998. Council Directive 98/58/EC of 20 July 1998 concerning the protection of animals kept for farming purposes. Off. J. Eur. Communities 23–27.

Cuesta, A., Laiz-Carrión, R., Arjona, F., Del Rio, M.P.M., Meseguer, J., Mancera, J.M., Esteban, Mª Ángeles, 2006. Effect of PRL, GH and cortisol on the serum complement and IgM levels in gilthead seabream (*Sparus aurata* L.). Fish Shellfish Immunol. 20, 427–432. doi:10.1016/j.fsi.2005.05.010

Cuesta, A., Meseguer, J., Esteban, M.A., 2004. Total serum immunoglobulin M levels are affected by immunomodulators in seabream (*Sparus aurata* L.). Vet. Immunol. Immunopathol. 101, 203–210. doi:10.1016/j.vetimm.2004.04.021

Dalum, A.S., Austbø, L., Bjørgen, H., Skjødt, K., Hordvik, I., Hansen, T., Fjelldal, P.G., Press, C.M., Griffiths, D.J., Koppang, E.O., 2015. The interbranchial lymphoid tissue of Atlantic Salmon (*Salmo sala*r L) extends as a diffuse mucosal lymphoid tissue throughout the trailing edge of the gill filament. J. Morphol. 276, 1075–1088. doi:10.1002/jmor.20403

Dawkins, M.S., 2012. Why animals matter, Ethics and Animals An Introduction. Oxford University Press.

Deane, E., Li, J., Woo, N., 2004. Modulated heat shock protein expression during pathogenic Vibrio alginolyticus stress of sea bream. Dis. Aquat. Organ. 62, 205–215. doi:10.3354/dao062205

De Vico, G., Cataldi, M., Carella, F., Marino, F., Passantino, A., 2008. Histological, histochemical and morphometric changes of splenic melanomacrophage centers (SMMCs) in Sparicotyle-infected cultured sea breams (*Sparus aurata*). Immunopharmacol. Immunotoxicol. 30, 27–35. doi:10.1080/08923970701812290

Dezfuli, B.S., Giari, L., 2008. Mast cells in the gills and intestines of naturally infected fish: Evidence of migration and degranulation. J. Fish Dis. 31, 845–852. doi:10.1111/j.1365- 2761.2008.00961.x

Dezfuli, B.S., Giari, L., Lui, A., Lorenzoni, M., Noga, E.J., 2011. Mast cell responses to Ergasilus (Copepoda), a gill ectoparasite of sea bream. Fish Shellfish Immunol. 30, 1087–1094. doi:10.1016/j.fsi.2011.02.005

Dezfuli, B.S., Squerzanti, S., Fabbri, S., Castaldelli, G., Giari, L., 2010. Cellular response in semi- intensively cultured sea bream gills to *Ergasilus sieboldi* (Copepoda) with emphasis on the distribution, histochemistry and fine structure of mucous cells. Vet. Parasitol. 174, 359–365. doi:10.1016/j.vetpar.2010.08.024

Dhabhar, F.S., 2014. Effects of stress on immune function: The good, the bad, and the beautiful. Immunol. Res. 58, 193–210. doi:10.1007/s12026-014-8517-0

Dhabhar, F.S., 2008. Enhancing versus Suppressive Effects of Stress on Immune Function : Implications for Immunoprotection versus. Allergy, Asthma, Clin. Immunol. 4, 2–11. doi:10.2310/7480.2008.00001

Dhabhar, F.S., 2002. Stress-induced augmentation of immune function - The role of stress hormones, leukocyte trafficking, and cytokines. Brain. Behav. Immun. 16, 785–798. doi:10.1016/S0889-1591(02)00036-3

Dhabhar, F.S., 2000. Acute Stress Enhances While Chronic Stress Suppresses Skin Immunity: The Role of Stress Hormones and Leukocyte Trafficking. Ann. N. Y. Acad. Sci. 917, 876– 893. doi:10.1111/j.1749-6632.2000.tb05454.x

Dhabhar, F.S., Mcewen, B.S., 1997. Acute Stress Enhances while Chronic Stress Suppresses Cell- Mediated Immunityin Vivo:A Potential Role for Leukocyte Trafficking. Brain. Behav. Immun. 11, 286–306. doi:10.1006/brbi.1997.0508

Dhabhar, F.S., Miller, A.H., Mcewen, B.S., Spencer, R.L., 1995. Effects of Stress on Immune Cell Distribution.

Donaldson, E.M., 1981. The pituitary-interrenal axis as an indicator of stress in fish., in: Stress and Fish. Academic Press, New York, pp. 11–47.

Douglas-Helders, G.M., Tan, C., Carson, J., Nowak, B.F., 2003. Effects of copper-based antifouling treatment on the presence of *Neoparamoeba pemaquidensis* Page, 1987 on nets and gills of reared Atlantic salmon (*Salmo salar*). Aquaculture 221, 13–22. doi:10.1016/S0044-8486(02)00517-3

Dürr, S., Watson, D.I., 2010. Biofouling and Antifouling in Aquaculture. Biofouling 267–287. doi:10.1002/9781444315462.ch19

Edwards, C.D., Pawluk, K.A., Cross, S.F., 2015. The effectiveness of several commercial antifouling treatments at reducing biofouling on finfish aquaculture cages in British Columbia. Aquac. Res. 46, 2225–2235. doi:10.1111/are.12380

Ekman, D.R., Skelton, D.M., Davis, J.M., Villeneuve, D.L., Cavallin, J.E., Schroeder, A., Jensen, K.M., Ankley, G.T., Collette, T.W., 2015. Metabolite profiling of fish skin mucus: A novel approach for minimally-invasive environmental exposure monitoring and surveillance. Environ. Sci. Technol. 49, 3091–3100. doi:10.1021/es505054f

Ellis, A.E., 1990. Lysozyme Assays, in: Stolen, J.S., Fletcher, T.C., Anderson, D.P., Roberson,

B.S. and Van Muiswinkel, W.B., E. (Ed.), Techniques in Fish Immunology. Fair Haven, SOS Publications, pp. 101–113.

Esteban, M.A., Cuesta, A., Ortuño, J., Meseguer, J., 2001. Immunomodulatory effects of dietary intake of chitin on gilthead seabream (*Sparus aurata* L.) innate immune system. Fish Shellfish Immunol. 11, 303–15. doi:10.1006/fsim.2000.0315.

European Commission, 2006. Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. Off. J. Eur. Communitites L364, 5–24. doi:10.2203/dose-response.06-012. Hanekamp

Evans, P., Halliwell, B., 2001. Micronutrients: oxidant/antioxidant status. Br. J. Nutr. 85, S67. doi:10.1049/BJN2000296

EY, 2017. The Norwegian Aquaculture Analysis 2016.

FAO, 2016. the State of World Fisheries and Aquaculture.

Feidantsis, K., Pörtner, H.O., Lazou, A., Kostoglou, B., Michaelidis, B., 2009. Metabolic and molecular stress responses of the gilthead seabream *Sparus aurata* during long-term exposure to increasing temperatures. Mar. Biol. 156, 797–809. doi:10.1007/s00227-009- 1135-z

Fernandes, D., Bebianno, M.J., Porte, C., 2009. Assessing pollutant exposure in cultured and wild sea bass (*Dicentrarchus labrax*) from the Iberian Peninsula. Ecotoxicology 18, 1043–1050. doi:10.1007/s10646-009-0368-4

Finkel, T., Holbrook, N.J., 2000. Oxidants, oxidative stress and the biology of ageing. Nature 408, 239–247. doi:10.1038/35041687

Fitridge, I., Dempster, T., Guenther, J., de Nys, R., 2012. The impact and control of biofouling in marine aquaculture: a review. Biofouling 28, 649–69. doi:10.1080/0 8927014.2012.700478

Flos, R., Reig, L., Torres, P., Tort, L., 1988. Primary and secondary stress responses to grading and hauling in rainbow trout, *Salmo gairdneri*. Aquaculture 71, 99–106. doi:10.1016/0044- 8486(88)90277-3

Fraser, D., Weary, D.M., Pajor, E.A., Milligan, B.N., 1997. A scientific concept of animal welfare that reflects ethical concerns. Anim. Welf. 6, 187–205.

Fredriksson, D.W., DeCew, J.C., Tsukrov, I., 2007. Development of structural modeling techniques for evaluating HDPE plastic net pens used in marine aquaculture. Ocean Eng. 34, 2124–2137. doi:10.1016/j.oceaneng.2007.04.007

Ghedira, J., Jebali, J., Bouraoui, Z., Banni, M., Guerbej, H., Boussetta, H., 2010. Metallothionein and metal levels in liver, gills and kidney of *Sparus aurata* exposed to sublethal doses of cadmium and copper. Fish Physiol. Biochem. 36, 101–107. doi:10.1007/s10695-008-9295- 1

González, L., 1998. The life cycle of Hysterothylacium aduncum (Nematoda: Anisakidae) in Chilean marine farms. Aquaculture 162, 173–186. doi:10.1016/ S0044-8486(97)00303-7

Greene, J.K., Grizzle, R.E., 2007. Successional development of fouling communities on open ocean aquaculture fish cages in the western Gulf of Maine, USA. Aquaculture 262, 289– 301. doi:10.1016/j.aquaculture.2006.11.003

Geist, J., Werner, I., Eder, K.J., Leutenegger, C.M., 2007. Comparisons of tissue-specific transcription of stress response genes with whole animal endpoints of adverse effect in striped bass (*Morone saxatilis*) following treatment with copper and esfenvalerate. Aquat. Toxicol. 85, 28–39. doi:10.1016/j.aquatox.2007.07.011

Grieg Seafood, 2017. Grieg Seafood Annual Report 2016.

Griffitt, R.J., Hyndman, K., Denslow, N.D., Barber, D.S., 2009. Comparison of molecular and histological changes in zebrafish gills exposed to metallic nanoparticles. Toxicol. Sci. 107, 404–415. doi:10.1093/toxsci/kfn256

Grinde, B., 1989. Lysozyme from rainbow trout, *Salmo gairdneri* Richardson, as an antibacterial agent against fish pathogens. J. Fish Dis. 12, 95–104. doi:10.1111/j.1365- 2761.1989.tb00281.x

Grosell, M., 2011. Copper 31, 53–133. doi:10.1016/S1546-5098(11)31002-3

Grosell, M., Blanchard, J., Brix, K. V., Gerdes, R., 2007. Physiology is pivotal for interactions between salinity and acute copper toxicity to fish and invertebrates. Aquat. Toxicol. 84, 162–172. doi:10.1016/j.aquatox.2007.03.026

Grosell, M., McDonald, M.D., Walsh, P.J., Wood, C.M., 2004. Effects of prolonged copper exposure in the marine gulf toadfish (*Opsanus beta*) II: Copper accumulation, drinking rate and Na+/K +-ATPase activity in osmoregulatory tissues. Aquat. Toxicol. 68, 263–275. doi:10.1016/j.aquatox.2004.03.007

Grosell, M., Wood, C.M., 2002. Copper uptake across rainbow trout gills: mechanisms of apical entry. J. Exp. Biol. 205, 1179–1188.

Grosell, M., Wood, C.M., Walsh, P.J., 2003. Copper homeostasis and toxicity in the elasmobranch Raja erinacea and the teleost *Myoxocephalus octodecemspinosus* during exposure to elevated water-borne copper. Comp. Biochem. Physiol. - C Toxicol. Pharmacol. 135, 179–190. doi:10.1016/S1532-0456(03)00089-9

Guardiola, F.A., Cuesta, A., Esteban, Mª Ángeles, 2016. Using skin mucus to evaluate stress in gilthead seabream (*Sparus aurata* L.). Fish Shellfish Immunol. 59, 323–330. doi:10.1016/j.fsi.2016.11.005

Guardiola, F.A., Cuesta, A., Arizcun, M., Meseguer, J., Esteban, M.A., 2014. Comparative skin mucus and serum humoral defence mechanisms in the teleost gilthead seabream (*Sparus aurata*). Fish Shellfish Immunol. 36, 545–551. doi:10.1016/j.fsi.2014.01.001

Guenther, J., Misimi, E., Sunde, L.M., 2010. The development of biofouling, particularly the hydroid *Ectopleura larynx*, on commercial salmon cage nets in Mid-Norway. Aquaculture 300, 120–127. doi:10.1016/j.aquaculture.2010.01.005

Hall, L.W., Anderson, R.D., 1995. The Influence of Salinity on the Toxicity of Various Classes ofChemicals to Aquatic Biota. Crit. Rev. Toxicol. 25, 281–346. doi:10.3109/10408449509021613

Halliwell, B., Gutteridge, J.M.C., 1984. Free Radicals, Lipid Peroxidation, and Cell Damage. Lancet. doi:10.1016/S0140-6736(84)91530-7

Haugarvoll, E., Bjerkås, I., Nowak, B.F., Hordvik, I., Koppang, E.O., 2008. Identification and characterization of a novel intraepithelial lymphoid tissue in the gills of Atlantic salmon. J. Anat. 213, 202–209. doi:10.1111/j.1469-7580.2008.00943.x

Hedley, C., Huntington, T., 2009. Regulatory and legal constraints for European aquaculture 102 pp.

Henry, M.A., Nikoloudaki, C., Tsigenopoulos, C., Rigos, G., 2015. Strong effect of long-term *Sparicotyle chrysophrii* infection on the cellular and innate immune responses of gilthead sea bream, *Sparus aurata*. Dev. Comp. Immunol. 51, 185–193. doi:10.1016/j.dci.2015.03.010

Hernández, A. and Tort, L., 2003. Annual variation of complement, lysozyme and haemagglutinin levels in serum of the gilthead sea bream *Sparus aurata*. Fish Shellfish Immunol. 15, 479– 481. doi:10.1016/S1050-4648(03)00024-X

Hernández, A. and Tort, L., 2003. Annual variation of complement, lysozyme and haemagglutinin levels in serum of the gilthead sea bream *Sparus aurata*. Fish Shellfish Immunol. 15, 479– 481. doi:10.1016/S1050-4648(03)00024-X

Hil, M. a T., Vi, A.M., 1998. Heat shock protein expression in fish. Rev. Fish Biol. Fish. 56. doi:10.1023/a:1008812500650

Hodson, S.L., Lewis, T.E., Burke, C.M., 1997. Biofouling of fish-cage netting: Efficacy and problems of in situ cleaning. Aquaculture 152, 77–90. doi:10.1016/S0044-8486(97)00007- 0

Hofmann, G.E., 2005. Patterns of Hsp gene expression in ectothermic marine organisms on small to large biogeographic scales. Integr. Comp. Biol. 45, 247–255. doi:10.1093/icb/45.2.247

Hole, W., 1952. Marine Fouling and Its Prevention Bureau of Ships , Navy Department. Holland, M.C.H., Lambris, J.D., 2002. The complement system in teleosts. Fish Shellfish

Immunol. 12, 399–420. doi:10.1006/fsim.2001.0408

Hopkins, K.D., 1992. Reporting Fish Growth: A Review of the Basics. J. World Aquac. Soc. 23, 173–179. doi:10.1111/j.1749-7345.1992.tb00766.x

Huntingford, F. a., 2006. Issues in Fish Welfare 44, 332–372. doi:10.1111/j.1095-8649.2005.01046.x

Ibarz, A., Padrós, F., Gallardo, M.Á., Fernández-Borràs, J., Blasco, J., Tort, L., 2010. Low- temperature challenges to gilthead sea bream culture: Review of cold-induced alterations and "Winter Syndrome." Rev. Fish Biol. Fish. 20, 539–556. doi:10.1007/s11160-010-9159- 5

IKamunde, C., Grosell, M., Higgs, D., Wood, C.M., 2002. Copper metabolism in actively growing rainbow trout (*Oncorhynchus mykiss*): interactions between dietary and waterborne copper uptake. J. Exp. Biol. 205, 279–290.

IMO, 2002. Focus on IMO: Anti-fouling systems. Int. Marit. Organ. 44, 1–31. doi:10.1097/TA.0b013e31817de3f4

Iwama, G.K., Pickering, A.D., Sumpter, J.P., Schreck, C.B., 1997. Fish Stress and Health in Aquaculture. Society for Experimental Biology Seminar Series, vol. 62. Cambridge University Press, UK.

Iwama, G.K., Vijayan, M.M., Forsyth, R., Ackerman, P. a, 1999. Heat Shock Proteins and Physiological Stress in Fish. Am. Zool. 39, 901–909.

Iwasaki, A., Medzhitov, R., 2010. Regulation of Adaptive Immunity by the Innate Immune System. Science (80-.). 327, 291–295. doi:10.1126/science.1183021

Jungmann, H.-A. Reins, C. Schobert, S.J., 1993. Resistance to cadmium mediated by ubiquitin- dependent proteolysis. Nature 361, 369–371.

Jensen, P., 2002. The ethology of domestic animals: an introductory text. CABI. doi:10.1079/9780851996028.0000

Kammer, A.R., Orczewska, J.I., O'Brien, K.M., 2011. Oxidative stress is transient and tissue specific during cold acclimation of threespine stickleback. J. Exp. Biol. 214, 1248–1256. doi:10.1242/jeb.053207

Klebert, P., Lader, P., Gansel, L., Oppedal, F., 2013. Hydrodynamic interactions on net panel and aquaculture fish cages: A review. Ocean Eng. 58, 260–274. doi:10.1016/j.oceaneng.2012.11.006

Knox, K.M.G., Reid, S.W.J., Love, S., Murray, M., Gettinby, G., 1998. Application of probability techniques to the objective interpretation of veterinary clinical biochemistry data. Vet Rec. 142, 323–327. doi:https://doi.org/10.1016/S0167-5877(97)00040-8

Kokou, F., Adamidou, S., Karacostas, I., Sarropoulou, E., 2016. Sample size matters in dietary gene expression studies - A case study in the gilthead sea bream (*Sparus aurata* L.). Aquac. Reports 3, 82–87. doi:10.1016/j.aqrep.2015.12.004

Laflamme, J.-S., Couillard, Y., Campbell, P.G.C., Hontela, A., 2000. Interrenal metallothionein and cortisol secretion in relation to Cd, Cu, and Zn exposure in yellow perch, Perca flavescens, from Abitibi lakes. Can. J. Fish. Aquat. Sci. 57, 1692–1700. doi:10.1139/f00- 118

Lambooij, E., Pilarczyk, M., Bialowas, H., van den Boogaart, J.G.M., van de Vis, J.W., 2007.

Electrical and percussive stunning of the common carp (*Cyprinus carpio* L.): Neurological and behavioural assessment. Aquac. Eng. 37, 171–179. doi:10.1016/j.aquaeng.2007.04.004

Lane, A., Willemsen, P., 2004. Collaborative effort looks into biofouling. Fish Farming Int. 44, 34–35.

Lauriano, E.R., Calò, M., Silvestri, G., Zaccone, D., Pergolizzi, S., Lo Cascio, P., 2012. Mast cells in the intestine and gills of the sea bream, Sparus aurata, exposed to a polychlorinated biphenyl, PCB 126. Acta Histochem. 114, 166–171. doi:10.1016/j.acthis.2011.04.004

Lehninger, A. L., Nelson, D. L. 1., & Cox, M.M., 2008. Lehninger principles of biochemistry, 5th ed. New York ; New Delhi.

Lerøy Seafood, L., 2017. Leroy Seafood Annual Report 2016.

Lieschke, G.J., Trede, N.S., 2009. Fish immunology. Curr. Biol. 19, 678–682. doi:10.1016/j.cub.2009.06.068

Linder, M.C., 1991. Chemistry of Copper, 1st ed. Springer US, New York. doi:10.1007/978-1- 4757-9432-8

Magnadottir, B., 2010. Immunological control of fish diseases. Mar. Biotechnol. 12, 361–379. doi:10.1007/s10126-010-9279-x

Marri, V., Richner, H., 2015. Immune response, oxidative stress and dietary antioxidants in great tit nestlings. Comp. Biochem. Physiol. Part A Mol. Integr. Physiol. 179, 192–196. doi:10.1016/j.cbpa.2014.10.013

Martínez-Álvarez, R.M., Morales, A.E., Sanz, A., 2005. Antioxidant defenses in fish: Biotic and abiotic factors. Rev. Fish Biol. Fish. 15, 75–88. doi:10.1007/s11160-005-7846-4

Mauri, I., Romero, A., Acerete, L., MacKenzie, S., Roher, N., Callol, A., Cano, I., Alvarez, M.C., Tort, L., 2011. Changes in complement responses in Gilthead seabream (*Sparus aurata*) and European seabass (*Dicentrarchus labrax*) under crowding stress, plus viral and bacterial challenges. Fish Shellfish Immunol. 30, 182–188. doi:10.1016/j.fsi.2010.10.006

Mazeaud, M.M., Mazeaud, F., Donaldson, E.M., 1977. Primary and Secondary Effects of Stress in Fish: Some New Data with a General Review. Trans. Am. Fish. Soc. 106, 201–212. doi:10.1577/1548-8659(1977)106<201:PASEOS>2.0.CO;2

McGeer, J.C., Szebedinszky, C., McDonald, D.G., Wood., C.M., 2000. Effects of chronic sublethal exposure to waterborne Cu, Cd or Zn in rainbow trout. 1: Iono-regulatory disturbance and metabolic costs. Aquat. Toxicol. 50, 231–243. doi:10.1016/S0166-445X(99)00105-8

McKenna, L.A., Gehrsitz, A., Soder, S., Eger, W., Kirchner, T., Aigner, T., 2000. Effective isolation of high-quality total RNA from human adult articular cartilage. Anal Biochem 286, 80–85. doi:10.1006/abio.2000.4779

Maricchiolo, G., Mirto, S., Caruso, G., Caruso, T., Bonaventura, R., Celi, M., Matranga, V., Genovese, L., 2011. Welfare status of cage farmed European sea bass (*Dicentrarchus labrax*): A comparison between submerged and surface cages. Aquaculture 314, 173–181. doi:10.1016/j.aquaculture.2011.02.001

Marine Harvest, 2017. Marine Harvest Annual Report 2016.

Minganti, V., Drava, G., Pellegrini, R. De, Siccardi, C., 2010. Trace elements in farmed and wild gilthead seabream, *Sparus aurata*. Mar. Pollut. Bull. 60, 2022–2025. doi:10.1016/j.marpolbul.2010.07.023

Minghetti, M., 2009. Characterisation and expression of copper homeostasis genes in sea bream (Sparus aurata). University of Stirling.

Mitchell, S.O., Baxter, E.J., Holland, C., Rodger, H.D., 2012. Development of a novel histopathological gill scoring protocol for assessment of gill health during a longitudinal study in marine-farmed Atlantic salmon (*Salmo salar*). Aquac. Int. 20, 813–825. doi:10.1007/s10499-012-9504-x

Moe, H., Olsen, A., Hopperstad, O.S., Jensen, Ø., Fredheim, A., 2007. Tensile properties for netting materials used in aquaculture net cages. Aquac. Eng. 37, 252–265. doi:10.1016/j.aquaeng.2007.08.001

Mommsen, T.P., Vijayan, M.M., Moon, T.W., 1999. Cortisol in teleosts: Dynamics, mechanisms of action, and metabolic regulation. Rev. Fish Biol. Fish. 9, 211–268. doi:10.1023/A:1008924418720

Monshouwer, M., Hoebe, K.H.N., 2003. Hepatic (dys-)function during inflammation, in: Toxicology in Vitro. Pergamon, pp. 681–686. doi:10.1016/S0887-2333(03)00104-8

Montero, D., Izquierdo, M.S., Tort, L., Robaina, L., Vergara, J.M., 1999a. High stocking density produces crowding stress altering some physiological and biochemical parameters in gilthead seabream, *Sparus aurata*, juveniles. Fish Physiol. Biochem. 20, 53–60. doi:10.1023/A:1007719928905

Montero, D., Marrero, M., Izquierdo, M.S., Robaina, L., Vergara, J.M., Tort, L., 1999b. Effect of vitamin E and C dietary supplementation on some immune parameters of gilthead seabream (*Sparus aurata*) juveniles subjected to crowding stress. Aquaculture 171, 269–278. doi:10.1016/S0044-8486(98)00387-1

Montero, D., Tort, L., Izquierdo, M.S., Robaina, L., Vergara, J.M., 1998. Depletion of serum alternative complement pathway activity in gilthead seabream caused by α-tocopherol and n-3 HUFA dietary deficiencies. Fish Physiol. Biochem. 18, 399–407. doi:10.1023/a:1007734720630

Montero, D., Tort, L.L., Izquierdo, M.S., Socorro, J., Vergara, J.M., Robaina, L., Fernandez- Palacios, H., 1995. Hematological recovery in *Sparus aurata* after bleeding. A time course study. Rev. española Fisiol. 51, 219—226.

Moves, C.D., West, T.G., 1995. Exercise metabolism of fish. Biochem. Mol. Biol. Fishes 4, 367– 392. doi:10.1016/S1873-0140(06)80019-6

Nash, C.E., 2011. The Story of Aquaculture. Blackwell Publishing Ltd., Ames, Iowa : doi:10.1002/9780470958971

Navarro, I., Gutiérrez, J., 1995. Fasting and starvation. Biochem. Mol. Biol. Fishes 4, 393–434. doi:10.1016/S1873-0140(06)80020-2

NOAA, 2017. Sources of copper pollution [WWW Document]. URL https://www.ecologycenter.us/population-dynamics-2/sources-of-copper-pollution.html

Nor, Y.M., 1987. Ecotoxicity of copper to aquatic biota: A review. Environ. Res. 43, 274–282. doi:10.1016/S0013-9351(87)80078-6

O'Gorman, J., Humphreys, H., 2012. Application of copper to prevent and control infection. Where are we now? J. Hosp. Infect. doi:10.1016/j.jhin.2012.05.009

Ortun, J., Esteban, M.A., Meseguer, J., 2001. Effects of short - term crowding stress on the gilthead seabream (*Sparus aurata* L .) innate immune response. Fish Shellfish Immunol. 11, 187–197. doi:10.1006/fsim.2000.0304

Pagés, T., Gómez, E., Súñer, O., Viscor, G., Tort, L., 1995. Effects of daily management stress on haematology and blood rheology of the gilthead seabream. J. Fish Biol. 46, 775–786. doi:10.1111/j.1095-8649.1995.tb01601.x

Parliament. of the United Kingdom, 1986. Animals (Scientific Procedures) Act 1986 Working with animals taken from the wild.

Peres, H., Santos, S., Oliva-Teles, A., 2013. Selected plasma biochemistry parameters in gilthead seabream (*Sparus aurata*) juveniles. J. Appl. Ichthyol. 29, 630–636. doi:10.1111/j.1439-0426.2012.02049.x

Perugini, M., Visciano, P., Manera, M., Zaccaroni, A., Olivieri, V., Amorena, M., 2014. Heavy metal (As, Cd, Hg, Pb, Cu, Zn, Se) concentrations in muscle and bone of four commercial fish caught in the central Adriatic Sea, Italy. Environ. Monit. Assess. 186, 2205–2213. doi:10.1007/s10661-013-3530-7

Pickering, A.D., 1981. Introduction: The concept of biological stress, in: (Ed.)., A.D.P. (Ed.), Stress and Fish. Academic Press, New York, pp. 1–9.

Playle, R.C., Dixon, D.G., Burnison, K., 1993. Copper and Cadmium Binding to Fish Gills: Estimates of Metal–Gill Stability Constants and Modelling of Metal Accumulation. Can. J. Fish. Aquat. Sci. 50, 2678–2687. doi:10.1139/f93-291

Plouffe, D.A., Hanington, P.C., Walsh, J.G., Wilson, E.C., Belosevic, M., 2005. Comparison of select innate immune mechanisms of fish and mammals. Xenotransplantation 12, 266–277. doi:10.1111/j.1399-3089.2005.00227.x

Post, G.B., 1987. Text book of fish disease. THF Publications, Neptune, New Jersey.

Reed, G.B., Spence, C.M., 1929. the Intestinal and Slime Flora of the Haddock: a Preliminary Report. Contrib. to Can. Biol. Fish. 4, 257–264. doi:10.1139/f29-019

Roncarati, A., Melotti, P., Dees, A., Mordenti, O., Angellotti, L., 2006. Welfare status of cultured seabass (*Dicentrarchus labrax* L.) and seabream (*Sparus aurata* L.) assessed by blood parameters and tissue characteristics. J. Appl. Ichthyol. 22, 225–234. doi:10.1111/j.1439- 0426.2006.00741.x

Rose, J.D., 2007. Anthropomorphism and "mental welfare" of fishes. Dis. Aquat. Organ. 75, 139– 154. doi:10.3354/dao075139

Rose, J.D., 2002. The Neurobehavioral Nature of Fishes and the Question of Awareness and Pain. Rev. Fish. Sci. 10, 1–38. doi:10.1080/20026491051668

Rotllant, J., Balm, P.H., Pérez-Sánchez, J., Wendelaar-Bonga, S.E., Tort, L., 2001. Pituitary and interrenal function in gilthead sea bream (*Sparus aurata* L., Teleostei) after handling and confinement stress. Gen. Comp. Endocrinol. 121, 333–342. doi:10.1006/gcen.2001.7604

Rotllant, J., Tort, L., 1997. Cortisol and glucose responses after acute stress by net handling in the sparid red porgy previously subjected to crowding stress. J. Fish Biol. 51, 21–8. doi:10.1111/j.1095-8649.1997.tb02510.x

Sala, A., Lucchetti, A., Buglioni, G., 2004. The change in physical properties of some nylon (PA) netting samples before and after use. Fish. Res. 69, 181–188. doi:10.1016/j.fishres.2004.05.005

Sala-Rabanal, M., Sánchez, J., Ibarz, A., Fernández-Borràs, J., Blasco, J., Gallardo, M.A., 2003. Effects of low temperatures and fasting on hematology and plasma composition of gilthead sea bream (*Sparus aurata*). Fish Physiol. Biochem. 29, 105–115. doi:10.1023/B:FISH.0000035904.16686.b6

Salinas, I., Zhang, Y.A., Sunyer, J.O., 2011. Mucosal immunoglobulins and B cells of teleost fish. Dev. Comp. Immunol. 35, 1346–1365. doi:10.1016/j.dci.2011.11.009

Sanchez, W., Palluel, O., Meunier, L., Coquery, M., Porcher, J.M., Aït-Aïssa, S., 2005. Copper- induced oxidative stress in three-spined stickleback: Relationship with hepatic metal levels. Environ. Toxicol. Pharmacol. 19, 177–183. doi:10.1016/j.etap.2004.07.003

Savan, R., Sakai, M., 2006. Genomics of fish cytokines. Comp. Biochem. Physiol. - Part D Genomics Proteomics 1, 89–101. doi:10.1016/j.cbd.2005.08.005

Schneider, C.A., Rasband, W.S., Eliceiri, K.W., 2012. NIH Image to ImageJ: 25 years of image analysis. Nat. Methods 9, 671–675. doi:10.1038/nmeth.2089
Seyle, H., 1953. The present state of stress conception. Munchener Medizinische Wochenschrift 95, 426–433.
Shelbourne, J.E., 1975. Marine fish cultivation: pioneering studies on the culture of the larvae of the plaice (*Pleuronectes platessa* L.) and the sole (*Solea solea* L.) 29.
Shephard, K.L., 1994. Functions for fish mucus. Rev. Fish Biol. Fish. 4, 401–429. doi:10.1007/BF00042888
Shimada, T., Oda, Y., Gillam, E.M., Guengerich, F.P., Inoue, K., 2001. Metabolic activation of polycyclic aromatic hydrocarbons and other procarcinogens by cytochromes P450 1A1 and P450 1B1 allelic variants and other human cytochromes P450 in *Salmonella typhimurium* NM2009. Drug Metab Dispos 29, 1176–1182.
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. Aquac. Res. 41, 939–944. doi:10.1111/j.1365- 2109.2009.02369.x
Sitjà-bobadilla, A., Alvarez-Pellitero, P., 2009. Experimental transmission of *Sparicotyle chrysophrii* (Monogenea:Polyopisthocotylea) to gilthead seabream (*Sparus aurata*) and histopathology of the infection. Folia Parasitol. (Praha). 56, 143–151.
Sitjà-Bobadilla, A., Mingarro, M., Pujalte, M.J., Garay, E., Alvarez-Pellitero, P., Pérez-Sánchez, J., 2003. Immunological and pathological status of gilthead sea bream (*Sparus aurata* L.) under different long-term feeding regimes. Aquaculture 220, 707–724. doi:10.1016/S0044- 8486(02)00615-4
Stanley, E.C., 1985. What Constitutes Animal Well-Being?, in: Moberg, G.P. (Ed.), Animal Stress. Springer. doi:10.1007/978-1-4614-7544-6_1
Statistics, N., 2016. Annually final figures. Aquaculture [WWW Document]. URL www.ssb.no Stickney, R.R., 2011. A Review of "The History of Aquaculture." Rev. Fish. Sci. 19, 316–319. doi:10.1080/10641262.2011.589541
Sunyer, J.O., 2013. Fishing for mammalian paradigms in the teleost immune system. Nat. Immunol. 14, 320–326. doi:10.1038/ni.2549
Sunyer, J.O., Tort, L., Lambris, J.D., 1997. Structural C3 diversity in fish: characterization of five forms of C3 in the diploid fish Sparus aurata. J. Immunol. 158, 2813–21.
Suzuki, K.T., Someya, A., Komada, Y., Ogra, Y., 2002. Roles of metallothionein in copper homeostasis: Responses to Cu-deficient diets in mice. J. Inorg. Biochem. 88, 173–182. doi:10.1016/S0162-0134(01)00376-2
Swain, G., Shinjo, N., 2014. Comparing biofouling control treatments for use on aquaculture nets. Int. J. Mol. Sci. 15, 22142–22154. doi:10.3390/ijms151222142
Swift, M.R., Fredriksson, D.W., Unrein, A., Fullerton, B., Patursson, O., Baldwin, K., 2006. Drag force acting on biofouled net panels. Aquac. Eng. 35, 292–299. doi:10.1016/j.aquaeng.2006.03.002
Tan, C.K.F., Nowak, B.F., Hodson, S.L., 2002. Biofouling as a reservoir of *Neoparamoeba pemaquidensis* (Page, 1970), the causative agent of amoebic gill disease in Atlantic salmon. Aquaculture 210, 49–58. doi:10.1016/S0044-8486(01)00858-4
Taylor, R.S., Muller, W.J., Cook, M.T., Kube, P.D., Elliott, N.G., 2009. Gill observations in Atlantic salmon (*Salmo salar*, L.) during repeated amoebic gill disease (AGD) field exposure and survival challenge. Aquaculture 290, 1–8. doi:10.1016/j.aquaculture.2009.01.030

Templeton, D.M., Cherian, M.G., 1991. Toxicological Significance of. Methods Enzimol. 205, 11–24.
The European Comission, 1998. DIRECTIVE 98/8/EC OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 16 February 1998 concerning the placing of biocidal products on the market. Off. J. Eur. Communities 63.
The International Organization for Standadization ISO 1806, 2002. Fishing nets—Determination of mesh breaking force of netting.
Tomanek, L., 2010. Variation in the heat shock response and its implication for predicting the effect of global climate change on species' biogeographical distribution ranges and metabolic costs. J. Exp. Biol. 213, 971–979. doi:10.1242/jeb.038034
Tort, L., 2011. Stress and immune modulation in fish. Dev. Comp. Immunol. 35, 1366–1375. doi:10.1016/j.dci.2011.07.002
Tort, L., Balasch, J.C., Mackenzie, S., 2003. Fish immune system. A crossroads between innate and adaptive responses. Immunologia. doi:10.1016/S1074-7613(04)00084-6
Tort, L., Montero, D., Robaina, L., Fernandez-Palacios, H., Izquierdo, M.S., 2001. Consistency of stress response to repeated handling in the gilthead sea bream *Sparus aurata* Linnaeus, 1758. Aquac. Res. 32, 593–598. doi:10.1046/j.1365-2109.2001.00607.x
Tort, L., Rotllant, J., Liarte, C., Acerete, L., Hernández, A., Ceulemans, S., Coutteau, P., Padros, F., 2004. Effects of temperature decrease on feeding rates, immune indicators and histopathological changes of gilthead sea bream *Sparus aurata* fed with an experimental diet. Aquaculture 229, 55–65. doi:10.1016/S0044-8486(03)00403-4
Tort, L., Padrós, F., Rotllan, J., Crespo, S., 1998. Winter syndrome in the gilthead sea breamSparus aurata. Immunological and histopathological features. Fish Shellfish Immunol. 8, 37–47. doi:10.1006/fsim.1997.0120
Tort, L., Rotllant, J., Rovira, L., 1998. Immunological suppression in gilthead sea bream *Sparus aurata* of the North-West Mediterranean at low temperatures. Comp. Biochem. Physiol. Part A Mol. Integr. Physiol. 120, 175–179. doi:10.1016/S1095-6433(98)10027-2
Tort, L., Sunyer, J.O., Gómez, E., Molinero, A., 1996. Crowding stress induces changes in serum haemolytic and agglutinating activity in the gilthead sea bream *Sparus aurata*. Genesis.
United Nations, Department of Economic and Social Affairs, P.D., 2017. The 2017 Revision, Key Findings and Advance Tables. ESA/P/WP/248. doi:10.1017/CBO9781107415324.004
Uribe1, C., Folch2, H., Enriquez1, R., G. Moran1, 2011. Innate and adaptive immunity in teleost fish: a review. Vet. Med. (Praha). 56, 486–503.
Varo, I., Nunes, B., Amat, F., Torreblanca, a, Guilhermino, L., Navarro, J.C., 2007. Effect of sublethal concentrations of copper sulphate on seabream Sparus aurata fingerlings. Aquat. Living Resour. 20, 263–270. doi:10.1051/alr
Vera, R., Vergara, A.M., 2016. Efectos del lavado in situ de redes en sedimentos asociados a la acuicultura en el sur de Chile. Rev. Biol. Mar. Oceanogr. 51, 505–514. doi:10.4067/S0718- 19572016000300003
Vijayan, M.M., Prunet, P., Boone, A.N., 2005. Chapter 13 Xenobiotic impact on corticosteroid signaling. Biochem. Mol. Biol. Fishes 6, 365–394. doi:10.1016/S1873-0140(05)80016-5

Wang, T., Long, X., Cheng, Y., Liu, Z., Yan, S., 2014. The potential toxicity of copper nanoparticles and copper sulphate on juvenile *Epinephelus coioides*. Aquat. Toxicol. 152, 96–104. doi:10.1016/j.aquatox.2014.03.023

Watanabe, T., Kiron, V., Satoh, S., 1997. Trace minerals in fish nutrition, in: Aquaculture. Elsevier, pp. 185–207. doi:10.1016/S0044-8486(96)01503-7

Wendelaar-Bonga, S.E., 1997. The stress response in fish. Physiol. Rev. 77, 591–625.

Wiens, G.D., Glenney, G.W., 2011. Origin and evolution of TNF and TNF receptor superfamilies. Dev. Comp. Immunol. 35, 1324–1335. doi:10.1016/j.dci.2011.03.031

Willemsen, P., Science, T.N.O., 2016. Collective Research on Aquaculture Biofouling Acronym : CRAB 1–2.

Willemsen, P.R., 2005. Biofouling in European Aquaculture: Is There an Easy Solution? Eur. Aquac. Soc. Spec. Publ. 82–87.

Williams, A.E., 2012. Cytokines. Immunol. Mucosal Body Surf. Defenses 68–92.

Xu, Z., Parra, D., Gomez, D., Salinas, I., Zhang, Y.-A., von Gersdorff Jørgensen, L., Heinecke, R.D., Buchmann, K., LaPatra, S., Sunyer, J.O., 2013. Teleost skin, an ancient mucosal surface that elicits gut-like immune responses. Proc. Natl. Acad. Sci. U. S. A. 110, 13097– 13102. doi:10.1073/pnas.1304319110.

Yada, T., Nagae, M., Moriyama, S., Azuma, T., 1999. Effects of prolactin and growth hormone on plasma immunoglobulin M levels of hypophysectomized rainbow trout, *Oncorhynchus mykiss*. Gen. Comp. Endocrinol. 115, 46–52. doi:10.1006/gcen.1999.7282

Annexes

I. Commission implementing regulation (EU) 2016/1089 approving di-copper oxide as an existing active substance for use in biocidal products of product.

II. On-site cleaning of form.

III. Experimental location and nets technical drawings.

IV. Products safety data sheet.

V. Netting specifications table.

VI. Description of the criteria for gill score analysis used as a reference in this work.

VII. Feeding, growth and conversion rates for sea bream culture in cages.

COMMISSION IMPLEMENTING REGULATION (EU) 2016/1089

of 5 July 2016

approving dicopper oxide as an existing active substance for use in biocidal products of product-type 21

(Text with EEA relevance)

THE EUROPEAN COMMISSION,

Having regard to the Treaty on the Functioning of the European Union,

Having regard to Regulation (EU) No 528/2012 of the European Parliament and of the Council of 22 May 2012 concerning the making available on the market and use of biocidal products ([1]), and in particular the third subparagraph of Article 89(1) thereof,

Whereas:

(1) Commission Delegated Regulation (EU) No 1062/2014 ([2]) establishes a list of existing active substances to be evaluated for their possible approval for use in biocidal products. That list includes dicopper oxide.

(2) Dicopper oxide has been evaluated for use in products of product-type 21, antifouling products, as described in Annex V to Regulation (EU) No 528/2012.

(3) France was designated as evaluating competent authority and submitted the assessment report together with its recommendations on 31 October 2014.

(4) In accordance with Article 7(2) of Delegated Regulation (EU) No 1062/2014, the opinion of the European Chemicals Agency was formulated on 9 December 2015 by the Biocidal Products Committee, having regard to the conclusions of the evaluating competent authority.

(5) According to that opinion, biocidal products of product-type 21 and containing dicopper oxide may be expected to satisfy the criteria of Article 19(1)(b) of Regulation (EU) No 528/2012, provided that certain specifications and conditions concerning their use are complied with.

(6) The acceptability of the risks related to the use of antifouling products, as well as the suitability of the proposed risk mitigation measures, should however be further confirmed. In order to facilitate, at the time of the renewal of the approvals of existing antifouling active substances, the review and comparison of the risks and benefits of those substances as well as of the risk mitigation measures applied, the expiry date of approval of all those substances should be the same.

(7) It is therefore appropriate to approve dicopper oxide for use in biocidal products of product-type 21 subject to compliance with certain specifications and conditions.

(8) A reasonable period should be allowed to elapse before an active substance is approved in order to permit interested parties to take the preparatory measures necessary to meet the new requirements.

(9) The measures provided for in this Regulation are in accordance with the opinion of the Standing Committee on Biocidal Products,

([1]) OJ L 167, 27.6.2012, p. 1.

([2]) Commission Delegated Regulation (EU) No 1062/2014 of 4 August 2014 on the work programme for the systematic examination of all existing active substances contained in biocidal products referred to in Regulation (EU) No 528/2012 of the European Parliament and of the Council (OJ L 294, 10.10.2014, p. 1).

HAS ADOPTED THIS REGULATION:

Article 1

Dicopper oxide is approved as an active substance for use in biocidal products of product-type 21, subject to the specifications and conditions set out in the Annex.

Article 2

This Regulation shall enter into force on the twentieth day following that of its publication in the *Official Journal of the European Union*.

This Regulation shall be binding in its entirety and directly applicable in all Member States.

Done at Brussels, 5 July 2016.

For the Commission
The President
Jean-Claude JUNCKER

ANNEX

Common Name	IUPAC Name Identification Numbers	Minimum degree of purity of the active substance (1)	Date of approval	Expiry date of approval	Product type	Specific conditions
Dicopper oxide	IUPAC Name: Copper (I) oxide EC No: 215-270-7 CAS No: 1317-39-1	94,2 % w/w	1 January 2018	31 December 2025	21	The product assessment shall pay particular attention to the exposures, the risks and the efficacy linked to any uses covered by an application for authorisation, but not addressed in the Union level risk assessment of the active substance. In the event that products containing dicopper oxide are subsequently authorised for use by non-professional users, persons making products available on the market for non-professional users shall ensure that the products are supplied with appropriate gloves. For biocidal products, authorisations are subject to the following conditions: 1. For industrial or professional users, safe operational procedures and appropriate organisational measures shall be established. Where exposure cannot be reduced to an acceptable level by other means, products shall be used with appropriate personal protective equipment. 2. Labels and, where provided, instructions for use shall indicate that children shall be kept away until treated surfaces are dry. 3. Labels and, where provided, safety data sheets of products authorised shall indicate that application, maintenance and repair activities shall be conducted within a contained area, on an impermeable hard standing with bunding or on soil covered with an impermeable material to prevent losses and minimise emissions to the environment, and that any losses or waste containing dicopper oxide shall be collected for reuse or disposal.

Common Name	IUPAC Name Identification Numbers	Minimum degree of purity of the active substance [1]	Date of approval	Expiry date of approval	Product type	Specific conditions
						4. For products that may lead to residues in food or feed, the need to set new or to amend existing maximum residue levels (MRLs) in accordance with Regulation (EC) No 470/2009 of the European Parliament and of the Council [2] or Regulation (EC) No 396/2005 of the European Parliament and of the Council [3] shall be verified, and any appropriate risk mitigation measures shall be taken to ensure that the applicable MRLs are not exceeded.

[1] The purity indicated in this column was the minimum degree of purity of the active substance used for the evaluation made in accordance with Article 89(1) of Regulation (EU) No 528/2012. The active substance in the product placed on the market can be of equal or different purity if it has been proven technically equivalent with the evaluated active substance.

[2] Regulation (EC) No 470/2009 of the European Parliament and of the Council of 6 May 2009 laying down Community procedures for the establishment of residue limits of pharmacologically active substances in foodstuffs of animal origin, repealing Council Regulation (EEC) No 2377/90 and amending Directive 2001/82/EC of the European Parliament and of the Council and Regulation (EC) No 726/2004 of the European Parliament and of the Council (OJ L 152, 16.6.2009, p. 11).

[3] Regulation (EC) No 396/2005 of the European Parliament and of the Council of 23 February 2005 on maximum residue levels of pesticides in or on food and feed of plant and animal origin and amending Council Directive 91/414/EEC (OJ L 70, 16.3.2005, p. 1).

ON SITE CLEANING OF AQUACULTURE NETS

All this information will be treated confidentially

1. Personal Information

Name

Position

Company

ON SITE CLEANING OF AQUACULTURE NETS

* Necessari

2. Operations

Do you use on site cleaning in your site? (If your answer is NO, please jump on to Section 6) *

- [ ] YES
- [ ] NO

When did you start using it?

- [ ] Less than 1 year ago
- [ ] Between 1-2 years ago
- [ ] More than 2 years ago

How often each net needs to be cleaned?

- [ ] Once a week
- [ ] Once every second week

- [ ] Once every three weeks
- [ ] Monthly
- [ ] Less than once per month

How long does the operation take per cage?

- [ ] less than 2 hours
- [ ] 2 to 3 hours
- [ ] 3 to 4 hours
- [ ] more than 4 hours

Which resources are usually used for on site cleaning? (human & equipment)

Approximately which cleaning % is reached?

- [ ] 0-25%
- [ ] 26-50%
- [ ] 51-75%
- [ ] 76-100%

Which is your perception about how fast is the re-growth rate?

What does affect it?

How many nets are used per cycle?

- [ ] 1 net
- [ ] 2 nets
- [ ] 3 nets
- [ ] More than 3 nets

Do you think cleaning operation affects nearby cages?

- [ ] YES
- [ ] NO
- [ ] N/A

How?

How do you think on site cleaning affects netting integrity? (BS)

Do you think net coatings help preventing netting damage after cleaning?

- [ ] YES
- [ ] NO
- [ ] Not enough
- [ ] Altres:

ON SITE CLEANING OF AQUACULTURE NETS

3. Feeding and feeding behaviour

Does feeding stop during on site cleaning?

- [ ] YES
- [ ] NO
- [ ] N/A

If so, when?

- [ ] The previous day
- [ ] Just before cleaning
- [ ] 0-2 hours before cleaning
- [ ] 2-4 hours before cleaning
- [ ] More than 4 hours before cleaning

When feeding re-starts after on site cleaning?

- [ ] immediately after cleaning
- [ ] 1-2 hours after cleaning

- [ ] 2-4 hours after cleaning
- [ ] 4-24 hours after cleaning
- [ ] The next day

Do you think on site cleaning affects feeding?

- [ ] YES
- [ ] NO
- [ ] N/A

In which way?

ON SITE CLEANING OF AQUACULTURE NETS

4. Performance

Have you seen any effect/trend (positive or negative) on growth and/or mortality on those fish batches reared in on site cleaned nets along the growth cycle?

ON SITE CLEANING OF AQUACULTURE NETS

5. Fish Health

Have you detected any behavioural change or stress indicator among the fish during on site cleaning?

Do you think the debris cloud generated during on site cleaning can affect fish health?

- [ ] YES
- [ ] NO
- [ ] N/A

If YES, in which way?

ON SITE CLEANING OF AQUACULTURE NETS

6. Personal Opinion

What is you general opinion (PROS AND CONS) of on site cleaning strategy vs net cleaning on land?

Which are, according to your experience, those points that should be enhanced?

MØRENOT
AQUACULTURE

Delta Aqua Redes, s.l.u.

Ctra.de Canal Amposta-St. Jaume, km1,5
43870 AMPOSTA

NET DRAWINGS

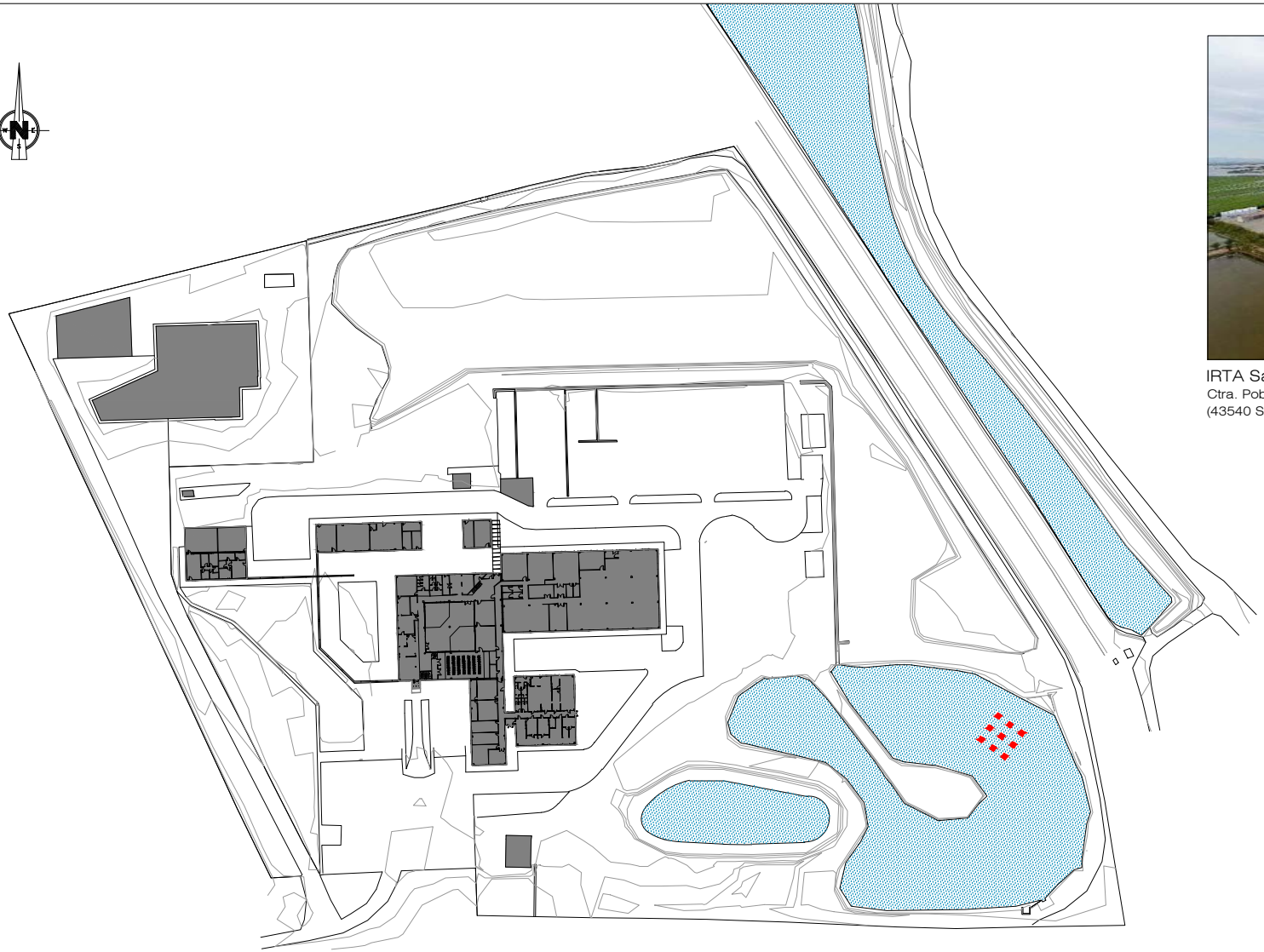
Drawed by:
MERCEDES PARDO
(from Delta Aqua Redes s.l.u.)

Date: OCTOBER 2015

Order nr	Net ID	Customer
----	----	----

NET SPECIFICATIONS

Circumf. top:	Circumf. bottom:	Height jumpnet:	Depth to bottom rope:	Depth to center base:	Total sides:	Thread number:	Half Mesh size:	Breaking strenght:
6 m	5.20 m	0 m	1.20 m	0 m	4 sides	NYLON 32 uk	18 mm	118 kg



IRTA Sant Carles de la Ràpita
Ctra. Poble Nou, km 5,5
(43540 Sant Carles de la Ràpita - Tarragona)

MØRENOT AQUACULTURE

Order nr:	----
Net ID:	----
Type of net:	SQUARED NET 6x6x1.20m
Customer:	----
Netting:	NYLON
Thread nr:	32
Thread type:	Uk
Half Mesh:	18 mm

Drawing name: SITE PLAN

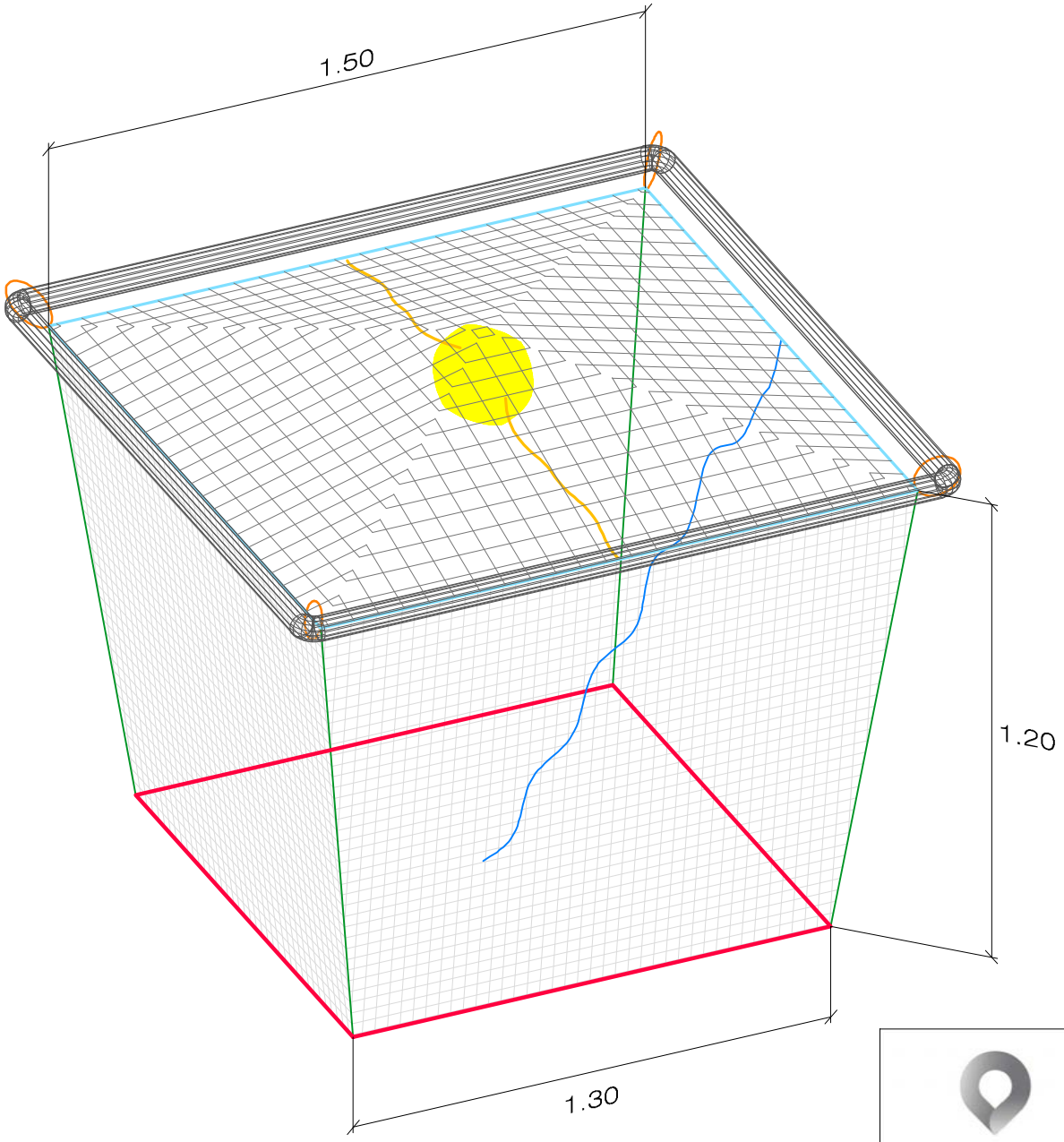
Drawed by: MERCEDES PARDO - Delta Aqua Redes s.l.u.

Date: OCT 2015

revision:

Scale: 1/2000

Drawing number: 01 /06



SYMBOL	
	LOOPS
	WATERLINE ROPE Danline 16mm, 1 piece
	LIFTING ROPE Danline 16mm, 4 pieces
	LEADLINE 1kg/m, 4 pieces
	Danline rope 12mm, 1 piece
	Danline rope 22mm, 2 pieces
	FLOAT 20L, 1 piece
	POLYETHYLENE TUBE Ø60mm
	HDPE NETTING

DIMENSIONS	
CIRCUMFERENCE AT WATERLINE ROPE	6 m
CIRCUMFERENCE AT BOTTOM ROPE	5.2 m
NUMBER OF SIDES	4
LENGTH SIDES	1.50 m
ANGLE ON BASE	0° (FLAT BASE)
HEIGHT JUMPNET	0 m
DEPTH TO BOTTOM ROPE	1.20 m
DEPTH FROM BOTTOM ROPE TO CENTER BASE	0 m (FLAT BASE)

NETTING:
NET- NYLON Thread nr. 210/96 Half mesh 18mm B.S.: 118kg
TOP NET- HDPE 75mm

All dimensions are exact size. Dimensions in meters.
NYLON NETTING MUST BE OVERSIZED BY 3% TO GIVEN DIMENSIONS

MØRENOT AQUACULTURE

Order nr:	----
Net ID:	----
Type of net:	SQUARED NET 6x6x1.20m
Customer:	----
Netting:	NYLON
Thread nr:	32
Thread type:	Uk
Half Mesh:	18 mm

Drawing name: 3D VIEW

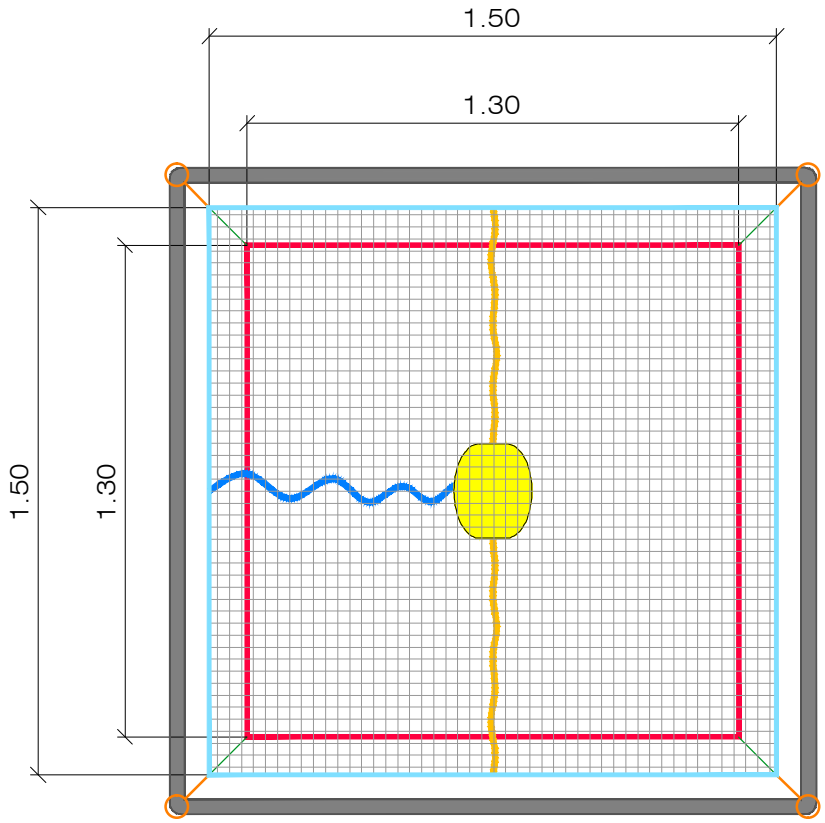
Drawed by: MERCEDES PARDO - Delta Aqua Redes s.l.u.

Date: OCT 2015

revision:

Scale: ----

Drawing number: 02/06



SYMBOL	
	LOOPS
	WATERLINE ROPE Danline 16mm, 1 piece
	LIFTING ROPE Danline 16mm, 4 pieces
	LEADLINE 1kg/m, 4 pieces
	Danline rope 12mm, 1 piece
	Danline rope 22mm, 2 pieces
	FLOAT 20L, 1 piece
	POLYETHYLENE TUBE Ø60mm
	HDPE NETTING

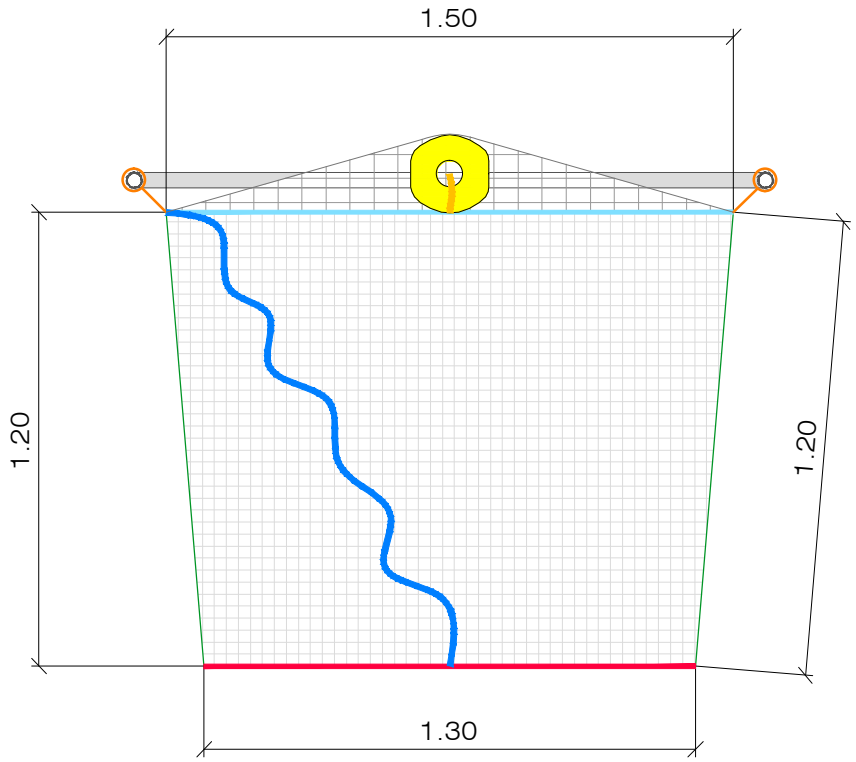
DIMENSIONS	
CIRCUMFERENCE AT WATERLINE ROPE	6 m
CIRCUMFERENCE AT BOTTOM ROPE	5.2 m
NUMBER OF SIDES	4
LENGTH SIDES	1.50 m
ANGLE ON BASE	0º (FLAT BASE)
HEIGHT JUMPNET	0 m
DEPTH TO BOTTOM ROPE	1.20 m
DEPTH FROM BOTTOM ROPE TO CENTER BASE	0 m (FLAT BASE)

NETTING:
NET- NYLON Thread nr. 210/96 Half mesh 18mm B.S.: 118kg
TOP NET- HDPE 75mm

All dimensions are exact size. Dimensions in meters.
NYLON NETTING MUST BE OVERSIZED BY 3% TO GIVEN DIMENSIONS

MØRENOT AQUACULTURE

Order nr:	----
Net ID:	----
Type of net:	SQUARED NET 6x6x1.20m
Customer:	----
Netting:	NYLON
Thread nr:	32
Thread type:	Uk
Half Mesh:	18 mm
Drawing name:	TOP VIEW
Drawed by:	MERCEDES PARDO - Delta Aqua Redes s.l.u.
Date:	OCT 2015
revision:	
Scale:	1/20
Drawing number:	03/06



SYMBOL	
	LOOPS
	WATERLINE ROPE Danline 16mm, 1 piece
	LIFTING ROPE Danline 16mm, 4 pieces
	LEADLINE 1kg/m, 4 pieces
	Danline rope 12mm, 1 piece
	Danline rope 22mm, 2 pieces
	FLOAT 20L, 1 piece
	POLYETHYLENE TUBE Ø60mm
	HDPE NETTING

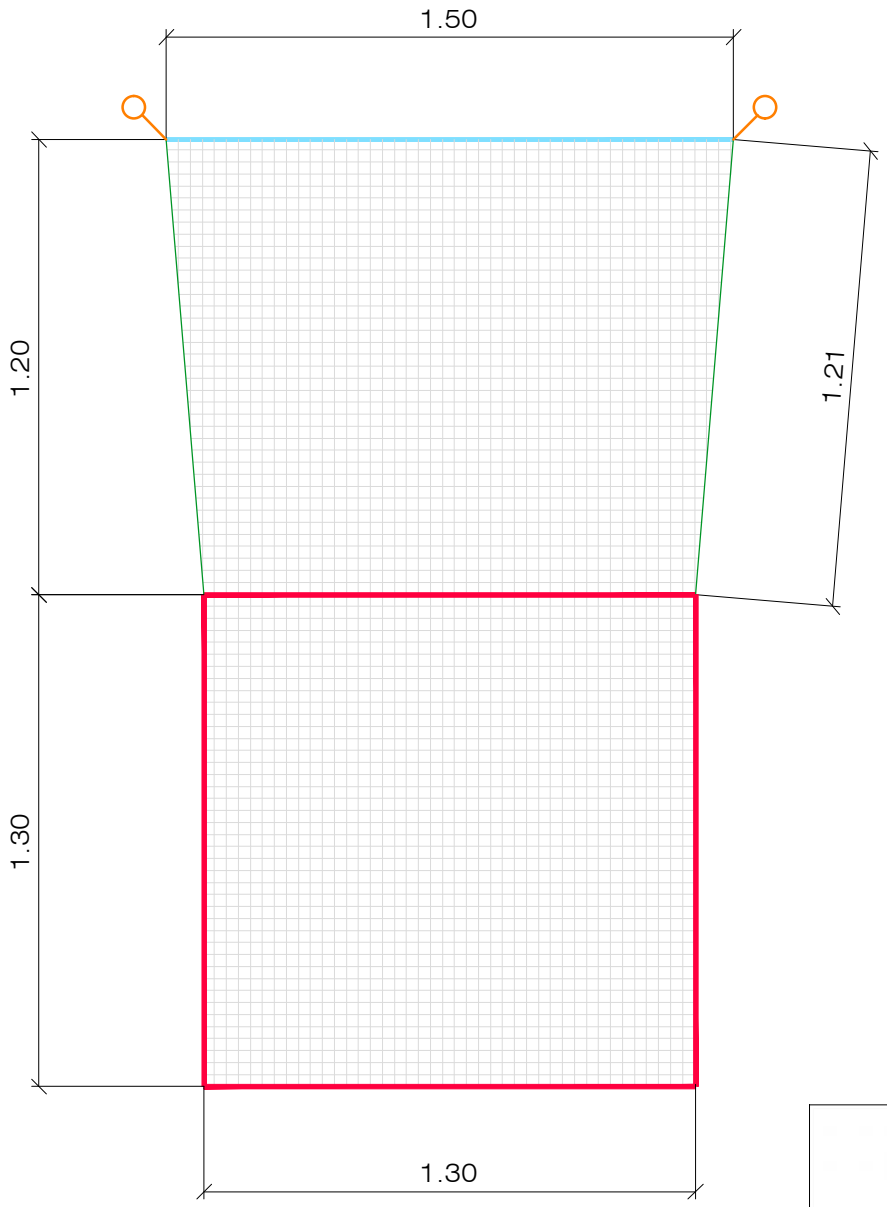
DIMENSIONS	
CIRCUMFERENCE AT WATERLINE ROPE	6 m
CIRCUMFERENCE AT BOTTOM ROPE	5.2 m
NUMBER OF SIDES	4
LENGTH SIDES	1.50 m
ANGLE ON BASE	0º (FLAT BASE)
HEIGHT JUMPNET	0 m
DEPTH TO BOTTOM ROPE	1.20 m
DEPTH FROM BOTTOM ROPE TO CENTER BASE	0 m (FLAT BASE)

NETTING:
NET- NYLON Thread nr. 210/96 Half mesh 18mm B.S.: 118kg
TOP NET- HDPE 75mm

All dimensions are exact size. Dimensions in meters.
NYLON NETTING MUST BE OVERSIZED BY 3% TO GIVEN DIMENSIONS

MØRENOT AQUACULTURE

Order nr:	----
Net ID:	----
Type of net:	SQUARED NET 6x6x1.20m
Customer:	----
Netting:	NYLON
Thread nr:	32
Thread type:	Uk
Half Mesh:	18 mm
Drawing name:	FRONT VIEW
Drawed by:	MERCEDES PARDO - Delta Aqua Redes s.l.u.
Date:	OCT 2015
revision:	
Scale:	1/20
Drawing number:	04 /06



SYMBOL	
	LOOPS
	WATERLINE ROPE Danline 16mm, 1 piece
	LIFTING ROPE Danline 16mm, 4 pieces
	LEADLINE 1kg/m, 4 pieces
	Danline rope 12mm, 1 piece
	Danline rope 22mm, 2 pieces
	FLOAT 20L, 1 piece
	POLYETHYLENE TUBE Ø60mm
	HDPE NETTING

DIMENSIONS	
CIRCUMFERENCE AT WATERLINE ROPE	6 m
CIRCUMFERENCE AT BOTTOM ROPE	5.2 m
NUMBER OF SIDES	4
LENGTH SIDES	1.50 m
ANGLE ON BASE	0° (FLAT BASE)
HEIGHT JUMPNET	0 m
DEPTH TO BOTTOM ROPE	1.20 m
DEPTH FROM BOTTOM ROPE TO CENTER BASE	0 m (FLAT BASE)

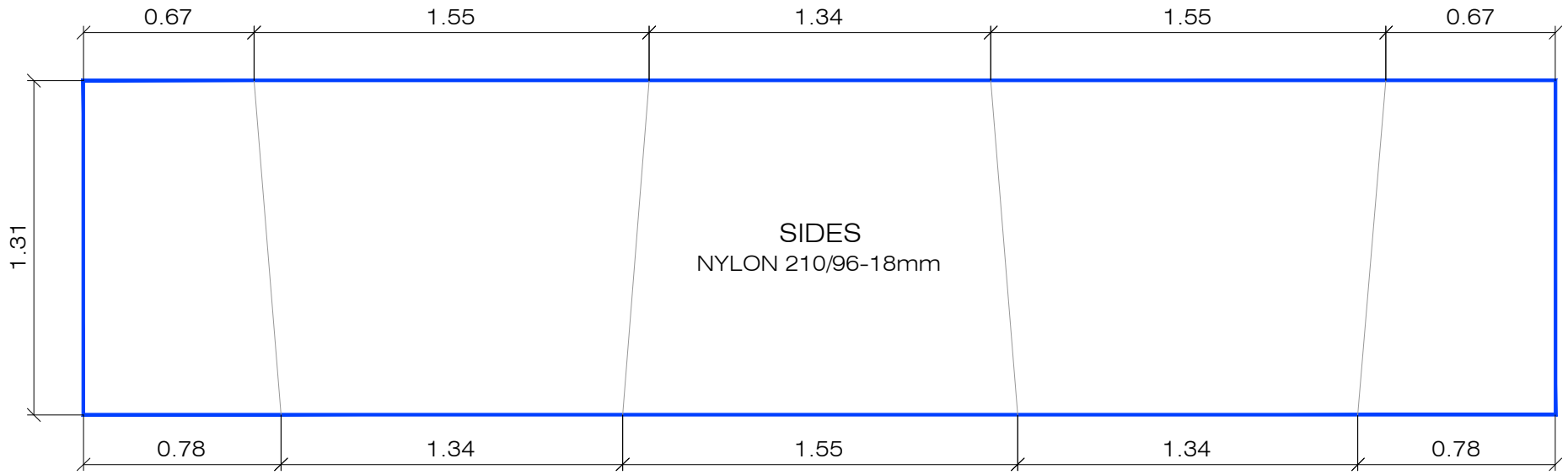
NETTING:
NET- NYLON Thread nr. 210/96 Half mesh 18mm B.S.: 118kg
TOP NET- HDPE 75mm

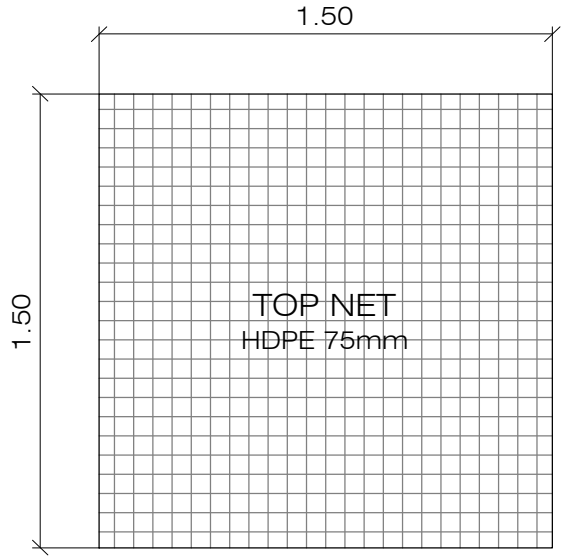
All dimensions are exact size. Dimensions in meters.
NYLON NETTING MUST BE OVERSIZED BY 3% TO GIVEN DIMENSIONS

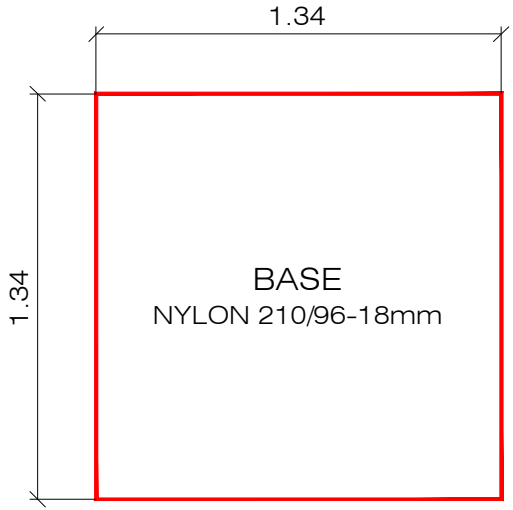
MØRENOT AQUACULTURE

Order nr:	----
Net ID:	----
Type of net:	SQUARED NET 6x6x1.20m
Customer:	----
Netting:	NYLON
Thread nr:	32
Thread type:	Uk
Half Mesh:	18 mm

Drawing name: ENVOLVENT
Drawed by: MERCEDES PARDO - Delta Aqua Redes s.l.u.
Date: OCT 2015
revision:
Scale: 1/50
Drawing number: 05/06







CUTTING RULE (12.48)	
1/13x1 - 1/12x1	
nylon slack 3%	dimensions in meters

MØRENOT AQUACULTURE

Order nr:	----
Net ID:	----
Type of net:	SQUARED NET 6x6x1.20m
Customer:	----
Netting:	NYLON
Thread nr:	32
Thread type:	Uk
Half Mesh:	18 mm

Drawing name: CUTTING DRAWING

Drawed by: MERCEDES PARDO - Delta Aqua Redes s.l.u.

Date: OCT 2015

revision:

Scale: 1/25

Drawing number: 06/06

SAFETY DATA SHEET
Netwax NI 3

SECTION 1: Identification of the substance/mixture and of the company/undertaking

1.1. Product identifier

Product name Netwax NI 3

1.2. Relevant identified uses of the substance or mixture and uses advised against

Applications Antifouling for aquaculture cage nets. For professional use only.

1.3. Details of the supplier of the safety data sheet

Supplier

NetKem AS
Slalåmveien 1
NO-1410 Kolbotn
Norway
Tel: +47 66 80 82 15
Fax: +47 66 80 25 21
www.netkem.no

Contact person Rune Antonsen (e-mail:rune.antonsen@netkem.no)

1.4. Emergency telephone number

Emergency telephone number 112 / The UK National Poisons Emergency number: +44 870 600 6266

SECTION 2: Hazards identification

2.1. Classification of the substance or mixture

Classification according to directive 1272/2008 (CLP)

GHS09
Aquatic Chronic 2: H411

2.2. Label elements

CLP

Hazard pictograms

Hazard statements Aquatic Chronic 2: H411 Toxic to aquatic life with long lasting effects.

Precautionary statements

P273 Avoid release to the environment.
P280 Wear protective gloves/protective clothing/eye protection/face protection.
P501 Dispose of contents/container to approved disposal plant in accordance with local regulations.

Contains dicopper oxide

2.3. Other hazards

Meets the criteria for vPvB No.

Meets the criteria for PBT No.

Other hazards which do not contribute to classification No known risks.

SECTION 3: Composition/information on ingredients

3.2. Mixtures

Ingredients

Name	CAS No.	REACH No.	Content	Classification	Symbol
water based fluid		N/A	60-100 %		
dicopper oxide	1317-39-1	01-2119513794-36	10-20 %	Acute Tox. 4: H302, Aquatic Acute 1: H400, Aquatic Chronic 1: H410	GHS09, GHS07, , Warning

Section 16 contains detailed classification phrases.

SECTION 4: First aid measures

4.1. Description of first aid measures

General Remove victim immediately from source of exposure. Provide rest, warmth and fresh air.

4.2. Most important symptoms and effects, both acute and delayed

Specific first aid treatment No specific first aid measures noted.

4.3. Indication of any immediate medical attention and special treatment needed

Inhalation If respiratory problems, artificial respiration/oxygen. Get medical attention if any discomfort continues. When unconscious, loosen tight clothing and position in secured recovery position.

Ingestion DO NOT INDUCE VOMITING! Rinse nose, mouth and throat with water. Immediately give a couple of glasses of water or milk, provided the victim is fully conscious. If vomiting occurs, keep head low so that stomach content doesn´t get into the lungs. Get medical attention if any discomfort continues.

Skin Immediately remove contaminated clothing. Wash skin with soap and water.

Eyes Important! Immediately rinse with water for at least 15 minutes. Immediately transport to hospital or eye specialist.

SECTION 5: Firefighting measures

5.1. Extinguishing media

Extinguishing media Fire can be extinguished using: Powder, foam or CO2.

5.2. Special hazards arising from the substance or mixture

Specific hazards Not known.

Hazardous combustion products Carbon monoxide (CO). Metal oxids,

5.3. Advice for firefighters

Protective measures in fire Firefighters exposed to combustion gases/decomposition products should use a respiratory protective device.

SECTION 6: Accidental release measures

6.1. Personal precautions, protective equipment and emergency procedures

Personal protection Use requisite protective equipment - refer to section 8.

6.2. Environmental precautions

Environmental protection Avoid discharge into drains, water courses or onto the ground.

6.3. Methods and material for containment and cleaning up

Spill cleanup methods Absorb in vermiculite, dry sand or earth and place into containers. Collect spillage in

containers, seal securely and deliver for disposal according to local regulations.

6.4. Reference to other sections

See section 13 for waste handling.

SECTION 7: Handling and storage

7.1. Precautions for safe handling

Usage precautions Wear appropriate personal protective equipment - see Section 8. Avoid spilling, skin and eye contact.

7.2. Conditions for safe storage, including any incompatibilities

Storage precautions Store above freezing. Store at temperature, °C: >4.

7.3. Specific end use(s)

Specific use(s) Contact supplier for more information.

SECTION 8: Exposure controls/personal protection

8.1. Control parameters

Ingredient name	CAS no.	Reference	LT Exp 8 Hrs	ST Exp 15 Min	Date
Copper I oxide as copper fumes	1317-39-1	WEL.	0,2 mg/m3		
Copper I oxide as copper dust and smoke	1317-39-1	WEL.	1 mg/m3		
Oil smoke (mineral oil particles)		WEL.	5 mg/m3	10 mg/m3	

Ingredient comments WEL = Workplace exposure limits. SK= Skin absorbance, Rep= Reproduction, Carc= Carcinogenic, Senz= Sensitisers, Mut= Carcinogenic

Protective equipment

Ventilation No particular ventilation requirements.

8.2. Exposure controls

Respirators Respiratory protection not required.

Protective gloves Use protective gloves made of: Neoprene. Nitrile. Thickness: >0,2mm Braketrough time, hours:>4 EN 374 standard.

Eye protection Wear approved safety goggles. EN 166 standard.

Other Protection No specific protective equipment noted, but may be required anyway.

Hygienic work practices No specific hygiene procedures noted, but good personal hygiene practices are always advisable, especially when working with chemicals.

DNEL No data.

PNEC No data.

SECTION 9: Physical and chemical properties

9.1. Information on basic physical and chemical properties

Appearance Water based fluid.

Colour Reddish.

Odour Mild (or faint).

Solubility description	Miscible with water.		
Boiling point (°C, interval)	> 100	**Pressure**	
Melting/freezing point (°C, interval)	65 - 70		
Density (g/cm3)	1,15	**Temperature (°C)**	15
Vapour density (air=1)	> 2		
Vapour pressure	< 20 mmHg	**Temperature (°C)**	20
pH-value, conc. solution	~ 7,5		
Flash point (°C)	> 200	**Method**	

9.2. Other information

Safety information Not known.

SECTION 10: Stability and reactivity

10.1. Reactivity

No incompatible groups noted.

10.2. Chemical stability

Normally stable.

10.3. Possibility of hazardous reactions

Hazardous polymerisation Unknown.

10.4. Conditions to avoid

Avoid heat.

10.5. Incompatible materials

Materials to avoid Oxidising agents.

10.6. Hazardous decomposition products

Hazardous decomp. products Carbon monoxide (CO). Metal oxides,

SECTION 11: Toxicological information

11.1. Information on toxicological effects

Toxic dose - LD 50:	> 2000 mg/kg (oral rat)
Toxic dose - LD 50 (skin):	> 2000 mg/kg (skin rabbit)
Sensitization	No known information.
Genotoxicity	No known information.
Carcinogenicity	No evidence of carcinogenic properties.
Reproduction toxicity	No known information.
Health hazards, general	No specific health warnings noted.
Inhalation	No specific health warnings noted.
Ingestion	No specific health warnings noted.
Skin	Liquid may irritate skin.
Eyes	Spray and vapour in the eyes may cause irritation and smarting.
COMPONENT:	**dicopper oxide**
Toxic dose - LD50:	470 mg/kg (oral rat)
Toxic dose - LD50 (skin):	> 2000 mg/kg (skin rat)
Toxic conc. - LC50:	> 50 mg/l/4h (inhalation rat)

SECTION 12: Ecological information

12.1. Toxicity

Ecotoxicity Toxic to aquatic life with long lasting effects.

12.2. Persistence and degradability

The degradability of this product has not been stated.

12.3. Bioaccumulative potential

No bioaccumulation expected.

12.4. Mobility in soil

Mobility The product has low water mobility.

12.5. Results of PBT and vPvB assessment

PTB/vPvB Component(s) is not identified as PBT or vPvB substance(s).

12.6. Other adverse effects

No known adverse affects.

COMPONENT: **dicopper oxide**
LC 50, 96 Hrs, Fish mg/l: > 0,173 Art: Cyprinodon variegatus
EC 50, 48 Hrs, Daphnia, mg/l: 0,51 Art: D.magna

SECTION 13: Disposal considerations

13.1. Waste treatment methods

General/cleaning Hazardous waste.

Disposal methods Dispose of in accordance with Local Authority requirements.

Waste class 08 01 11* waste paint and varnish containing organic solvents or other dangerous substances
The given EWC-code is a guiding, and the code depends on how the waste is formed. User must evaluate the choice of correct code.

SECTION 14: Transport information

General No dangerous goods (ADR/RID, IMDG, IATA/ICAO)

14.1. UN number

14.2. UN proper shipping name

14.3. Transport hazard class(es)

TRANSPORT BY INLAND WATERWAYS (ADN):

14.4. Packing group

14.5. Environmental hazards

Transport by inland waterways notes Not applicable.

14.6. Special precautions for user

No particular precautions.

14.7. Transport in bulk according to Annex II of MARPOL73/78 and the IBC Code

No IBC-code for bulk transport offshore (MARPOL).

SECTION 15: Regulatory information

15.1. Safety, health and environmental regulations/legislation specific for the substance or mixture

EU directives EC-regulation 453/2010/EC, 1907/2006/EC (REACH), 1272/2008/EC (CLP), 790/2009/EC. Tranport of dangerous goods (ADR/RID, IMDG, IATA/ICAO). Workplace exposure limits.
Directive 98/8/EC (Biocides), Directive 2009/107/EC (amendments).

Specific regulations (Norway) Product type (biocide): TP21 - Antifouling products.

Product declaration number 76554

15.2. Chemical safety assessment

Chemical Safety Assessment	Chemical Safety Report (CSR) has not been carried out for this product.

SECTION 16: Other information

Explanations to classification in section 3	H302 Harmful if swallowed. H400 Very toxic to aquatic life. H410 Very toxic to aquatic life with long lasting effects.
*** Information revised since the previous version of the SDS**	
Revision comments	Revision 31.05.2010 no. 1: superseed safetydatasheet of 06.05.2009. No change of composition or product classification. Revision 30.06.2011 no. 2: superseed safetydatasheet of 31.05.2010. No change of composition or product classification. Revision 03.09.2012 no. 3: supersede SDS of 30.06.2011. Prepared in CLP format. No change of composition or product classification. Revision 2013.08.14 no. 4: supersede SDS of 2012.09.03. No change of composition or product classification. Revision 2014.06.25 no. 5: supersede SDS of 2013.08.14. No change of composition or product classification. Revision 15.07.2015 no. 6: supersede SDS of 25.06.2014. No changes of composition or product classification.
Issued by	Essenticon AS, Leif Weldingsvei 18, N-3208 Sandefjord, Norway. E-mail: post@essenticon.no Phone: +47 33 42 34 50 Fax: +47 33 42 34 59 www.essenticon.com.
Date of issue	06.05.2009
Revision date	15.07.2015
Revision no.	5
Rev. no./repl. SDS generated	14.08.2013
Safety Data Sheet status	CLP 04 ATP
Signature	K.Dyreskog

Vicrapore
porous ptfe technology

Safety Data Sheet

VICOTE

1 IDENTIFICATION OF THE SUBSTANCE/PREPARATION AND THE COMPANY

PRODUCT NAME: **VICOTE**

APPLICATIONS:	**Coating Polymer**
SUPPLIER:	**Vicrapore**
	45A Ardrossan Road, Seamill,
	West Kilbride, Ayrshire,
	Scotland, KA23 9 NB
TEL:	**07980 606952**
FAX:	**07977 103059**

2 COMPOSITION/INFORMATION ON INGREDIENTS

INGREDIENT NAME/CAS number	CONTENT
Water / 7732-18-5	50-70%
Polyurethane Polymer	30-40%

3 HAZARDS IDENTIFICATION

Main risks: CAUTION odourless , off white liquid which may be coloured with a pigmented dye (blue)
Inhalation: Non hazardous under normal circumstances.
Skin contact: May cause mild skin irritation.
Contact with eyes: May cause eye irritation.
Ingestion: May be harmful if swallowed.

4 FIRST AID MEASURES

Inhalation: Remove from source of contamination to fresh air
Skin Contact: Wash off with plenty of soap and water. Remove contaminated clothing and launder before re-use.
Eye Contact: Flush eyes with copious amounts of water for 15 minutes.If redness persists seek medical attension.
Ingestion: If swallowed give 1-3 glasses of water, but do not induce vomitting.Do not give anything by mouth to an unconcious or convulsing person.Get medical attention.

5 FIRE FIGHTING MEASURES

Extinguishing Media : Water fog,carbon dioxide,foam,dry chemical.
Special exposure Hazards: The dried powder is capable of burning.
Protection of fire-fighters: Full protective clothing and approved self-contained breathing apparatus required for fire fighting personal.

6 ACCIDENTAL RELEASE MEASURES

Personal precautions: Wear suitable protective clothing. Do not attempt to take action without suitable protective clothing – see section 8 of MSDS.

Environmental precautions: Do not discharge into drains or rivers. Contain the spillage using bunding.

Clean-up procedures: Soak up residue with an absorbant such as sand or clay.Place in a non-leaking container fpr disposal according to local authority regulations.
Do not discharge into waterways or sewage systems.

7 HANDLING AND STORAGE

Storage containers: Containers can be stainless steel, rubber lined mild steel or plastic tanks. Store between 10-27°C (50 -80 °F)

Storage stability: Keep from freezing

Technical protective measures: Handle in accordance with good industrial hygiene and safety practice.Wash after handling.

8 EXPOSURE CONTROLS AND PERSONAL PROTECTION

Engineering measures: When handling, the working area should be well ventilated.

Respiratory protection: As with most industrial chemicals , use well ventilated area.

Eye protection: Safety goggles.(with side shields)

Skin protection: Protective clothing, rubber or plastic gloves
Handle in accordance with good industrial hygiene and safety practice.

9 PHYSICAL AND CHEMICAL PROPERTIES

Form	Liquid
Odour:	Minimal
%Solids by Weight	**35%**
%Volitile by Volume	**56%**
pH	**7.0-8.0**
Specific Gravity	**1.04**
Density	**8.65**
Solubility in Water	**Dispersible**
Molecular weight	**Not determined**
VOCs (lbs/gallon)	**<0.1 lbs/gal**

Evapouration rate	Slower than water
Boiling temp (°F):	>212F
Flash point(°C):	**>**212F

10 STABILITY AND REACTIVITY

Chemical Stability:	Stable under normal conditions of handling,use and transportation.
Hazardous Polyimerisation:	Will Not Occur
Conditions to avoid:	Do not expose to high heat or store at temperatures above 100F(38C).Store above 50 F (10C)
Materials to avoid:	Acids and cationic materials will cause the dispersion to separate.
Hazardous Decomposition Products:	Combustion of the dried polymer may release: Carbon Dioxide,Carbon Monoxide,Oxides of Nitrogen , Traces of HCN.
Addition Guildlines:	Not Applicable

11 TOXICOLOGICAL INFORMATION

Acute Effects: Acute Health Effects of this product have not been determined. The following information is available on major components: None available

Chronic Effects: Chronic Health Effects of this product have not been determined. The following information is available on major components: None available

Aggravated Conditions: Not determined

Carcinogenicity: Carcinogenic effects of this product have not been determined. The following information is available on major components: None available

Reproductive/Developmental Toxicity: Reproductive/Developmental health effects of this product have not been determined. The following information is available on major components: None available

Mutagenicity: Mutagenicity of this product has not been determined. The following information is available on major components: None available

Other: Not determined

12 ECOLOGICAL INFORMATION

No data available

13 DISPOSAL CONSIDERATIONS

Other Disposal Considerations: None

Contaminated Packaging: If empty container retains product residues, all label precautions must be observed. Transport with all closures in place. Return for reuse or dispose according to national or local regulations.

14 TRANSPORT INFORMATION

NOT REGULATED

5 REGULATORY INFORMATION

LABEL FOR SUPPLY:

SIGNAL WORD:
WARNING

HAZARD STATEMENTS

PRECAUTIONARY STATEMENTS

Precautionary statement:
P302 P352 – IF ON SKIN Wash with plenty of soap and water
P301 P312 – IF SWALLOWED Call a poison centre or doctor/physician if you feel unwell
P305 P351 P338 – IF IN EYES Rinse continuously with water for several minutes. Remove contacts if present and easy to do – continue rinsing.

16 OTHER INFORMATION

Additional Information:

Not Applicable

Important Note:

The company makes no warranty regarding the safety of this product when used incorrectly.

*******END OF MSDS*******

WEIGHT AND BREAKING STRENGTH

Knotless ripple free netting

Net nr:	Thread diam.:	Mesh size mm 1/2-msk:	Width mesh:	Weight kg pr m:	Weight gr pr m2:	Solidity	Breaking strength kg	Bale size meter
210/30 (10)		10	960	2,69	286		42	160
210/30 (10)		12,5	960	2,50	213		42	180
210/36 (12)		10	960	3,45	367		55	130
210/36 (12)		13	960	3,20	262		55	130
210/36 (12)		14	960	3,10	235		55	140
210/36 (12)		15,5	960	3,05	209		55	140
210/42 (14)	1,45	13	960	3,80	311	0,22	61	110
210/42 (14)	1,45	14	960	3,75	285	0,21	61	110
210/42 (14)	1,45	15,5	960	3,70	254	0,19	61	110
210/48 (16)	1,51	13	1008	4,75	373	0,23	67	95
210/48 (16)	1,51	14	1008	4,60	335	0,22	67	95
210/48 (16)	1,51	15,5	1008	4,40	290	0,19	67	95
210/48 (16)	1,51	18	1008	4,30	244	0,17	67	95
210/48 (16)	1,51	19,5	1008	4,15	217	0,15	67	110
210/48 (16)	1,51	22,5	1008	4,10	186	0,13	67	110
210/48 (16)	1,51	25	1008	4,05	165	0,12	67	110
210/60 (20)	1,70	14	1008	5,85	426	0,24	89	75
210/60 (20)	1,70	15,5	1008	5,75	379	0,22	89	75
210/60 (20)	1,70	16,5	1008	5,65	349	0,21	89	75
210/60 (20)	1,70	18	1008	5,45	309	0,19	89	75
210/60 (20)	1,70	19,5	1008	5,40	283	0,17	89	75
210/60 (20)	1,70	22,5	1008	5,20	236	0,15	89	80
210/60 (20)	1,70	25	1008	5,05	206	0,14	89	80
210/60 (20)	1,70	28,5	1008	4,95	177	0,12	89	80
210/72 (24)	1,73	15,5	1008	6,40	421	0,22	96	70
210/72 (24)	1,73	16,5	1008	6,30	390	0,21	96	70
210/72 (24)	1,73	18	1008	6,20	351	0,19	96	70
210/72 (24)	1,73	19,5	1008	5,95	311	0,18	96	70
210/72 (24)	1,73	22,5	1008	5,60	254	0,15	96	80
210/72 (24)	1,73	25,5	1008	5,50	220	0,14	96	80
210/72 (24)	1,73	28,5	1008	5,40	193	0,12	96	80
210/96 (32)	1,87	18	1008	7,15	405	0,21	118	60
210/96 (32)	1,87	19,5	1008	6,95	364	0,19	118	60
210/96 (32)	1,87	22,5	1008	6,85	311	0,17	118	60
210/96 (32)	1,87	25,5	1008	6,70	268	0,15	118	60
210/96 (32)	1,87	29	1008	6,35	223	0,13	118	60
210/108 (36)	1,98	19,5	1008	8,65	453	0,20	138	50
210/108 (36)	1,98	22,5	1008	8,25	374	0,18	138	50
210/108 (36)	1,98	25,5	1008	7,95	318	0,16	138	55
210/108 (36)	1,98	27	1008	7,85	297	0,15	138	55
210/108 (36)	1,98	29	1008	7,80	274	0,14	138	55
210/108 (36)	1,98	35	1008	7,60	222	0,11	138	55
210/120 (40)	2,15	22,5	1008	9,70	440	0,19	154	50
210/120 (40)	2,15	25,5	1008	9,15	366	0,17	154	50
210/120 (40)	2,15	27	1008	8,80	333	0,16	154	50
210/120 (40)	2,15	29	1008	8,60	303	0,15	154	50
210/120 (40)	2,15	32	1008	8,40	268	0,13	154	50
210/138 (46)	2,23	25,5	768	7,75	402	0,17	172	60
210/138 (46)	2,23	27	768	7,40	362	0,17	172	60
210/138 (46)	2,23	29	768	7,15	326	0,15	172	60
210/138 (46)	2,23	32	768	7,10	293	0,14	172	60
210/156 (52)	2,35	29	768	8,55	390	0,16	192	50
210/156 (52)	2,35	32	768	8,40	347	0,15	192	50
210/156 (52)	2,35	35	768	8,30	314	0,13	192	50

Table 1 Description of index and ancillary criteria and relevant scores for the gill scoring protocol

Pathology (score)	Lamellar hyperplasia	Lamellar fusion	Cellular anomalies	Lamellar oedema
Index criteria				
None (0)	Not significant—none or very minor	Not significant—none or very minor	None	None
Mild (1)	Mild increase in epithelial cells (low-level of focal or widespread hyperplasia, affecting <10% of gill tissue)	Occasional focal fusion of filaments (<10% of gill tissue affected)	Scattered occasional degenerating or necrotic cells or focal areas of sloughing	Epithelio—capillary separation with proteinacous fluid in the epithelio-capillary space: <10% of gill tissue affected
Moderate (2)	Moderate widespread or multifocal hyperplasia, affecting 10–50% of the tissue	Multifocal areas of fusion (moderate widespread hyperplasia affecting 10–50% of gill tissue) interspersed with normal gill tissue	Degenerating cells, necrotic cells and/or cell sloughing in multifocal areas throughout the tissue	10–50% of gill tissue affected
Severe (3)	Extensive lamellar hyperplasia, >50% of gill tissue affected	Extensive fusion and loss of normal architecture, >50% of gill tissue affected	Widespread necrosis, degeneration and/or sloughing visible throughout section	>50% of gill tissue affected
Ancillary criteria—score for presence (1) or absence (0)				
Inflammation (presence of inflammatory cells outside the blood vessels)				
Eosinophilic granular cells (EGCs) (higher than normal numbers of ECGs in gill filament)				
Circulatory disturbances (thrombi, telangiectasis, congestion), score = 1 if >10% tissue is affected				
Cellular hypertrophy (hydropic degeneration of lamellar cells)				
Bacteria—*Tenacibaculum* spp. (mats of filamentous bacteria on lamellar surfaces)				
Bacteria—Epitheliocystis				
Other bacteria				
Protist parasites—*Neoparamoeba*				
Protist parasites—*Costia*				
Protist parasites—*Trichodina*				
Other parasites				

Common feeding, growth and conversion rates in sea bream cage farming

Data obtained from 150 batches reared in commercial cages in the Mediterranean coast of Spain.

SGR%day	10.1 a 20	20.1 a 35	35.1 a 50	50.1 a100	100.1 a 150	150.1 a 200	200.1 a 250	250.1 a 300	300.1 a 350	350.1 a 400	400.1 a 450
<14.1	0,37	0,22	0,12	**0,03**	**0,03**	0,02	0,02	0,02	0,02	0,01	0,01
14.1 a 15	0,79	0,49	0,27	0,13	0,09	0,08	0,07	0,06	0,05	0,05	0,04
15.1 a16	1,06	0,68	0,37	0,23	0,14	0,12	0,10	0,09	0,08	0,08	0,06
16.1 a17	1,21	0,84	0,49	0,29	0,22	0,20	0,19	0,17	0,15	0,13	0,09
17.1 a 18	1,44	1,02	0,68	0,45	0,35	0,29	0,24	0,21	0,19	0,17	0,13
18.1 a 19	1,74	1,22	0,87	0,68	0,54	0,44	0,34	0,28	0,24	0,21	0,16
19.1 a 20	2,03	1,42	1,03	0,74	0,62	0,53	0,44	0,37	0,33	0,27	0,20
20.1 a 21	2,67	1,80	1,27	0,91	0,77	0,67	0,55	0,46	0,42	0,37	0,30
21.1 a 22	3,30	2,33	1,59	1,10	0,95	0,83	0,75	0,63	0,54	0,47	0,37
22.1 a 23	3,60	2,52	1,79	1,23	1,04	0,91	0,82	0,68	0,58	0,49	0,37
23.1 a 24	3,93	2,83	2,07	1,47	1,22	1,04	0,91	0,75	0,65	0,54	0,40
24.1 a 25	4,33	3,34	2,32	1,76	1,37	1,19	1,04	0,88	0,76	0,63	0,48
25.1 a 26	4,54	3,71	2,52	1,91	1,56	1,30	1,10	0,95	0,80	0,69	0,55
>26	3,77	2,73	2,15	1,66	1,29	1,09	0,96	0,82	0,70	0,57	0,44

SFR%day	10.1 a 20	20.1 a 35	35.1 a 50	50.1 a100	100.1 a 150	150.1 a 200	200.1 a 250	250.1 a 300	300.1 a 350	350.1 a 400	400.1 a 450
<14.1	1,67	1,10	0,66	0,22	0,19	0,18	0,17	0,15	0,14	0,11	0,10
14.1 a 15	1,92	1,32	0,83	0,44	0,33	0,28	0,25	0,22	0,21	0,20	0,18
15.1 a16	2,19	1,59	0,99	0,66	0,44	0,37	0,34	0,32	0,31	0,29	0,27
16.1 a17	2,31	1,74	1,20	0,78	0,61	0,58	0,55	0,52	0,48	0,44	0,35
17.1 a 18	2,55	1,95	1,53	1,09	0,89	0,78	0,65	0,60	0,56	0,50	0,44
18.1 a 19	2,83	2,26	1,79	1,45	1,20	1,04	0,85	0,71	0,64	0,57	0,51
19.1 a 20	3,22	2,53	2,07	1,61	1,40	1,21	1,06	0,91	0,86	0,75	0,64
20.1 a 21	3,50	2,65	2,00	1,50	1,32	1,20	1,03	0,90	0,87	0,81	0,75
21.1 a 22	4,02	3,19	2,40	1,80	1,60	1,45	1,34	1,20	1,07	0,99	0,89
22.1 a 23	4,10	3,28	2,60	1,90	1,70	1,51	1,40	1,22	1,10	1,00	0,90
23.1 a 24	4,20	3,40	2,77	2,10	1,83	1,65	1,50	1,32	1,20	1,08	0,96
24.1 a 25	4,33	3,67	2,97	2,50	2,06	1,86	1,67	1,47	1,33	1,19	1,07
25.1 a 26	4,45	3,82	3,07	2,65	2,28	1,96	1,70	1,54	1,36	1,24	1,18
>26	4,28	3,55	2,90	2,36	1,93	1,75	1,60	1,40	1,27	1,10	1,00

FCR	10.1 a 20	20.1 a 35	35.1 a 50	50.1 a100	100.1 a 150	150.1 a 200	200.1 a 250	250.1 a 300	300.1 a 350	350.1 a 400	400.1 a 450
<14.1	4,57	5,06	5,72	6,38	6,88	7,26	7,65	7,92	8,20	8,69	9,90
14.1 a 15	2,43	2,70	3,04	3,42	3,53	3,64	3,73	3,77	4,02	4,23	4,90
15.1 a16	2,06	2,33	2,69	2,91	3,03	3,09	3,29	3,40	3,62	3,73	4,27
16.1 a17	1,91	2,07	2,45	2,69	2,81	2,86	2,94	3,06	3,20	3,29	3,77
17.1 a 18	1,78	1,91	2,24	2,40	2,54	2,64	2,78	2,84	2,94	3,00	3,43
18.1 a 19	1,63	1,85	2,06	2,14	2,23	2,35	2,49	2,55	2,64	2,71	3,13
19.1 a 20	1,58	1,78	2,01	2,19	2,25	2,29	2,39	2,47	2,65	2,74	3,14
20.1 a 21	1,31	1,47	1,58	1,65	1,72	1,79	1,86	1,96	2,07	2,16	2,51
21.1 a 22	1,22	1,37	1,51	1,63	1,69	1,74	1,79	1,89	1,99	2,09	2,43
22.1 a 23	1,14	1,30	1,45	1,55	1,63	1,66	1,70	1,80	1,90	2,04	2,46
23.1 a 24	1,07	1,20	1,34	1,43	1,50	1,58	1,65	1,75	1,85	1,99	2,40
24.1 a 25	1,00	1,10	1,28	1,42	1,50	1,56	1,60	1,68	1,76	1,88	2,25
25.1 a 26	0,98	1,03	1,22	1,39	1,46	1,51	1,55	1,62	1,69	1,80	2,14
>26	1,14	1,30	1,35	1,42	1,50	1,60	1,67	1,70	1,82	1,93	2,29