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Application of Quality, Safety and Traceability system procedures in the aquaculture production of new species in the Madeira Autonomous Region (RAM)

DOCTORAL THESIS

Jorge Dinis Câmara Freitas
DOCTORATE IN CHEMISTRY

UNIVERSIDADE da MADEIRA
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Abbreviations

AQbD - analytical quality-by-design

BMD - biomarker discovery analysis

BTS - bacterial test standard

CN/CNL - Caniçal

CAR/PDMS - carboxen/polydimethylsiloxane

CMAs - critical method attributes

CMPs - critical method parameters

CMP - critical method parameters

CE - capilar electroforese

CFU - colony forming units

CpK - process capability index

DMA - dimethylamine

DVB/CAR/PDMS - divinylbenzene/carboxen/polydimethylsiloxane

DoE - design of the experiment

EI - electron impact

FA - formaldehyde

FFD - fractional factorial design

GC-MS - gas chromatography-mass spectrometry

GSB - gilthead seabream

GrA - greater amberjack

HCA - hierarchy clustering analysis

HCCA - cyano-4-hydroxycinnamic acid

HS-SPME - headspace solid-phase microextraction

HPLC - high-performance liquid chromatography

HL - high concentration level

IP - intermediate precision

Ir - iron agar

IPx - Ilhapeixe SA

IUU - illegal, unreported, and unregulated

ISO - International Standard Organization

IR - infra red

KS - knowledge space

LOD - limit of detection

LOQ - limit of quantification

LSL - lower specification limit

LL - Low concentration level

mID - microorganisms identification

MBT - MALDI BioTyper

MODR - method operable design region

ME - matrix effects

McA - MacConkey agar

MALDI-TOF MS - Matrix-Assisted Laser Desorption Ionization-Time-of-Flight Mass Spectrometry

ML - medium concentration level

Nt - nutrient agar

NMR - nuclear magnetic resonance

NCBI - National Center for Biotechnology Information

PR - peak resolution

PCA - principal component analysis

PDMS/DVB - polydimethylsiloxane/divinylbenzene

QIM - quality index method

QI - quality index

QRA - quality risk assessment

QDA - quality descriptive analysis

qPCR - quantitative polymerase chain reaction

RB - Ribeira Brava

RBP - Ribopeaks

RSD - relative standard deviation.

RFID - Radio Frequency Identification

STCZ - Santa Cruz

SF - symmetry Factor

SSO - specific spoilage organisms

TFA - trifluoro acetic acid

TCA - trichloroacetic acid

TMA - trimethylamine

TVB-N - total volatile base-nitrogen

TMAO - trimethylamine oxide

TMAOase - trimethylamine oxide demethylase

TVC - total viable counts

TPA - total peak area

TF - tailing factor

TRM - Torrymeter

USL - upper specification limit

WSN - Wireless Sensor Networks

3M - three months

6M - six months

12M - twelve months

Abstract

The resolution of issues related with sustainability and food safety by the industry requires transformation and adaptation to new monitoring/evaluation procedures, to assure consumers about the proclaimed quality.

Therefore, this work aims to support the knowledge transference to the industry assisting in the adaptations required to this new paradigm of the aquaculture sector. Three main areas were identified to demonstrate overall quality: freshness, traceability, and safety.

Product freshness was studied to improve knowledge about spoilage evolution of the fish species produced in the aquaculture facilities. During this study it was possible to develop a sensory approach based on QIM methodology for data collection and treatment. A timeline for the product spoilage was determined for the studied species, for gilthead seabream it was established 9 days (±1 day) and for greater amberjack 12 days (±1 day).

Product traceability studies were focused on the development of a methodology to determine the fish origin. The method studied was based on protein mass fingerprint of the fish mucus, acquired using MALDI-TOF equipment. As result it was possible to discriminate between samples with different origins, resulting in a list of 35 potential biomarkers.

The bacterial community around the production cages was selected as a safety concern. Therefore, the MALDI-TOF equipment capabilities were studied to accomplish the microorganisms' identification. As a main result, it was confirmed the possibility to use the Ribopeaks database to assess the bacterial community diversity around sea cages and its seasonal variation.

The multi-disciplinary approach used in this project was very useful in the improvement of the company knowledge and management procedures. It also contributes with indications on how to address relevant issues on the aquaculture sector, such as traceability, safety, and freshness, from the methodologies that can be used to how to gather and treat the data.

Keywords: Quality, Freshness, Safety, Traceability, Authenticity, Aquaculture

Resumo

A resolução das questões relacionadas com fraude, sustentabilidade ou segurança alimentar requerem por parte da indústria adaptação a novos procedimentos de monitorização/avaliação, assegurando aos consumidores a qualidade declarada.

O objetivo deste trabalho é a transferência de conhecimento para a indústria auxiliando nas adaptações necessárias a este novo paradigma do setor da aquacultura. Três áreas principais foram identificadas para demonstração da qualidade total: frescura, rastreabilidade e segurança.

O estudo da frescura do produto teve como objetivo melhorar o conhecimento da evolução da deterioração das espécies de peixe produzidas nas instalações aquícolas. Durante este estudo foi desenvolvida uma abordagem sensorial baseada na metodologia QIM para a recolha e tratamento de dados. Foi estabelecido o tempo de deterioração para a dourada cerca de 9 dias (± 1 dia), e para o charuteiro, 12 dias (± 1 dia).

O principal foco dos estudos de rastreabilidade foi o desenvolvimento de uma metodologia para determinar a origem dos peixes produzidos. O método estudado baseou-se na análise do padrão de massas iónicas da fração proteica do muco do peixe por MALDI-TOF. Como resultado foi possível discriminar entre amostras de diferentes origens, através de 35 biomarcadores.

A análise da comunidade bacteriana em torno das jaulas foi selecionada como ponto de interesse em termos de segurança. Foram estudadas as capacidades do equipamento MALDI-TOF para efetuar a identificação dos microrganismos, obtendo-se resultados promissores, além de confirmada a capacidade da base de dados Ribopeaks para analisar a diversidade bacteriana em torno das jaulas e a sua variação sazonal.

Deste modo a abordagem multidisciplinar utilizada neste projeto foi muito útil na melhoria dos conhecimentos e procedimentos de gestão da empresa. Também contribuiu com indicações sobre como abordar questões relevantes no setor da aquacultura, como rastreabilidade, segurança e frescura, desde as metodologias que podem ser utilizadas até à recolha e tratamento de dados.

Palavras-chave: Qualidade, Frescura, Segurança, Rastreabilidade, Autenticidade; Aquacultura

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Thesis Publications

Published works

Freitas, J.; Vaz-Pires, P.; Câmara, J.S. From aquaculture production to consumption: Freshness, safety, traceability and authentication, the four pillars of quality. **Aquaculture** 2020, 518, 734-857. doi: 10.1016/j.aquaculture.2019.734857. (IF: 4.240)

Freitas, J.; Vaz-Pires, P.; Câmara, J.S. Quality Index Method for fish quality control: Understanding the applications, the appointed limits and the upcoming trends. **Trends Food Sci. Technol**. 2021, 111, 333–345, doi: 10.1016/j.tifs.2021.03.011. (IF: 12.563)

Freitas, J.; Vaz-Pires, P.; Câmara, J.S. Freshness assessment and shelf-life prediction for *Seriola dumerili* from aquaculture based on the quality index method. **Molecules** 2019, 24, 3530. doi:10.3390/molecules24193530. (IF: 4.411)

Freitas, J.; Silva, P.; Vaz-Pires, P.; Câmara, J.S. A Systematic AQbD Approach for Optimization of the Most Influential Experimental Parameters on Analysis of Fish Spoilage-Related Volatile Amines. **Foods** 2020, 9, 1321. doi:10.3390/foods9091321. (IF: 4.350)

Freitas, J.; Silva, P.; Perestrelo, R.; Vaz-Pires, P.; Câmara, J.S. Improved approach based on MALDI-TOF MS for establishment of the fish mucus protein pattern for geographic discrimination of *Sparus aurata*. **Food Chemistry**. doi: 10.1016/j.foodchem.2021.131237 (IF: 7.514)

Submitted

Freitas, J.; Perestrelo, R.; Vaz-Pires, P.; Câmara, J.S. Bacterial diversity analysis of coastal superficial seawaters near aquaculture facilities, using MALDI-TOF approach and Ribopeaks database. Submitted to **Aquaculture**. (IF: 4.240)

Oral Communications

Mass Fingerprint based approach for origin discrimination of Madeira aquacultured *Sparus Aurata*. Freitas, J.; Silva, P.; Perestrelo, R.; Vaz-Pires, P.; Câmara, J.S. **Aquaculture Europe** 4th to 7th of October **2021.**

Fish quality evaluation from Madeira aquaculture production. Freshness analysis and rejection day estimation, through complementary approaches. Freitas, J.; Vaz-Pires, P.; Câmara, J.S. **7th CQM Annual Meeting**. 21st to 22nd of September **2020**.

Development of Quality Index Method (QIM) to evaluate the freshness *of Seriola dumerili*. Freitas, J.; Vaz-Pires, P.; Câmara, J.S. **6th CQM Annual Meeting**. 26th to 27th of April **2019**.

Poster presentation

Freitas, J.; Silva, P.; Perestrelo, R.; Vaz-Pires, P.; Câmara, J.S Fish mucus mass fingerprinting-based analysis for geographic origin discrimination of *Sparus aurata* from mariculture. 23rd International Symposium on Advances in Extraction Technologies. Spain. 30th June-2nd July **2021**.

J. Freitas, P. Silva, P. Vaz-Pires, J. Câmara. Development of a SPME/GC-MS method based on Quality by Design approach, for evaluation of fish deterioration progress through TMA and DMA quantification. Encontro Nacional de Cromatografia, Caparica, Portugal **2019**, December, nº [pag 106- P53].

J. Freitas, P. Vaz-pires, J. Câmara. Development of a Quality lndex Method as useful approach to evaluate freshness of Greater amberjack (*Seriola dumerili*). 32^{nd} European Federation of Food Science and Technology lnternational Conference, Nantes, France, 6^{th}-8^{th} November **2018**, ns [P1'0621'].

J. Freitas, P. Silva, P. Vaz-Pires, J. Câmara. Volatile amines as a valuable approach for fish freshness evaluation. 32^{nd} European Federation of Food Science and Technology lnternational Conference, Nantes, France, 6^{th}-8^{th} November **2018** ne tp1.0631.

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General Introduction

Introduction

In the current decade (2020-2030), one of the main goals of the European Union (EU) is to be refocused on the ocean's importance towards EU development. The Horizon Europe program, the European Union's research and innovation program for the period 2021-2027, is focused on four ocean dimensions: environmental, social, economic, and geopolitical. The aim is to make a significant contribution to the strategic EU Ecological Pact, part of the European commitment to implement the United Nations (UN) 2030 Agenda. In this case the sustainable exploration of the ocean is one of the main objectives to reap all the benefits generated in it and from it, including a circular and inclusive blue economy [1].

One of the sectors identified as having a great potential to contribute to achieving such objectives is aquaculture. Aquaculture is commonly defined as the breeding of aquatic organisms (*e.g.*: fish, algae, mollusks, and crustaceans) under controlled conditions, involving both marine and freshwater species. Due to the stagnation of fishing in the last 20-30 years, aquaculture emerged as one of the most important alternatives in supplying the nutritional needs of an expanding human population, becoming the fastest growing food-producing sector in terms of annual growth rate [2]. Such fast progression has been nominated as *Blue Revolution* and besides its benefits, has been under strong criticism due to environmental concerns (*e.g.*: pollution) and socio-economic pressures (*e.g.*: conflicts with other economic sectors) [3,4].

Therefore, to avoid past mistakes and protect the future (*e.g.*: agriculture and meat production) in a sustainable way, is necessary to continue to increase the development of knowledge as well to improve its transference to the important stakeholders and support governance regulatory measures [5].

Thesis Aims

The aim of this doctoral thesis was to transfer knowledge and research protocols, commonly used in aquaculture research, and adapt them to the corporative requirements, in order to improve procedures and information on the species with aquaculture interest. It also has as objective to prepare for the adaptations needed in the future, to correspond in terms of regulations/laws, consumer perspectives and demonstration of responsible/sustainable production.

To achieve the purposed aims four main areas of investigation were followed under the quality concept: freshness determination; product authenticity; product traceability; and product safety. Due to relative correlation between authenticity and traceability approaches, and company needs, it was given higher relevance to traceability . The studied fish species were the gilthead seabream (*Sparus aurata*) and greater amberjack (*Seriola dumerili*), produced in marine cage culture located at Madeira Autonomic Region (Região Autónoma da Madeira – RAM), Portugal.

The stipulated objectives under the main areas were:

Fish freshness:

i) Determine the main physical, chemical, and bacteriological alterations of seabream and amberjack during ice storage.

ii) Determine the time that gilthead seabream and greater amberjack sold as a whole, maintain its freshness.

iii) Develop a Quality index protocol for evaluation and remaining time of gilthead seabream and greater amberjack freshness.

Product authenticity and traceability:

iv) Determine if is possible to discriminate between the seabreams produced in different locations.

v) Establish a methodology to determine the fish origin.

Safety:

vi) Establish an approach for the determination of the bacterial community surrounding the aquaculture cage structure.

vii) Determine the bacterial community surrounding the facilities at different times of the year.

viii) Evaluate potential interferences from anthropogenic origin on the waters surrounding the facilities.

ix) Evaluate the possible environmental impact of the aquaculture exploration on the surrounding waters.

The employed methods are commonly used in the research field and with limited application on commercial organizations due to acquisition costs or personnel expertise. Therefore, they were used as potential approaches to obtain relevant data and knowledge transference to the real corporative environment and adapted to its specific characteristics.

Thesis outline and structure

This doctoral thesis was conceived in the form of scientific articles, where the reported research has been submitted, accepted or published in peer-reviewed scientific journals of the field.

Chapter 1 is a manuscript that comprises a specific introduction and bibliographic review on the topic of aquaculture quality, integrating four areas freshness, traceability, authentication, and safety.

Chapter 2 reviews the Quality Index Method protocol (QIM) used for freshness studies of the species of interest.

Chapter 3 is related to the development of the first QIM for the species *Seriola dumerili*.

Chapter 4 is focused on the *Sparus aurata* freshness using chemical analysis.

Chapter 5 evaluates the traceability and origin determination of *Sparus aurata*.

Chapter 6 addresses the analysis of the seawater bacterial community surrounding the sea cages.

The final chapter General Conclusion addresses the future perspectives and final considerations about the developed work.

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Chapter 1 – From aquaculture production to consumption: freshness, safety, traceability, and authentication, the four pillars of quality.

Freitas, J.; Vaz-Pires, P.; Câmara, J.S. *From aquaculture production to consumption: Freshness, safety, traceability and authentication, the four pillars of quality*. **Aquaculture** 2020, 518, 734-857. https://doi.org/10.1016/j.aquaculture.2019.734857

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Review

From aquaculture production to consumption: Freshness, safety, traceability and authentication, the four pillars of quality

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Abstract

Farmed aquatic products are among the most widely traded commodities and one of the sectors with the fastest growth in the last years. However, aquaculture is still affected by negative connotations in comparison with other agroindustry sectors. Markets, consumer preferences and concerns about food safety and sustainability are influencing the growth of the sector and are forcing the implementation of quality management systems. Modern management systems help to minimize the environmental impacts and the distribution of unsafe or poor-quality products, thereby reducing the potential for bad image, liability and recalls. This article presents a comprehensive overview of the status, relevance, and impact of the quality management systems in the development of marine aquaculture, with the focus on four of the most important criteria associated with these systems: freshness, safety, traceability, and authenticity.

Keywords

Seafood, Freshness, Safety, Traceability, Authenticity, Aquaculture

1.1 Introduction

Aquaculture production is steadily increasing every year, while capture production has almost stabilized in the last 20-30 years, meaning that aquaculture represents enormous potential to supplement the quantities from the catch while alleviating the pressure on natural stocks. In 2014, an important milestone was reached, for the first time 50% of the fish, for human consumption (excluding non-food uses such as fishmeal or fish oil), was from aquaculture production [1]. In 2016 the value for human consumption was 53% while considering the total global fish production aquaculture represented 47% [2]. It is expected that world aquaculture production becomes higher than that of capture fisheries by 2025 [3].

Regional or local supply no longer limits the retailers' and food industries' response to market demand. Technological improvements have altered the production, trade, and distribution in the seafood industry, allowing it to source their products from all over the world, transforming the industry into an interconnected system with a large variety of complex relationships [4]. As consequence, the inherent complexity of the seafood supply chain, allows the opportunity for the development of ''obscure paths'' that leads to illegal, unreported, and unregulated (IUU) activities, that also implicates quality control failures (*e.g.*: heavy metals), seafood fraud (*e.g.*: mislabelling), unsustainable fishery (*e.g.*: overfishing and pollution), and human rights (*e.g.*: unfair payment) [5,6].

Even though aquaculture has clear benefits in several domains, such as social (*e.g.*: employment, price stability), environmental (*e.g.*: bioremediation, habitat structure) or product quality (*e.g.*: freshness and safety), negative perceptions or assumptions about the sector still exist [7-9]. Consumer attitudes, and perceptions, regarding aquaculture or fisheries, can impact the success and acceptance of new food products [10,11].

Consumer decisions about a food product do not depend solely on the associated pleasure or on its organoleptic properties [12,13]. It depends on many personal expectations that differ from consumer to consumer as well as with the culture or geographical localization in which the product is marketed [7]. Even though seafood products seem to be safer than ever before, from a technical point of view and due to several quality control programs, the awareness and concerns of consumers are increasing and further redefining industry quality parameters [9,12,14]. The increasing interest in fish quality in all parts of the seafood chain is particularly true for aquaculture, in which the products and production processes have several specific characteristics that influence product safety and quality assurance throughout all production chains. Factors include product variation, production yields, and shelf-life, which are influenced by, for example, weather conditions, biological variation, cooling facilities and hygienic measures [15]. Some other specific hazards exist in the production and distribution of seafood, such as the sources of raw materials and the incorporation of many participants. In the last case, the large number of formal and informal relationships during captures, processing and transactions, as their multitude of specific activities and common practices, increase the difficulty of regulatory agencies and governments to control and oversee the sector [15–17]. National and international authorities are responding to this by implementing new legislation and regulations to ensure safe and animal-friendly production, sustainability and lower pollution levels [16;18]. Similar principles from the livestock industry are being adapted to aquaculture: 1) ensuring food safety and quality; 2) improving processing technology; 3) adding value to products; 4) expanding supplies and markets. These topics are closely interconnected and focused on ensuring quality for the consumer and on helping businesses to prosper [19]. The application of these principles in the aquaculture supply chain, besides being voluntary, is also becoming a common requirement for the main importer countries, in order to safeguard public health and demonstrate that the product originates from legal and sustainably managed aquaculture according to codes of best practices [4]. A complete understanding of the relationship between fish product attributes, social/ethical problems, environmental impact and issues underlying food traceability and authentication is very relevant in this context, not only to consumer quality judgments and perception of product value but also to all players in supply chain [15;18]. Therefore, the purpose of this review is to present an overview of the application of quality management principles in the aquaculture sector, giving relevance to important quality management attributes such as freshness, safety, traceability, authenticity and how they interact with each other in the final products quality perception.

1.2. Defining quality and its attributes for evaluation

It is generally known that seafood products are one of the most vulnerable and perishable food items. For seafood benefits to be fully realized, it must have to be produced and maintained in secure conditions and remain as fresh as possible to the point of purchase by the consumer.

According to Bremner (2000), the term ‘‘quality’’ in the food science literature is often misused, creating some confusion between the terms employed for conceptual discussion and the ones used for the practical report of measurable data. The problem with ‘quality’ definitions is that they do not clearly indicate which attributes or indicators should be measured, to assess quality in any particular production situation, the types of raw materials used, or in the final products [20]. In this regard, quality is defined by the International Standard Organization (ISO) as “*the totality of features and characteristics of a product that bear on its ability to satisfy stated or implied needs*”. In addition, quality can be defined as “conformance to requirement”, “fitness for use” or, more appropriately for foodstuffs, "fitness for consumption" [20]. Thus, quality is also described as the requirements necessary to satisfy the needs and expectations of the consumer [21].

In the food industry, quality is frequently defined using terms related to nutrition, microbiology, physicochemical characteristics or consumer acceptability [22]. These terms should be analyzed through an integrated vision since all of them contribute to quality assessment in different steps of the supply chain [22]. To overcome such undefined terminology, Bremner proposed a hierarchical approach that encompasses all the aspects (concepts, criteria, specifications of the criteria and methods to provide values for the criteria) [20]. The same methodology is proposed to be applied to other similar generic terms such as freshness and safety [23,24]. Although there are many different formulations of this concept, it is commonly agreed that quality can be generally categorized as: search qualities (*i.e.* attributes perceived before purchase); experience qualities (*i.e.* attributes only perceived when consuming the product); or credence qualities (*i.e.* attributes not readily perceived without explicit clues) [13]. Essentially, food quality is associated with a proactive policy and the creation of requirements to maintain an efficient and secure food supply.

In this work, the term "quality" will have the same meaning as defined by ISO - "*the totality of features and characteristics of a product that bear on its ability to satisfy stated or implied needs*". The chosen criteria to evaluate quality in the aquaculture sector will be freshness, safety, traceability, and authenticity.

1.2.1 Freshness

Freshness is a major basic contributor to quality and safety of fish and fishery products. The term 'fish freshness' is a concept that sums up many factors, (similar to quality as explained in section 1.2) and should be carefully described. According to Oehlenschläger "*Freshness is an ideal of perfect condition or state, when fish properties* *are close to the ones it had while living or immediately post-capture or harvest*" [25]. In fact, different stages of fish freshness can be described using multiple properties, that are known to be associated with the progression of fish spoilage [13]. Besides evaluating its optimal condition (after slaughter), it is possible to estimate its capability to retain those sets of characteristics until the time it is processed, cooked, presented, eaten or all of these, following capture [13,26,27]. These properties are commonly referred to as ‘‘freshness indicators’’, and where described by Bremner and Sakaguchi, as "*that measurable entity that should provide a monotonic response, which can be used to describe one or more of the post mortem changes that have occurred*’’ [23]. In this case, monotonic response refers to the ability of the analyzed parameter to provide specific data results, that are associated with one or more stages of freshness loss. For example, in the case of the measurement of total volatile amines (TVB-N), where the data results increase with the progression of time and is possible to associate a specific value to determine an acceptable condition of the fish for consumption. The most common studied indicators are associated to four specific fields:

- *Sensory – e.g.*: appearance, odor, taste, touch.
- *(Bio)chemical – e.g.*: volatile compounds, proteins, lipids, amino acids and adenosine triphosphate.
- *Physical- e.g.*: muscular structure and color changes.
- *Microbiological – e.g.*: microorganisms’ growth and identification [28,29].

Three main *post mortem* processes that influence these parameters are enzymatic autolysis, oxidation, and microbial degradation. All of them are responsible, to some extent, for the development or transformation of specific substances that contribute to alterations in the previous parameters, characteristic of spoiled fish [30]. Therefore, their evaluation through measurable indicators is a direct measure of time expired since catch and death under the prevailing circumstances [23]. According to Huss (1995), the mentioned changes occur more or less simultaneously but are more important in certain periods that can be divided into four phases, as shown in Figure 1.1 [31].

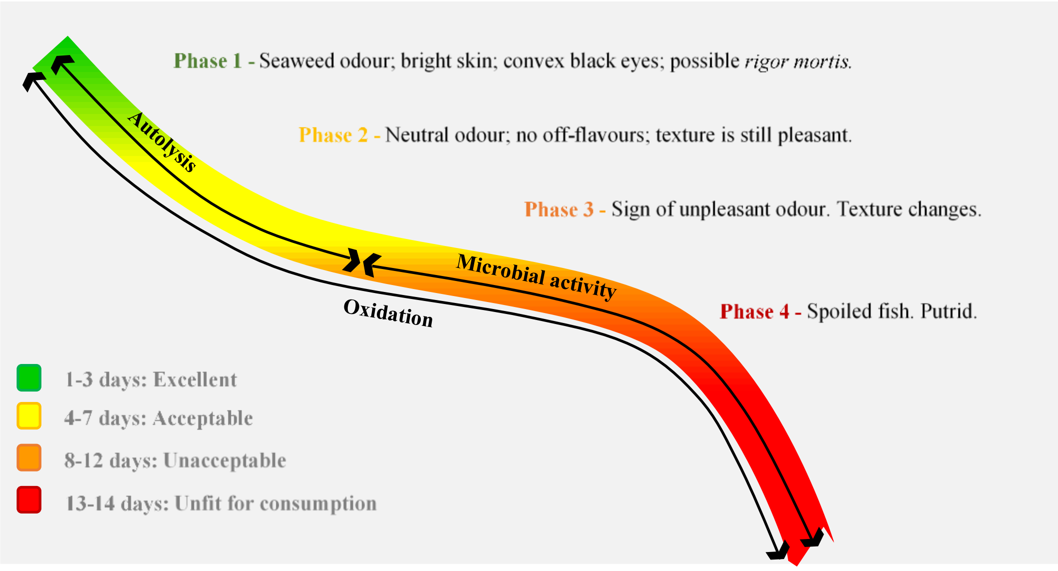

Figure 1.1 - Stages of fish degradation. Progression of fish degradation process, principal changes, common time frame and consumer acceptance, based on Huss (1995).

The shelf-life investigation is another research area, that shares some common principles with freshness. This area of investigation faces the same problems related with definitions as described previously for quality and freshness [24]. The term shelf-life is associated with methods used to delay the impact of *post mortem* processes on seafood characteristics, extending its storage time and consequently its suitability for human consumption. Several techniques are already in use and have been extensively reviewed [28,30,32], involving the use of chemicals (acids, sulfites, oils), modified atmosphere packaging, temperature or pH changes.

1.2.2 Safety

Safety, as quality criteria, is difficult to perceive. A product can appear to be of high-quality standards, but safety hazards may be hidden or go undetected (pathogenic organisms, toxic chemicals or physical hazards) until the product has been consumed. If detected, serious food safety threats may result in market access exclusion and major economic loss or costs [16,21]. Nowadays there is a consensus among specialists that safety is a very important quality pre-requisite. Since food safety hazards directly affect public health and economies, achieving proper food safety must always take precedence over achieving higher levels of other quality criteria [33].

The links between safety and quality can be established in the following ways: quality and safety are both interrelated and linked to trust/confidence. Quality is seen to lead to taste, health, care, and pleasure. Similarly, safety is seen to be the consequence of control, origin, best before date and excellence, while resulting in health and a feeling of calm [21]. On the other hand, food quality is primarily an economical issue decided by the consumer, while food safety is a governmental commitment to ensure that the food supply is harmless for consumers and meets regulatory requirements [21].

Seafood safety is based on conformity to not exceed predetermined levels of contaminants or freedom from foodborne pathogens (*e.g.*: *Salmonella* spp.; *Vibrio* spp.; *Anisakis* spp.); metals (*e.g.*: Hg, Cd, Pb), toxins, pesticides, food additives, preservatives, physical hazards and spoilage [34,35]. As the product moves through the supply chain, safety can also be defined as the style of production, harvesting, preparing, handling and storing, to prevent infection and to help ensure that the food maintains its nutritional value for the consumer [15]. In order to reduce directly or indirectly the presence of hazards, the European Union (EU) has elaborated several regulatory proposals. They stipulate limits in which different hazards can be present in the products and in the environment, restraining the proliferation of emerging diseases or exotic pathogens in the natural ecosystems of receiving countries (*e.g.:* Council Decisions - CD No 2010/221 (EU), CD No 2011/187/EU; Regulations - Reg. No 1143/2014, Reg. (EU) No 324/2011, Reg. (EU)

No 350/2011; Council Implementing Decisions – 2014/22/EU, 2013/706/EC, 2012/31/EU), [36,37]. In this context, food safety is a responsibility shared by producers, processors, distributors, retailers, and consumers.

1.2.3 Traceability

Consumers demand verifiable, authentic, and traceable information as evidence of food excellence and safety. Therefore, there is an increasing need for transparent information on the entire food chain, supported by modern traceability methods.

While food safety is an intrinsic part of food quality, traceability systems are an essential component of food safety and quality management systems [21,33,38]. Traceability systems neither produce safer/high-quality products nor determine liability. Traceability is not a type of information; it is the means by which information is retrieved and hence also stored and arranged [39]. Traceability systems can efficiently collect data and provide information about whether control points in the production or supply chain are operating correctly or not. The more precise the system, the faster a producer can identify and resolve food safety or quality problems [21].

Traceability could answer the questions of "who (*i.e.*, actor/product), what (*i.e.*, actor/product information), when (*i.e.*, time), where (*i.e.*, location) and why (*i.e.* cause/reasons)" with regard to food safety, quality and visibility [21]. In addition, depending on the direction in which information is recalled, supply chain traceability can be defined as tracing or tracking. Backward traceability or tracing is the ability, at every point of the supply chain, to find the origin and characteristics of a product based on one or several given criteria. Forward traceability or tracking is the ability, at every point of the supply chain, to find the product's localization from one or several given criteria [40]. It is important for an information system to support both types of traceability, as the effectiveness for one type does not necessarily imply the effectiveness for the other [21].

Also, the efficiency of a traceability system can be characterized by breadth (*i.e.* the amount of information collected), depth (*i.e.* how far back or forward the system tracks the relevant information) and precision (*i.e.* degree of assurance to pinpoint a particular movement of a food product) to be able to balance cost and benefits [40].

It is worth noting that since traceability is based on systematic recordings and record keeping, there is no guarantee that the recordings are true. Both errors and fraud may lead to untrue claims with respect to the properties of the food product [39].

Nowadays is not enough to have ecolabels or references to sustainable productivity. Consumers are perceiving it as marketing strategies and higher levels of food chain transparency are required in order to gain their confidence [17]. In this case, transparency means, allow to the public/organizations access to information perceived as relevant for them, such as about, sustainability practices, supplier's information or product production, which is something that companies may not be prepared to do, due to concerns about competitiveness loss and costs [17,41].

1.2.4 Authentication

Authentication can be defined as the act of establishing or confirming something (*e.g.*: food of animal origin) as authentic, that is, the claims made by or about the subject are true [42]. The main objectives for authenticity assessment include i) protection against fraud, to safeguard fair trade; ii) ensure correct labeling of products following EU regulations (EC 104/2000); iii) protect endangered species; and iv) support customs examinations [27,43,44]. In addition, the legislative protection of regional foods strengthens the importance of authenticity testing as a quality criterion for food and food ingredients. This might involve confirming the identity of a product (*e.g.*: which species), its geographical origin (*e.g.*: from which farm) and discrimination between production methods (*e.g.*: farmed or wild) [45]. Food authenticity testing does not serve only consumers but also the stakeholders who are seeking the opportunity to assure their food products labeling compliance and branding [45].

1.3. Application of quality attributes in aquaculture

Quality in fish products relates to attributes that fish possess, among distinct species, as well as in the same species. Such attributes vary due to interactions of endogenous factors (*e.g.*: age, proximate composition) as with environmental, nutritional and rearing conditions [13,22]. The most common key attributes associated to consumer conception of food quality and their associated decisions are related with organoleptic characteristics (*e.g.*: taste, odor, flavor); marketable traits (*e.g.*: freshness, size); safety (*e.g.*: parasites, hygiene) and nutritional value (*e.g.*: vitamins, fatty acids) [13].

However, in aquaculture production, to evaluate quality, specific characteristics have to be considered. Pre-harvesting, harvesting and post-harvesting factors will influence the main quality criteria (freshness, safety, traceability, and authentication). Operational parameters like quality of hatcheries that supply the juveniles, rearing conditions, feed quantity, food formulation ingredients, slaughter method and, product manipulation and storage, must be considered and controlled [15]. Nowadays, quality is increasingly being associated with Certification/Standards schemes, in order to: 1) control origin (*i.e.*, wild/farmed) and processing conditions (fresh/frozen, thawed fish); 2) ensure the product's safety; 3) ensure that food products are correctly labeled in terms of which animals are actually processed for consumption; and 4) recognize traceability systems from fish to fork (*e.g*. geographical origin) [21]. The adoption of quality standards/certification schemes (summarized in table 1.1) offers systematic approaches to incorporate the concept of continuous improvement and can be applied to any process in the food chain. This implies more emphasis on freshness and safety control, traceability of food products, environmental issues and animal friendliness [46]. Also, to achieve higher compliance with regulatory and customer requirements, it could be necessary to shift from the basic product control at end of the line, to control all steps in the supply chain [16,46]. This also means control from the feed constituents, hatchery quality, carbon footprint, sustainability and economic performance [47].

Adaptation of such schemes has to be correctly executed from the beginning since all these aspects are critical for the best maintenance of the original fish product excellence. In the following sections quality attributes, previously defined (section 1.2), will be described emphasizing aquaculture production and products.

1.3.1 Product Freshness

Several methods and techniques have been developed to determine and evaluate the alterations of fish freshness. Some of these methods have been extensively reviewed [22,29,48] and are summarized in table 1.2. Briefly, classical indicators to characterize freshness are related with the four groups of parameters: sensory, chemical, physical and microbiological. In recent years, the development of new techniques was focused on the fusion of several of the traditional methods. The aim is to develop sensors [49], spectroscopic techniques, computational methods [27] and/or mathematical models [50], to allow simultaneous analysis of different indicators to overcome the disadvantages associated with classical methods (*e.g.:* time consuming; specialized personal; high amount of sample).

Table 1.1 - Most representative private certification schemes for aquaculture (adapted from Potts et al., 2016).

Name	Species Scope	Global scope	Consumer label
Aquaculture Stewardship Council (ASC)	Abalone, bivalves, freshwater trout, pangasius, salmon, shrimp, tilapia.	Asia, Africa, Australia and Oceania, North America, South America, Central America and Caribbean; Europe.	
Naturland	Carp, salmonids, whitefish, mussels, shrimps, tropical freshwater fish, perch like fish, jack like and cod like fish; and macro algae.	Asia, Africa, Australia and Oceania, South America, Central America and Caribbean; Europe.	
China G.A.P	All species with specific control points for eel, crab, croaker, flounder, shrimp, tilapia.	China.	
Friends of the Sea (FOS)	All species of fish, abalone, bivalves, crustaceans.	Asia, Africa, Australia and Oceania, North America, South America, Central America and Caribbean; Europe.	
Global Aquaculture Alliance Best Aquaculture Practices (GAA BAP)	Barramundi, catfish, golden pompano, jade perch, mussels, pangasius, rainbow trout, salmon, shrimp, tilapia, trout.	Asia, Africa, Australia and Oceania, North America, South America, Central America and Caribbean; Europe.	
The Global Partnership for Good Agricultural Practice (GLOBAL G.A.P.)	35 species of finfish, crustaceans and mollusks (hatchery-based and passive collection of seedlings from the planktonic phase for mollusks).	30 countries from North, Central and South America; Europe; Asia; Australia and Oceania.	None (business to business)
International Federation of Organic Agriculture Movements (IFOAM)	All species for aquaculture.	Asia, Africa, Australia and Oceania, North America, South America, Central America and Caribbean; Europe.	None
Generic food quality and safety standards	HACCP	Systematic approach to the identification, evaluation and control of steps in food manufacturing that are critical to product safety.	
	ISO	International standards to achieve uniformity and to prevent technical barriers to trade throughout the world. Focuses on management.	

Table 1.2 - Summary of the principal methods used for fish freshness determination.

	Sensory			Microbial	Chemical				Physical		Spectroscopy
Method	QIM	Torry scheme	EU scheme	TVC	Volatile compounds	Protein analysis	Lipid oxidation	K-value	Texture Devices (*e.g.*:Torrymeter)	Colour Measurements	UV, VIS NIR; FTIR, Raman spec.; NRM.
Advantages	Human senses. Non-destructive Low cost for industry.			Detection and quantification of microorganisms.	Result of fish decomposition. Detect spoilage molecules. Most used in analytical laboratories.				Fast. Not destructive Suitable on field. Experience not required.	Non-destructive. Fast.	Rapid data acquisition. Parameters simultaneous determination.
Disadvantages	Trained personal. One species one scheme. Torry scheme is destructive.			Time consuming Not all organisms are detected.	Time consuming. Expensive for industry. Specialized personal and equipment. Influence of diverse variables.				Not used in: thawed fish or chilled seawater. Limited for: fillets, high salt content and damaged fish.	Lack standardization. Expensive.	Lack standardization. Relatively expensive.
References	[27]			[133]	[29,48]				[48]		[22]

However, even with the emergence of these technologies, they face some obstacles to be accepted, such as price, recognition as international standard methods or industrial applicability [22]. This means that some of the older methods still prevail in laboratories and companies.

Several works related to fish freshness analysis contributed to clarifying the impact of aquaculture common procedures on fish, leading to improvements on the methods utilized by the aquaculture producers. Examples are the analysis of capture methods [51–53] product processes like washing and gutting [54,55], storage conditions [32,56–58] and diet formulations [59–61]. Also, such studies contributed to clarify that time-temperature reference [62], by itself, does not perfectly describe the fish freshness state [23]. This means that low temperatures are not the only influence on fish freshness. Other factors, such as pre-harvest, harvest, slaughter, and post-slaughter techniques affect every major property of fish flesh (*i.e.*: texture or appearance) in the first few days of storage, contributing to initial freshness state condition and its duration. The inclusion of procedures like starvation period, slaughter in ice:water mixtures and temperature control, contributed to a much slower rate of change in properties of fish from aquaculture. As a consequence, no direct or indirect measure of any of the properties can provide a measure of 'freshness' unless the peri-mortal circumstances are known [23].

Besides temperature control methodologies (*e.g.*: chilling, superchilling, and freezing) [63] other methods were developed to reduce fish spoilage with minimal impact on sensory, physicochemical and nutritional value contributing to seafood shipment over large distances and extended periods of time [64]. Such examples are: processing methods (*e.g.*: drying or smoking) [65]; packaging (*e.g.*: vacuum, modified atmosphere, smart packaging) [66,67]; surface decontamination (*e.g.*: high pressure, irradiation, natural antimicrobials) [64]; edible films [68]; chemical methods [69] and transportation [70,71].

All the produced knowledge leads to a variety of methodologies to choose from, enabling companies to adapt their production methods according to their objectives and/or client demands. The preferable targets for such studies are the most profitable and produced species, like salmonids. Other species have been studied, as they gain relevance in the market, also contributing for diversification of aquaculture production (*e.g.*: *Dicentrarchus labrax* [72], *Sparus aurata* [59], *Seriola dumerili*, *Pagrus major* [73] and *Boops boops* [57]).

1.3.2 Safety in the aquaculture sector.

Although aquaculture has the potential to feed millions of people, improper facility management (*e.g.*: inappropriate stock density, cage location, and feed excess) may severely degrade aquatic ecosystems and pose health risks to consumers, through contamination with natural and man-made hazards [74]. Also, several pathogenic agents that can be found in the aquatic environment, in some cases can affect only aquatic life but in others also affect humans. Consequently, safety control in the aquaculture sector is a basic precondition of adequate quality of the fish, through quality management of the facilities and fish health management plan [75], (for occupational health and safety hazards see Guertler et al., 2016; Holen et al., 2017 or Moreau and Neis, 2009 [76–78]). These control plans will further impact the overall quality conception of the final product, from the consumer perspective (as explained in section 1.3.2.2).

In a broader analysis, as in the case of aquaculture quality, the preconditions for safety can be linked to sustainability, environmental concerns, and product consumption safety. In recent years, the risk of propagation of infectious or toxic agents and the occurrence of disease outbreaks have increased [79]. The principal reasons for disease risk increment are related to: i) consumption of raw or minimally processed fish; ii) international transactions of aquaculture products and their derivatives; iii) diagnosis methodologies; iv) changes in ecological balance; v) pollution and climatic changes [80–82]. To some extent, all contributed to alterations in the dynamics of the relationship between individual animals, infectious agents, and people, influencing pathogen rates of replication and proliferation, broadening transmission times, geographic distribution and host species [80].

In the following subsections, these themes will be discussed, giving emphasis to facilities management and fish diseases control (subsection 1.3.2.1), as to safety in seafood consumption (subsection 1.3.2.2.).

1.3.2.1 Facilities management and disease control.

The occurrence of disease outbreaks of aquatic origin has become one of the most important safety concerns in aquaculture production in the last decades [83]. The occurrence of contamination/infection of fishes or humans, due to bacterial, viral, parasitic or biotoxin infections, has been responsible for the interruption of the production cycles, heavy losses and unsustainable activity development [84].

Fish and other aquatic animals live in symbiosis with their environment which makes them especially sensitive to many substances found in water, both natural and anthropogenic [85]. Disease development in aquaculture can be a multifactorial occurrence that can be explained by the conceptual model of the disease triangle (Figure 1.2), a classic pathology concept [86,87]. This model examines the interactions between the environment [88–90], the host [85,91,92] and the infectious (or abiotic) agent [93–95], to understand how epidemics might be predicted, limited or controlled [86]. The relationship between the three are complex, as the presence of a pathogen does not necessarily lead to the development of disease [75]. Sitjà-Bobadilla and Oidtmann (2017) adapted this concept to aquaculture and resumed it in 13 factors (divided through the host, pathogen and environment) that intervene in the emergence and spread of diseases in the aquaculture industry (Figure 1.2).

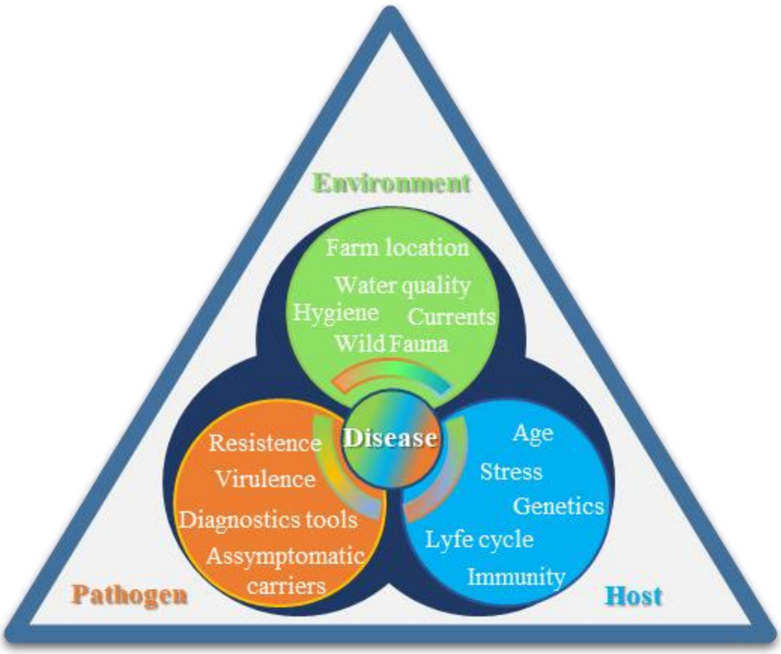

Figure 1.2 - Aquaculture disease triangle. Description of common parameters for evaluation of pathogen, host and environment interactions, based on Sitjà-Bobadilla and Oidtmann (2017) and Scholthof (2007).

Controlling the factors of one of the triangle vertices could impair the disease development, reducing the impact of the outbreak in the production [86]. The most common fish pathogens are presented in tables 1.3, 1.4 and 1.5, addressing virus, bacterial and parasitic diseases, respectively. The tables give a general overview of each type of infectious disease (causative agent, occurrence, symptoms) common in marine aquaculture. Other diseases that affect important economically species like salmonids (viral hemorrhagic septicemia (VHS); infectious hematopoietic necrosis (IHN); infectious salmon anemia (ISA); Koi herpes virus (KHV); epizootic hematopoietic necrosis (EHN); epizootic ulcerative syndrome) are not addressed in the tables. Specific information about them can be found at the European reference laboratory (www.eurl-fish.eu), world organization for animal health (www.oie.int).

Even with accumulated knowledge on pathogenic agents, many others will remain undescribed to science or will pass undetected, due to the vast number of fish species and diversity of pathogens that can infect them, but also due to lack of resources to carry out the necessary studies [93].

In recent years there was greater attention on the implementation of management, production, and biosafety regulations, intending to reduce the impacts of aquaculture in the ecosystems [94,96]. This implies control of the rearing conditions, the susceptibility to disease or pathogen occurrence and environmental degradation, avoiding in this context, an overall reduction in performance (*e.g.*: growth) [97]. The measures needed to mitigate these occurrences are well known both by policy makers as by the aquaculture sector, throughout not only adequate technical equipment installation [88] but also good quality water, well-balanced feed, and good aquaculture practices [35,98].

The implementation of these measures, as well as the development of protocols to monitor the aquaculture activity and its environmental impacts [75,99], are essential for detecting and alerting for relevant changes for the commercial activity [100]. The data generated can also be an important part, to be integrated into traceability management systems, contributing to the overall quality perception of the consumer and transparency [17,41].

Most of these surveillance measures are prophylactic since the treatment of outbreaks in marine aquaculture are not compatible with the environment (*e.g.*: antibiotics or chemicals), are economically unsustainable and are frequently not practical [75,97]. The epidemiological challenges to an effective surveillance system are related to rapid detection, representative reporting and accurate diagnosis [101]. This led to the development of sensitive techniques for monitoring and diagnosis, prompted by the rapid growth of the aquaculture sector. A summary of these methodologies advantages and disadvantages are present in table 1.6.

Table.1.3 - Common marine viral diseases in fish species other than salmonids.

Disease	Occurrence	Clinical signs	References
Lymphocystis disease	22 and 27◦C, Younger fish are more susceptible. Incubation time is 10 days at 25◦C. Detectable for at least 4 weeks after the fish recover. Surviving fish are apparently immune to reinfection	Macroscopic clusters of hypertrophic dermal fibroblasts (whitish, greyish or darker)	[134–136]
Viral encephalopathy and retinopathy	Juvenile and larval Adult chronic asymptomatic carriers Elevated temperatures Incubation time 4-30 days	Neuro invasive eyes and brain. Impaired coordination, loss, whirling swimming, blindness swim bladder hyperinflation, and hyper excitability in response to noise and light.	[134–136]
Red sea bream iridoviral disease (RSID)	When water temperature is >20°C	Lethargy, anemic, hemorrhagic petechial in the gills and splenomegaly. Formation of inclusion body bearing, basophilic, hypertrophic cells within infected organs.	[134–136]
Viral haemorrhagic septicaemia (VHS)	Disease rarely manifests above 16°C. Juvenile and adult fish under stress conditions Temperatures changes Wide species range	Lethargy, moderate exophthalmia. haemorrhagic in the ocular tissue, skin and fin bases	[134–136]

The implementation of surveillance systems, in which the maintenance of biosafety is preferred in all aspects of the activity, helps to reduce, and control the incidence or spread of diseases. Risk based surveillance systems [95,102] or integrated pathogen management strategies (IPMS) [75], will also enable the readiness of measures to be applied in case of contamination before becoming a significant problem to the farm [94,103]. It also provides relevant information that can be used by entrepreneurs or governments to sustain national and international protection laws [80,94].

In primary production (*e.g.*: aquaculture), farmers are responsible to assure their product safety. Consequently, control of environmental hazards or the ones with an animal occurrence at the time of catch is important. Therefore, implementation of disease surveillance and quality systems, in aquaculture primary production, will also help in the preparation of processes to control hazards that are directly associated with seafood consumption risks (section 1.3.2.2).

1.3.2.2 Seafood consumption risks

The incidence of foodborne illness or zoonosis involving farm-raised fish species as vectors [104] will differ from region to region and from habitat to habitat and will vary according to the method of production, management practices and environmental conditions [34,35,79,98,105]. Seafood related illnesses can be divided into three types: infectious, toxic and allergic, [106]. The causative agents associated with foodborne illness are: virus; bacteria; parasites; toxins and chemicals. Tables 1.7 and 1.8 summarize the causative agents, most common symptoms, risk factors, and are divided into infectious and toxic/allergic agents, respectively. The degree of risk differs for seafood eaten raw and those cooked before ingestion. Taking this into consideration, seafood can be classified into three main risk categories [107,108]:

1) High risk – fresh or frozen, mollusks and raw fish.
2) Medium risk - fresh or frozen crustaceans and fish, to be eaten after cooking.
3) Low risk - lightly preserved products (*e.g.*: marinated, fermented or cold smoked); semi-preserved fish (*e.g.*: caviar); heat-processed (*e.g.*: pasteurized; sterilized).

Shellfish are one of the main sources of foodborne diseases due to their nature. Being filter feeders, they concentrate pathogenic agents inside to levels that are harmful to humans [109]. Fish products contamination by infectious pathogenic agents occurs mainly on the skin and the gut.

Table 1.4 - Common marine bacterial diseases in fish species other than salmonids.

Disease	Causative agent	Occurrence	Clinical signs	References
Vibriosis	Vibrionaceae family	Facultative pathogens Cold-water vibriosis occurs below 10°C. Also, at temperate and warm waters.	Systemic hemorrhagic septicemia. Lethargy, skin darkening, corneal thickening, anemic gills, internally, congested visceral blood vessels, intestinal hemorrhage.	[135–137]
Photobacteriosis or Pasteurellosis	*Photobacterium damselae* ssp. *piscicida*	Temperatures above 25◦C increase the probability and severity of the outbreaks. Young fish more susceptible. At lower temperatures, mortality may decrease, but fish become carriers. Pathogen can enter a viable but not-cultivable state and survive in the water column for long periods.	Splenomegaly. Granulomatous-like lesions in the spleen and kidney at more advanced stages appear darkened and, in some cases, petechiae are visible on the head, gills, operculum and fin bases.	[135–137]
	Photobacterium damselae ssp. *damselae*		Skin ulcers or systemic disease.	[135–137]
Pseudomonas infections	*Pseudomonas* spp.	Associated in several occasions with the "winter syndrome". The optimum temperature for disease outbreaks is below 16°C and outbreaks in the Mediterranean area consequently occur during the winter months.	External signs: moderate abdominal distension and keratitis. Occasional petechial hemorrhages on skin and liver. Histopathologic ally, granulomatous inflammation of connective tissues surrounding the skeleton/cartilage of the head region is observed.	[135–137]
"Flexibacteriosis", "gliding bacterial disease", "eroded mouth syndrome" and "black patch necrosis"	*Tenacibaculum* spp.	Opportunistic pathogen. Wild and cultured fish of any size can be affected, more severe in younger fish. Prevalent and severe in temperatures above 15ºC. Various stressors and skin abrasions. More frequent during summer than in winter	The mouth appears eroded and hemorrhagic, lesions may open in the skin, fins and tail appear frayed, and foci of gill rot may develop. A yellow pigmentation at the edges of the lesions is often seen due to an accumulation of *T. maritimum.*	[135–137]
Epitheliocystis	Obligate intracellular prokaryotes related to the genus *Chlamydia*	Heavy infections and mortalities occur mainly in juvenile fish. Bluegill showed increased epitheliocyst at 12°C compared with temperatures above 20°C.	Formation in the host gills and skin, of spherical or ellipsoid "cysts". Flared opercula and respiratory distress.	[135–137]
Mycobacteriosis	*M. marinum* and *M. salmoniphilum*	The use of uncontrolled fish feed. Horizontal transmission from wild-living fish. Invasion through damaged skin or gill tissue.	Skin ulcers and granuloma formation in the form of greyish-white nodules in kidney, spleen and liver, with hemorrhage in musculature.	[136,137]
Streptococcosis	*Streptococcus iniae S. parauberis, S. agalactiae, Lactococcus garvieae*	Have both a sporadic and epizootic character. Transmission from wild fish to farmed fish fresh trash fish used in the fish diet.	Lethal septicaemia. Fish become sluggish. Whirling, spiral, or erratic movements reveal (meningoencephalitis). Exophthalmos with hyphaemia. Superficial haemorrhages. Pale liver and dark red spleen.	[136,137]
Edwardsiellosis	*Edwardsiella tarda*	Opportunistic pathogen.	Skin ecchymosis and ulceration, fin and tail erosion; pale and inflamed gills. Internal organs appear haemorrhagic and oedematous. The kidney appears enlarged.	[135–137]

Table 1.5 - Common marine parasites in fish species other than salmonids.

Disease	Causative agent		Occurrence	Symptoms	References
	Ichthyophonus hoferi		Feeding fish with untreated fish is a main route for transmission.	Cutaneous ulcers and granulomatous lesions in internal organs. occurrence of creamy white nodules on the heart as the most predominant lesions.	[136,138]
	Exophiala spp.		Temperatures of 12–14°C.	Fish dark and lethargic, erratic and whirling swimming behavior. Dermal nodules. round yellow to white granulomas in visceral organs.	[136,138]
Kudoa thyrsites.	*Kudoa* spp.		Transferred from native wild populations to infect introduced cage-farmed stocks.	The muscle of the infected fish gets very soft, almost liquefied, during storage.	[135,138]
Enteromyxosis	*Enteromyxum* spp.		Transmitted directly from fish to fish, through host ingestion of excrement containing vegetative developmental stages.	Inducing severe, chronic desquamate enteritis, emaciation, poor growth, bloated abdomen, sunken eyes and death.	[135,138]
Protistan ectoparasites	*Trichodina* *Brooklynella1* *Uronema*		Occurring on the skin and gills of fish cosmopolitan, opportunistic pathogens with a tendency to proliferate on stressed or debilitated hosts.	Cause irritation, leading to mucus hyper production and hyperplasia. Gills damage.	[136,138]
Amylodiniosis ('velvet disease')	*Amyloodinium ocellatum*		Presence of these organisms on cage cultured fish suggests that the cages have been deployed in water too shallow.		[136,138]
Cryptocaryonosis white spot disease	*Cryptocaryon irritans*			Pinhead-sized whitish vesicles, mucus hyper production, epithelial hyperplasia, corneal cloudiness, skin discoloration, and, with the disruption of the gill lamellar structure, severe respiratory distress.	[136,138]
Protistan endoparasites	Microsporidian*s*		Transmitted directly between fish through ingestion of infective spores.	Causing hypertrophy of the host cell and forming a xenoma or pseudotumor.	[136,138]
	Apicomplexans			Wide range of cell types and target organs are invaded.	
Sea Lice and other parasitic crustaceans	*Copepoda & Isopoda*		Copepod on fish has rarely been associated with mortalities, can proliferate and spread to epizootic proportions. Wild species can act as vectors. Some found paired, attached to the buccal and branchial cavities.	All cause irritation, infiltration of macrophages and lymphocytes and induce epithelial proliferation. Damaged in gill filaments. Skin erythema and hemorrhage typically in heavy infestations.	[135,138]
Flat worms	*Turbellaria*		Mostly free-living organisms, capable of horizontal transmission.	Shallow pouches in the host's gills or body wall.	[135,138]
	Digenea		Endoparasites. Transmission from fresh not treated feed.	Inflammation on internal organs. Found on intestinal tract, peritoneum, swim bladder. Can proliferate in circulatory system.	[136,138]
	Monogean	*Polyopisthocotylea*	Hematophagous and principally stationary.		[135,136,138]
		Monopisthocotylea	Fins and body surface, juvenile fish; characteristically motile.	Hyper melanosis, lethargy, anorexia and weight loss.	

Table 1.6 - Principal methods used for diagnostic and surveillance of fish diseases.

	Classical methods	Immune & Serological methods	Nucleic acids-based methods	Commercial kits
	Histopathology; cell culture; morphological, phenotypic, or biochemical characteristics	ELISA and its variants	PCR based methods; LAMP; DNA arrays; Restriction enzymes	
Advantages	Fundamental in development of new diagnostic method. Detected new diseases with no other methods for detections are available. Used as confirmation when no other methods are available.	Versatility, simplicity, speed, and possibility to quantify the target pathogen.	Very Specific & sensitive. Allow differentiation to subspecies level; fast comparing to classical methods. No need to cultivate pathogens.	Availability of tests. Easy application.
Disadvantages	Phenotypical close bacteria differentiation is difficult. Depend on competent (taxonomical) expertise Time-consuming and labor intensive. Ability of the organism to be cultured *in vitro* culture medium, cell lines (for viruses). Needs pure isolation of the pathogens.	Difficult to obtain specific and sensitive antibodies antiserum for detection of a pathogen. Expensive to obtain antibodies.	Necessary previous knowledge of the DNA sequence of target species to develop primers. Prone to contamination with other DNA molecules.	Cost per unit. Number of individuals needed for a relevant degree of trust.
References	[103]	[101,103]	[139,140]	[103]

Table 1.7 - Most common pathogenic agents associated with infections from seafood.

Origin	Causative agent		Common Symptoms	Risk factor	References
Bacteria	*Vibrio* spp.		Gastroenteritis; septicemia; necrotizing wound infection.	Consumption of raw seafood from contaminated waters.	[106,108,141–143]
	Salmonella spp.		Diarrhea, abdominal pain, muscle aches, fever.	Foodborne or waterborne. Environment of processing plants.	
	Aeromonas spp.		Diarrhea; a chronic enterocolitis fever and vomiting, cellulitis, muscle necrosis, septicemia.	Raw seafood; fresh and brackish water; refrigerated food products with extended shelf-life.	
	Plesiomonas spp.		Diarrhea; abdominal cramps.	Foodborne or waterborne. Cutaneous contact with infected materials, including fishing-related injuries.	
	Listeria monocytogenes		Septicemia and central nervous system infection; fever, myalgia, headache.	Production lines and the environment of seafood processing plants.	
	Mycobacterium spp.		Granulomatous inflammation of the skin and deeper tissues.	Exposure of wounds and skin abrasions to contaminated water; injuries contracted during seafood processing.	[104]
Virus	Caliciviruses (*e.g*. Norovirus);		Gastroenteritis.	Sewage-polluted area; Shellfish consumption.	[106,144]
	Hepatitis A virus		Fatigue, myalgia, anorexia, nausea, abdominal discomfort., icterus and dark urine.	Consumption of raw seafood, water; person-to-person contact: foods via the fecal-oral route.	
Parasites	Nematodes	*Anisakis* spp.	Abscess or eosinophilic granuloma, epigastric distress, nausea.	Seafood open marine waters eating raw, inadequately cooked, poorly salted or smoked.	[138,145,146]
	Trematodes	*Opisthorchis* spp. *Metagonimus* spp.	Abdominal pain, fever, diarrhea, headache, nausea, and back pain; anemia, dizziness; inflammation, ulceration, and necrosis of small intestine.	Consumption of farmed freshwater fish and crustaceans; eaten raw, marinated or improperly cooked.	
	Cestodes	*Diphyllobothrium latum*	Abdominal pain and diarrhea and had eosinophilia.	Seafood from cold water habitats; eaten raw, marinated, or undercooked.	
	Protozoan	*Cryptosporidium* *Giardia*	Diarrhea. Pulmonary and tracheal cryptosporidiosis. Nausea, chills, fever, epigastric pain, and foul-smelling diarrhea.	Seafood from cold water habitats; eaten raw, marinated, or undercooked.	

Table.1.8 - Most common pathogenic agents associated with toxins and allergic reactions from seafood.

Origin	Causative agents		Common symptoms	Risk factor	References
Biotoxins	Amnesic shellfish poisoning		Abdominal pain, vomiting, disorientation, seizures, permanent short-term memory loss. Excessive respiratory secretions. Coma and death.	Mollusks and squid. Viscera of scallops, sardines, anchovies, crab.	[111]
	Ciguatera fish poisoning		Diarrhea, nausea vomiting, parathesis, reversal of temperature sensation; metallic taste, itching, dizziness.	Large, predatory tropical reef fish (barracuda, grouper, red snapper, amberjack); some types of eels; farm-raised fish feed with contaminated fish.	
	Diarrheic shellfish poisoning		Diarrhea, vomiting, abdominal pain, headache, fever.	Mussels, oysters, scallops, clams, cockles, some species of crabs.	
	Neurotoxic shellfish poisoning		Consumption: diarrhea, abdominal cramps, reversal temperature sensation; slurred speech, pupil dilation, overall fatigue, involuntary muscle spasms. Inhalation: allergen like; throat irritation, sneezing, coughing, itchy and watery eyes, burning of upper respiratory tract.	Mussels, clams, whelks, conch, coquinas, oysters, scallops; liver and stomach contents of some planktivorous fish; inhalation of toxin aerosolized by coastal wind and waves.	
	Paralytic shellfish poisoning		Parathesis, nausea, respiratory distress. Muscular weakness, drowsiness, incoherent speech.	Scallops, mussels, clams, geoducks, cockles, puffer fish, some fish, gastropods, crustaceans.	
	Clostridium botulinum		Vomiting, diarrhea, blurred vision, muscle weakness, dysphagia, dysarthria, and hypoglossal weakness, respiratory failure.	Water sediment. retail seafood products, depending on the processing and packaging methods.	[106]
	Staphylococcus aureus		Food-intoxication syndrome.		[147]
Chemicals	Veterinary drug residues		Antimicrobial resistance, carcinogenic agents.	Exceed the maximum level or cases where forbidden drugs have been found.	[109]
	Environmental	*e.g.*: mercury, cadmium, PCBs; dioxins	Neurobehavioral deficits, neuronal loss, ataxia, visual disturbances, impaired hearing, paralysis and death.	Accumulation in the tissues of marine organisms. natural occurrence and from industrial and agricultural sources.	
	Biogenic amines	*e.g.*: histamine	Allergy-like form of food poisoning.	Time-Temperature abuse or natural occurrence in fish muscle. produced during bacterial growth.	
	Additives	*e.g.*: nitrites	Cause hypersensitivity reactions, or food intolerances.	Processing treatments.	
	Allergens	*e.g.*: parvalbumins	Cause hypersensitivity reactions, or food intolerances.	Individual sensitivity.	
	PAHs; Nitrosamines		Carcinogenic agents.	Processing treatments (*e.g.*: smoking; salting, fermented).	

Muscle contamination in the case of infectious agents occurs by mishandling the product or in the case of toxins and chemicals due to the accumulative effect [109]. However, in some cases, infection agents (*e.g.*: parasites, bacteria, and viruses) with origin in the environment or processing plants, pose a lesser risk for human health, if seafood product is properly handled, stored or cooked before consumption [110]. In the case of the presence of toxins or chemicals since they are difficult to remove from the product and can be heat resistant, more restrict control is necessary (*e.g.*: mandatory label information, chemical routine analysis) once the majority of times they are only perceived after ingestion and when symptoms are presented [111]. Therefore depuration (in the case of shellfish), environment quality control and compliance with certified quality programs (*e.g.*: HACCP) are of utmost importance, in reducing the occurrence of seafood borne diseases [109].

1.3.3 Tracing and tracking in aquaculture

Traceability is a tool for achieving different objectives, such as: assuring food safety and public health, (identify hazards, manage safety warnings, product recalls); to provide reliable information to consumers (protection against fraud; ensure fair trade; prevent unfair competition, environmental performance) and to improve process and product overall quality (stock management; costs reduction) [17,19,41].

In aquaculture production, it could be applied the same group of six basic principles for an integrated agro-food chain traceability, they are: 1) product traceability; 2) process traceability; 3) genetic traceability; 4) inputs traceability; 5) disease traceability and 6) traceability of measurements [19,40]. All these principles required adequate knowledge on multiple seafood properties, as traceability systems can be composed by multiple datasets related to each of the six principles. According to Olsen and Borit (2013), there are two major definitions of traceability related with the type of information recorded.

One is related with the online location tracking system, for food products and all their ingredients; the other is the accurate analysis of all analytically verifiable properties a food sample may have [39]. The first definition is related to paper documentation and more recently the electronic traceability and condition monitoring using RFID (Radio Frequency Identification) or WSN systems (Wireless Sensor Networks) [112–114]. The second relates to the perishable nature and the variability of fresh food, like seafood. It involves the measurement of parameters, typically physicochemical, genetic or microbiological, able to identify and discriminate products [27]. For each type of data, there is a diverse range of methodologies; table 1.9 review their main advantages and disadvantages within the aquaculture sector. Utilization of limited or out of date traceability methods (*e.g.*: paper records) hinders the efficiency of traceability in the supply chain because they could difficult the communication between participants (*i.e.*: bureaucratic barriers), interaction with other systems and lack transparency [17,41,115]. In comparison, the incorporation of the most recent traceability methods will allow the integrations of the information on online platforms or blockchain applications, which consequently will improve traceability, transparency, and access to all participants [17,41,115].

Also according to Dabbene et al., (2014), measurements of the parameters should be used to validate the information of the traceability system which is related to authenticity. However, even with a large amount of information possible to gather using both approaches, there are aspects of “history, application or location” relating to a food product that it is not possible to get through tracking movement and instantaneous measurements. These include data on yield and economics, properties relating to ethics, sustainability, and legality [39].

1.3.4 Fish product authentication

There is a clear need to verify the trueness of the records and claims gathered for the implementation of traceability methods. Authenticity is the field responsible for validating that information. Through the use of analytical methods and instruments to determine specific food properties, it is possible to relate them, for example, with specific geographical location, organism species or production method [39]. Therefore regulatory authorities are asking for an extended and updated list of the analytical methods to support law enforcement and confirm the authenticity of food products [117].

This is also related to the mislabelling of food products which is very common in the seafood sector, despite the clear set of regulations from the EU for this topic [118]. The large numbers of participants throughout the seafood sector, the nature of the information transmitted and the supports used in supply chain transitions increase the possibility of errors and the risk of counterfeits [42].

In fact, it was only through the use of DNA based methodologies for identifying species that some recent food frauds were detected [117].

As in the meat industry, identification of fish species is important also to ascertain commercial frauds. Seafood authenticity is mostly based on morphological characterization. However, there are very similar species that are difficult to differentiate through morphology [119]. Another problem is related with replacing valuable species with others of lower value, especially in transformed foodstuffs (for example, breaded fillets) [119].

Therefore molecular methods are becoming one of the prospective standards in the near future to overcome the limits of the conventional labeling and analytical procedures [120,121].

Table 1.9 - Common types of information that are part of traceability systems.

Techniques		Advantages	Disadvantages	References
Conventional	Bar codes RFID WSN	Simple to use and most are economic. High data input. Easy integration in present manages software.	Unreadable for damaged labels. Absent or limited environmental information. Prone to mislabelling and fraud.	[21]
Analytical	NMR IR GC-MS HPLC CE	Analysis of physicochemical attributes. Chemometrics provides the ability to detect compounds patterns, related to origins.	Physicochemical properties in fish can be affected by farming systems, processing methods, environmental conditions and industrial procedures. Stability of the nutritional contents affected by feeds supplied by international companies. Do not provide historical information.	[123]
Genetic	DNA RNA PCR qPCR	Stability of genetic under production and processing techniques. Very specific identification.	Farmed fish present high gene flow between populations in abundant and widely distributed marine fish species. Fish farmers of diverse locations may use the same maternity fish stock.	[38,148]

DNA amplification by Polymerase Chain Reaction (PCR) is a very powerful technique to overcome traceability and authentication hurdles due to DNA properties since: i) it is present in all cells and tissues; ii) it is unique for each animal (except for monozygotic individuals); iii) it is stable for long periods and to physical treatments, and iv) it can be easily isolated and analysed [42,121]. Molecular based techniques have a very high degree of reliability to confirm or deny the origin, descent or strain of animals or products and can also be used as evidence in court [122]. Other techniques have also been applied to address the authentication of food products [45,123]. Among the main fields of study are: genomic; immunology; proteomic; lipidomic; chromatography; isotopes; vibrational & fluorescence spectroscopy; sensory analysis & biomimetic sensors. The main techniques associated with each group are summarised in table 1.10.

The selection of the most suitable approach for traceability or authentication depends on the question being addressed, in which part of the supply chain it is located, the amount and type of sample and available funds [4,121]. In addition, multi-analytical capabilities are essential for food authentication studies providing more descriptors and facilitating better classification. However, the ability to manage and analyse these data are falling behind the ability to generate them. To overcome this, various chemometric or data analysis techniques are crucial for the successful development of models [45].

The perfect traceability and authenticity tool for seafood products should be fast, simple, cheap, reliable and be applied without major financial burdens or logistical restrictions [114,123]. Currently, while such a method has yet to be developed and validated, the best approach will be the combination of multiple tools that complement each other, therefore maximizing its accuracy and reliability. Future implementation of authentication protocols for traceability or certification should avoid past mistakes and capitalize on previous successes recorded in the implementation of similar methods [4].

Table 1.10 - Principal methods for seafood authentication.

Field	Main techniques	References
Genomic	PCR based techniques (SSCP; DGGE; RFLP; RAPD; AFLP; SSCP; ISSR; SCAR; PNA; FINS; Multiplex; DNA fingerprinting; Real Time).	[44,149]
	Isothermal nucleic acid amplification (LAMP; HDA; MDA; RPA).	[150]
	Next generation sequencing (454 Pyrosequencing Technology, Roche Diagnostics; HiSeq 2000 Sequencer, Illumnia; Ion Personal Genome Machine System, Thermo Fisher).	[120]
	DNA barcoding; DNA microarray.	[120,151]
Immunology	ELISA; immuno- precipitation; immuno-diffusion; immuno-electrophoresis.	[45,152]
Proteomic	Electrophoretic techniques (2-DE; CE; IEF; Urea-IEF; SDS-PAGE; DIGE).	[152,153]
Chromatography	Separation techniques (GC; LC; HPLC; UHPLC).	[45]
Spectroscopy	PTR-MS; MALDI-TOF-MS; DART-MS.	[45]
Isotopes	Isotopic techniques (IRMS; MC-ICP-MS; TIMS).	[45,123]
Vibrational and fluorescence	NIR; MIR; NMR; Fluorescence; Atomic; ICP-MS; ICP-AES.	[45,123]
Sensory analysis & biomimetic sensors	Organoleptic test panels; (e-tongue), (e-nose), (e- eye).	[45,123]

1.4. Future Perspectives

The principal paths suggested to achieve integrated aquaculture quality systems, are based on sustainable practices, product diversification and transparent traceability [124]. The ability of firms to internationalize, comply with environmental commitment and innovate will determine their survival capacity [125]. An integrated multi-disciplinary chain approach to food quality and safety is necessary, since in the future the most innovative systems, addressing technological, logistical, economic, environmental and organizational aspects, will probably vary with species, country, region and policies [126,127].

Several advances have been made in the reduction of the adverse impacts of the aquaculture sector. Most of them through various combinations of technological developments, improvements in existing technology, better management practices and site selection, feed technologies and species development [9,128]. An example in other areas is the strategic utilization of aquaculture by-products to increase margins and improve the sustainability of the industry. Stevens et al, (2018) present a case study of the Scotland salmon industry for the implementation of such an approach. Aquaculture by-products could be applied to products such as protein powders and hydrolysates, oil supplements, collagen supplements, pharmaceutical or animal feeds [129].

However, the principal driver of aquaculture industry development will probably be related to quality-control based policies and a "farm/sea-to-table" policy [114]. Due to globalization in the food trade, quality assurance in the food industry has become a reality. Food chain integrity not only includes concerns with production and product freshness but also origin, fraud, and safety [21]. The high complexity of interactions between all participants, along the supply chain, demands an effective way to address quality demands. To achieve the full potential, it must be scientifically based and responsive to the changes in the seafood production chain [15]. On the other hand, from the point of view of the producers/suppliers, the variety of assurance systems and the implementation costs arouse doubts about the effectiveness of such systems [16,130]. The limited global dissemination of the assurance, standards and certification schemes may imply more difficult access to some of the biggest markets. The offer is diverse, and the choice should be done as to whether a given standard is "fit for purpose", rather than whether it covers all categories to the highest degree [46]. Therefore, considering the risks involved in certification processes and maintenance costs, they could be offset by the benefits. Also, international standards, have to start considering regional specificities, such as traditional market dynamics characteristic of country or region. The lack of sensibility to those issues could impair its implementation, compliance or maintenance, of such schemes [131]. There is a need for correct implementation of innovative technology, standardize quality schemes and protocols, as faster international agreements on methods for validating technologies.

In the case of complex supply chains, such as seafood, assuring a high quality is a difficult endeavor. The efficiency of traceability systems is becoming more dependent on the level of transparency throughout the supply chain [17,41]. Digital technologies and platforms (*e.g*. Amazon and blockchain) open new seas into seafood trade and logistics, having a great capability to change dramatically value chains approach [126]. They have the capability to decrease uncertainty and information errors between participants, as satisfy customers' interest in resource origin or sustainability performance [41,132]. The prerequisite for the application of such technologies is trust between the supply chain intervenient. However, some barriers are related to willingness to provide confidential information and system costs distribution between participants [41,115]. On the other hand is still necessary to debate issues that will arise in a more transparent world, more precisely accountability (*e.g.*: who is responsible to whom and over which time frame) [17].

1.5. Conclusions

In conclusion, an integrated multi-disciplinary approach to aquaculture development can help reconcile the human and environmental objectives of sustainable development. In order for future changes, become effective in the aquaculture sector, is also necessary an alteration from a consumer-oriented demand for sustainability to an industry motivation and regulatory monitoring [17]. The reasons for this are that in some cases, consumers may not be able to further generate meaningful impact for quality or sustainability. Other reasons are lack of motivation, willingness to pay premium prices, or how their change of habits translates into industry improvements [17]. Market forces, consumer demand and government regulations all should converge to push a new level of supply chain visibility and sustainability intensification, further redefining the industry.

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Chapter 2 - Quality Index Method for fish quality control: understanding the applications, the appointed limits and the upcoming trends.

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Quality Index Method for fish quality control: Understanding the applications, the appointed limits and the upcoming trends

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Abstract

The Quality Index Method (QIM) is a widely used approach for fish sensory grading, based on a structured scaling for freshness measurements, providing information concerning the fish freshness status, as a prediction of the remaining shelf-life for specific species or products. However, its tendency to be used in an oversimplified way and other common misapplications could lead to the discrediting of a methodology with great potential. The objective of this work is to review the principles of QIM methodology, discussing its concept, applications, and understand their limits, as a useful strategy to propose improvements, reinforce its predictive power and consequent acceptability.

Keywords: Seafood; Fish Sensory Analysis**;** QIM Concept; Freshness Attributes.

2.1. Introduction

Consumers' demands for foods with high nutritional value or certain specific sensory properties (*e.g.*: appearance) are a result of consciousness of the impact of food products on their health, pleasure, or preference. All seafood products are associated to highly perishable products, mostly the ones that are to be sold as fresh products. Degradation of fish products is related to three main post-mortem processes, responsible for their main sensory changes: oxidation, microbial degradation, and autolysis. They are responsible for the evolution of spoiled fish, and for the development of specific substances that contribute to fish spoilage [1,2]. The knowledge of the evolution of various descriptors and properties associated with the spoilage process allows the evaluation of fish optimal condition (after slaughter), as well the estimation of its capability to retain those sets of characteristics through time. The collection of this information could reflect the apparent elapsed time since the capture, contributing to the estimation of the rejection time for consumption [3,4]. In the case of fresh fish sold as a whole product, there is minimal industrial processing based on washing and consequent cold storage, more commonly in ice. Implementation of these procedures aims at the inhibition of bacterial growth, enzymatic action, and oxidation, contributing to its freshness retention, safety, and shelf-life extension [5]. Freshness and shelf-life assume crucial importance to industry and consumers, as they determine the product acceptability and, consequently, its commercial value. However, they are difficult terms to define due to their common basic principles, associated with food degradation and the methods used to study them [6–9]. Freshness is associated with an ideal condition of the product, related to the properties it had before capture/harvest or immediately after such activities and with methods used for evaluation [4,10]. Shelf-life will be related to the duration of product conformity with label declaration, nutritional data, chemical, microbial, physical, and sensory characteristics, as well as with methods used to delay the impact of the post-mortem process and extend storage time [1,11].

Consumer decisions about a food product do not depend solely on the associated pleasure or its organoleptic properties [4,12]. They are also dependent on personal expectations that vary among consumers as well as with the cultural or geographical influence [13,14]. In many cultures and countries, fish is frequently bought as a fresh whole product, with minimal labeling and without being packed. Since fresh fish is influenced by sensory changes (*e.g.*: appearance), also perceived by the buyers, it would help them made a more scrupulous choice in the purchase phase than at the consumption. This is normally credited to the idea that at the buying moment most consumers are considering storing the product for a period before consumption. At the expected consumption moment, they are more tolerant to defects to avoid wasting the product [9,15]. This scenario will influence the estimated product shelf-life. Instead of being related with sensory rejection due to unacceptable taste characteristics (maximum shelf-life), it will be based on alterations of other sensory attributes occurring in an early stage of fish degradation (product grading approach) [1,2]. Either way, this period is always smaller than the "maximum shelf-life" estimated for a fresh fish [15].

The determination of this middle point (rejection day) is normally of utmost interest for the fish industry. For industry, it is important to ensure the retention of the best characteristics during the time necessary for its distribution, acquisition, and consumption [9]. Consequently, the industry establishes the product shelf-life having in consideration the moment when the product is rejected by a percentage of the evaluation panelists, to which is subtracted a period that comprises the distribution, product purchase, and consumption. Also, at the industrial level, it might only be required to determine freshness, acceptance, or rejection on a basic level. Therefore, freshness characterization of fish products according to the factors of capture or post-production (handling, packaging/conservation, distribution) is of increasing relevance, to achieve better predictions of storage effects and distribution conditions on product shelf-life [3]. To avoid economic loss, the seafood industry should rely on accurate methods and procedures to ensure that the required quality parameters are met by all chain representatives [16]. The methods reflect the different species spoiling patterns and can be categorized in chemical analysis (*e.g.*: TVB-N and TMA analysis) [2], microbial counts [17], sensory analysis [18], spectroscopic techniques [16], and electronic sensors [19]. Some methods have common drawbacks such as being destructive and labor-intensive. Also, no single index can encompass all the complex changes occurring during spoilage [20]. However, they can complement each other and deliver acceptable estimations. Independently of the chosen method and the reasons behind it, sensory evaluations will remain a key factor, since sensory clues are the main parameters, a consumer can follow when buying fresh fish. Therefore, any chemical or instrumental analysis that is developed or used, must be in agreement with sensory results [21].

In Europe, it is usual to perform a sensory evaluation at different levels in the seafood industry such as after landing, at the fish plant and processing halls, or auction sites. Often fish is graded, priced, and sold based on freshness criteria accessed by fish inspectors using sensory analysis [22]. For this purpose, specific sensory methods were developed for fish sensory analysis, with a special application in the industry but also with a correlation to consumer acceptance. Such methods include the EU scheme, Torry sensory analysis, and Quality index Method (QIM) [23]. From these, QIM is more adaptable, once it is developed and validated, its application is fast, non-destructive on raw fish, is species-specific, allows direct measurement of the perceived attributes, estimation of the product shelf-life, and enables the collection of information for a better understanding of consumer responses [24]. Besides its general acceptance, several limitations have been pointed out, during its development phase and application, that could undermine its predictive power. Therefore, this work aims to review QIM basic principles, clarify its application and benefits, explain its limitations, and point out what its future could be.

2.2. Fish Sensory Quality Control

The sensory investigation is performed to create predictions about how product alterations (arising from ingredients, processes, packaging, and aging/shelf-life), will be perceived by human observers. Sensory science is described as a quantitative discipline that uses the human senses (*e.g.*: vision, smell, or taste) for interpretation, measurement, and analysis of different environmental, physiological, processing, or conservation factors on food products characteristics (*e.g.*: appearance, odor/aroma, texture, flavor/taste) [25]. To serve its purpose, the procedures for sensory evaluation must be very well defined. For proper result interpretation, a correct data analysis is critical. When a method has to be chosen the following conditions should be considered: the problem to be solved; the advantages it has; its accuracy, precision, and robustness; its adaptability to future requirements; its information value; its probability of adoption and costs; its correlation with actual knowledge and prediction capabilities [26].

The classical view about sensory analysis is that it can be associated with objective sensory questions (*e.g.*: performed in laboratories) influenced by food industry processes, and with consumer research, that deals with subjective parameters of quality (*e.g.*: led by marketing departments) [27]. However, it has as an objective to find answers for particular questions related to product quality perception. This topic has been discussed, in terms of its application in specific areas (*e.g.*: consumer research; marketing or shelf-life) and the methodologies appropriateness for stipulated objectives [28–31].

Frequent terms used in methods for seafood sensory analysis or quality control are scaling, ranking, and grading. However, in quality control, they can have similar meanings while in a pure sensory analysis approach they are different [30,32].

In the ranking, an ordinal scale is utilized to put in order the intensity/degree of an attribute (*e.g.*: the color of smoked salmon). They are best fitted to research but not so suitable for industry quality control applications [33]. Scaling emphasizes differences and degrees of change, that are usually higher than the limit level or with a noticeable difference. A specific approach is category scaling, where the panelists are requested to rate the stimulus intensity by being trained with standards of different intensities (inexistence to excess). Grading is the application of a categorical value to a product lot or group. In sensory grading, the grader needs to integrate different perceptions being requested to rate the simultaneous effect of negative and positive attributes, the mixture or balance between them, and compare the products with physical or written standards (structured scaling). The conclusions are confirmed by the correlation of measurable chemical or physical properties with statistical analysis. The advantage of grading is that it allows the selection of products for different ‘‘qualities’’[18,30].

From sensory science, the definition of grading is the one that integrates the industry perspective. However, the utilization of quality grading schemes should be done carefully. Common recommendations are to use it on products with a generalized consensus on its sector (*e.g.*: fish, dairy, and wine industry) [32].

For fresh fish, quality evaluation along the supply chain is governed by its sensory evaluation, frequently through the analysis of appearance and odor [34]. This marks a common point between industrial quality control and the consumer perspective on the product freshness that will influence his overall quality perception [35]. Recognition of freshness and the identification of defective characteristics is the basis of sensory analysis of fresh whole fish and maybe all that is necessary for routine industry decisions. In this case, it is clear which are the ideal product characteristics and the common sensory defects that arise from poor handling, processing, or storage [34]. Therefore, it is in the industry's interest to make available, products that correspond to the common sensory criteria also perceived by the consumer, to increase the probability of selling its products. This approach is related to the fulfillment of the quality standards criteria, stipulated by regulatory entities, and is also associated with grading methods [32,36].

The primary methods used for sensory fish quality control are presented in table 2.1, even though others can be used in different steps of the supply chain [36]. All of them have their strengths, weakness, and are used to rate, scale, or grade fish products [18,32,37].

2.2.1 Torry scale

The Torry scale is a detailed scheme for the freshness evaluation of cooked fish. It is frequently applied on cooked fish samples to evaluate odor and off-flavors, such as to establish the product's maximum shelf-life, which is determined when the eating qualities show evidence of off-flavors or poor taste. It is a descriptive scale of 10-points, developed for fat, medium fat, and lean fish. The value of 10 is considered the freshest fish possible. The limit for consumption is set at 5.5, at the value of 3 is considered spoiled and below this is considered unfit for consumption. Several adaptations of the scheme exist and are used by trained panels [21]. The method's drawbacks are the sample destruction and the time required for execution, which is not reliable for routine analysis in the industrial environment. However, the technique is correlated with other methodologies more suitable to industry requirements such as electrical conductivity, chemical, and microbial analysis [16,17,38].

Table 2.1 - Principal methods for sensory analysis.

Methodology		Test	Objective	Reference
Subjective	**Affective** - assess the acceptability of products or the relative preference among a set of products	Preference	Compare one product directly against another in situations such as product improvement or parity with the competition.	[24]
		Acceptance	Determine how well a product is liked by consumers.	
		Attribute	Determine the reasons for preference or rejection through questions about the sensory attributes.	
		Hedonic scales	Estimating hedonic quality of seafood using a scalar method.	
Objective	**Discriminative** - compare two products or stimuli looking for differentiation on a sensory basis	Triangle	Determine existing sensory differences between products.	[24]
		Paired comparison a) directional paired comparison method. b) difference paired comparison	a) - Determine whether they differ in a specific attribute. b) - Ask simply whether the samples differ.	
		Duo trio	Determine if two samples are perceptibly different, but the analyst will not know in which attributes the samples differ.	
		A-not-A	When the experimenter cannot make the two formulations have exactly the same color, shape or size.	
		Ranking	Arrange three or more samples according to the degree to which they exhibit some specified characteristics.	
	Descriptive - when a detailed description of the innate and added attributes of any given product is needed	Flavor profile		[39–41]
		Quantitative descriptive analysis	Detailed description of the sensory profile for a product.	
		Texture profile		
		Sensory spectrum		
		Generic descriptive analysis		
		Free choice profiling	Assessors use their own words instead of common vocabulary.	
		Difference from control	The objective is the combination of determining if a difference exists between samples and control, as estimating the size of such a difference.	

Therefore, it would be beneficial for the industry to have other methods that could also correlate very well with the ice storage time, but performed in whole fish, at early stages in the production chain, and capable to assist in product management (*e.g.*: shelf-life prediction).

2.2.2 European Union Scheme

The EU regulation (EC) No 2406/96 states the obligation to grade the product according to EEC guidelines at the first sale on landing points. According to it, all described fish species (whitefish, bluefish, selachii, cephalopods, and crustaceans) are categorized into 4 levels: E (Extra) – the highest level, A – good quality; B – acceptable for consumption; and C – unfit for consumption [36]. A weakness of this method is that there is no specification for training, sampling, and other procedures, making it difficult to be reliable and reproducible without extensive experience. The usage of unspecific parameters for fish sensory description increases the probability of overemphasis of one criterion as a discriminative parameter. Also, it does not consider species specific spoilage characteristics. The structure used does not allow to differentiate between intermediate points, since there are characteristics that are not described, or do not agree with the attributed grade (E, A, B, or C), influencing the amount of information provided in terms of shelf-life data, or to apply statistical analyses for reliability and reproducibility studies. Therefore the design of the EU scheme is not suitable for Quality Assurance control at the industry level [18,20]. The EU scheme is also often misused in research studies as a structured scale, to which is applied arithmetic analysis [17,42]. Despite the criticism, the scheme is still in use, and a useful multilingual glossary in 12 European languages for the EU grading scheme is available. It is better applied at the first sale to detect unacceptable fish, where fast decisions must be made, by experts and inspection authorities [23,24]. However, quality grading in the industry requires a more reliable and useful tool to grade fish freshness. One suggested method is the Quality Index Method that overcomes some of the limitations attributed to the EU scheme, making it possible to establish a correlation between QIM results and the other available methodologies.

As an objective attribute scoring procedure, QIM is based on Soudan scales [34]. Its specific features are: a) the higher number of attributes evaluated (10-15), with specific alterations during storage; b) the use of short scales with different lengths (0 to 3), according to the number of perceptible changes in each attribute; c) the overall quality index (QI) obtained from the summation of the scores. QIM measures the rate of change in important attributes used to describe freshness, making it possible to relate them with time-temperature integration and shelf-life estimation [22,43,44]. However, some doubts are also pointed out to the method, concerning whether all the attributes scored are needed, or the impact of the variability attributed to the fish samples and if the assessors' bias is adequately considered. Therefore, the merits and limits of the method will be reviewed in the following section (section 2.3).

2.3. Quality Index Method

QIM was originally developed by the Tasmanian Food Research Unit. It is a fast, simple, and non-destructive, descriptive method for seafood freshness evaluation. It provides all the users throughout the supply chain, with a standardized and reliable sensory measure of product freshness. Its standardization is supported by the ISO norms which are important in the development of new schemes and the establishment of common rules for research or industrial implementation [20,22,24]. It allows collecting specific information about the fish condition during storage considering the differences between fish species (see Section 2.3.1). Also, once established and developed, QIM schemes are easier to use than some other methods and only require as much equipment as the human senses. QIM is an objective method, well suited to train, teach, monitor, and evaluate new or experienced panelists. The descriptors of quality are well defined and complemented with illustrations, in most of the schemes [44,45].

The main disadvantages commonly associated with the method are: subjectivity connoted with sensory analysis; the time needed to train the personnel; and development of different schemes for each specific species or fish product (other specific limitations are discussed in section 2.3.3). It is necessary to refer that this sensory evaluation method has a well-established protocol for product evaluation and scheme development. Figure 2.1 describes the main steps for the development of the QIM scheme.

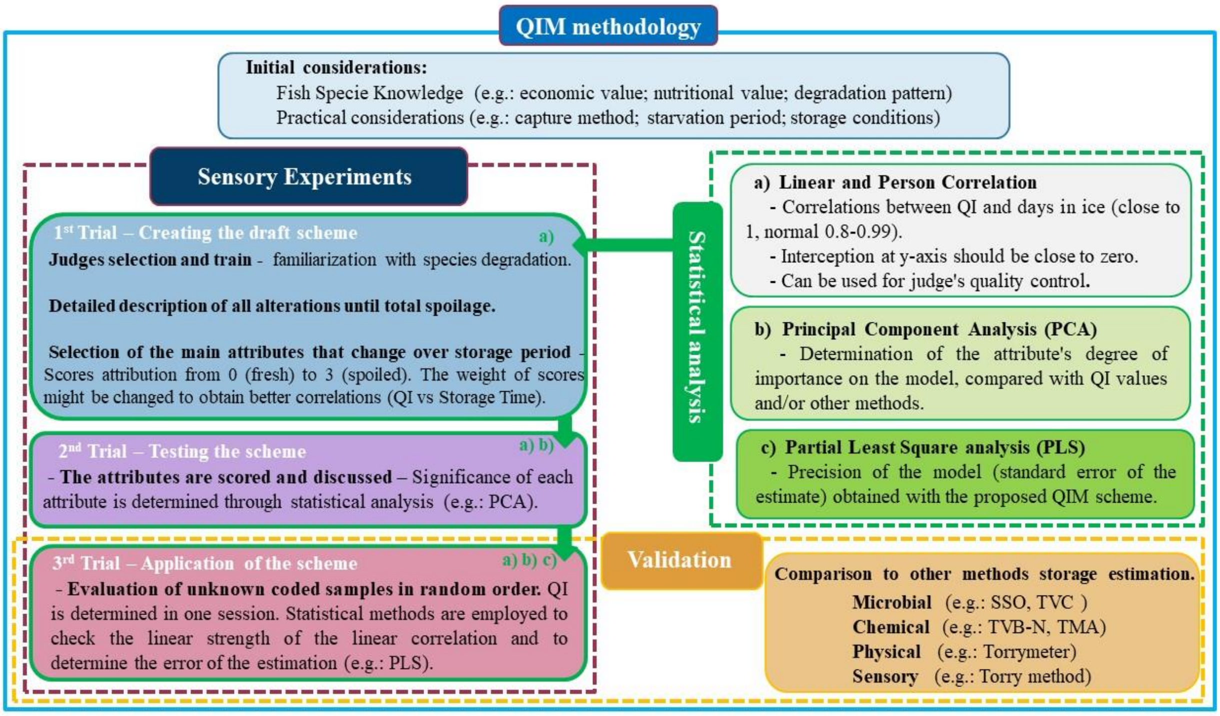

Figure 2.1 - Common steps for QIM development.

When selecting and training judges for sensory analysis, it is important to be aware that some people might have natural limitations as in tasting rancid flavors or iodine, possess low response to cold-storage flavor, or allergic reactions to different fish-proteins, shellfish, or histamine [24]. The utilization of truly specialized and trained panel members is important when the methodology is applied for new species or products, in which the

QIM must be rigorously established and validated, in order to be easily replicated by others (*i.e.*: factory workers). Once this validation is done, the implementation at companies or personal training is straightforward. The developed guidelines have only to be followed and checked. The analysis is based on specific and clear parameters, that suffer alterations during storage time, and any person (that does not have specific natural limitation) can detect them [25,46].

In the following sections, special attention will be given to the concept behind the QIM method (Section 2.3.1), the QIMs applications (Section 2.3.2), and the limitations appointed to the method (Section 2.3.3)

2.3.1. Concept

The implementation of good practices in the fisheries and aquaculture sector provided standardization of procedures (*e.g.*: temperature control) that allow the development of methods for expressing freshness, spoilage, or shelf-life. The proposition behind the QIM scheme is that evaluators cannot efficiently judge degrees of perfection but can very readily detect deviations or changes from it. This is due to the knowledge that during the storage of fish, changes occur, are detectable and often measurable.

The concept of the relative rate of spoilage states that most protein foods (*i.e.*: fish, meats, or milk) spoil at similar rates, expressed as a ratio to the rate they spoil at the reference temperature of zero degrees Celsius (0 °C). This means that spoilage at a variety of temperatures can be expressed in terms of equivalent days of storage at 0 °C and that the integrated effects of storage at different temperatures can be taken into account. It also results from the nature of this relationship, in the case of fish, that it spoils four times faster at 10 °C than at 0 °C, and twice as fast at 4 °C than at 0 °C [22,24].

However, besides the importance of the time-temperature reference, such relation by itself is not sufficient to perfectly describe the fish freshness state [8]. This means that other factors will also impact fish spoilage rates. In the case of aquaculture, the processes of pre-harvest (*i.e.*: starvation period), harvest (*i.e.*: slaughter method), and post-slaughter techniques (*i.e.*: evisceration) affect every major property of fish flesh (*i.e.*: texture or appearance) in the first few days of storage, contributing to the initial freshness state condition and its duration [3,47]. Therefore before QIM development, it is necessary to register the peri-mortal circumstances [8]. This knowledge will help to determine which conditions the scheme is better suited for application.

Having into consideration the previous pre-conditions, what the QIM measure is the rate and degree of alterations in important attributes during fish spoilage. Such attributes can be alteration in fish skin, mucus, smell, overall appearance, eyes, etc., (Figure 2.2). In this system, all the attributes are evaluated in each fish following the same order; besides that, no importance is given to any particular aspect and, therefore, errors and incongruences associated with one attribute assessment are reduced [20]. The resultant QIM scheme becomes a list of attributes, with associated parameters each with a specific change accompanied by a description. The terminology used for the description should state specific alterations related to a precise moment and not be dependent on previous or future states of the sample. Figure 2.2 represents a novel sensory wheel for QIM methodology, presented and proposed for the first time in this review, with the most common evaluated attributes for fish and some commonly used terminology.

Demerit points are attributed to the defects encountered in the product, associated with each parameter. The scores are based on a scale from 0-3, being 0 the best condition possible, and 3 the worst. Also, the scoring allotted to each parameter is such that no single parameter could dominate and that the scores values are easy to judge. Since the individual attributes can have different degradation patterns (when plotted the scores *vs* storage days), the individual scores are summed to provide a total, an overall evaluation denominated as Quality Index (QI).

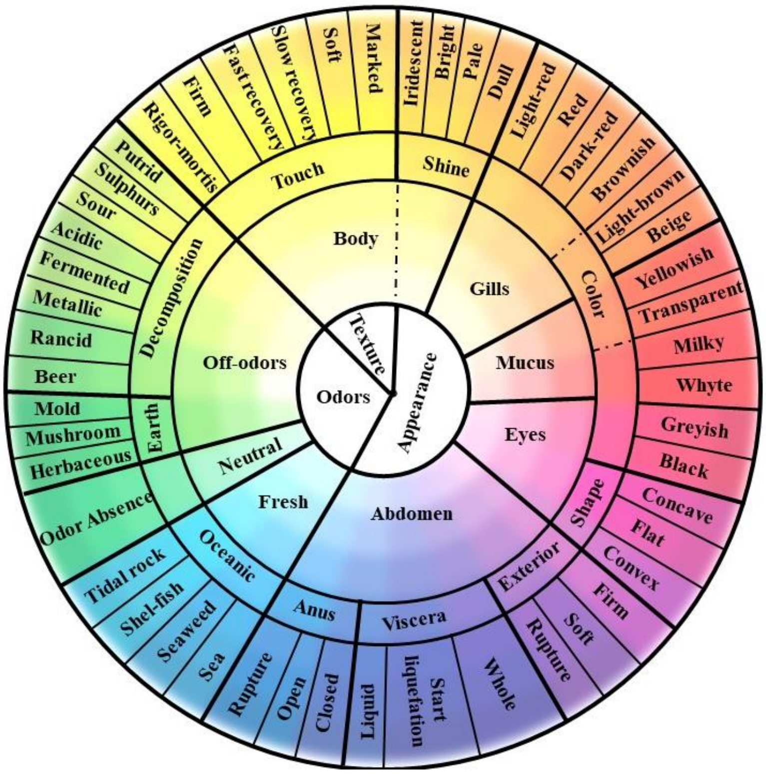

Figure 2.2 - QIM sensory wheel, with the most common attributes evaluated and terminology used.

The lower the total score, the fresher the fish. If, for example, the maximum of three demerit points are scored within the first five days, but it is generally known that the shelf-life is, in total, about 14 days, the description per demerit point needs to be adjusted in such a way that the scoring covers more of the complete shelf-life [20,44]. To avoid scoring irrelevant criteria or the imprecision of using only one, proper statistical treatment of the selected criteria should be performed in order to select the ones that significantly contribute to the relationship between degradation progress and storage time [7,48]. Figure 2.1 presents some of the most used methods for statistical treatment. Successful evaluation of QI models implies the following: the amount of data collected (number of selected attributes and data points) must be in sufficient number to give a possible score of reasonable magnitude [24]. The result obtained in the scheme can be used as an index of what the material should be for an appropriate end-use. A simple calculation can indicate: the equivalent to the number of days that the product has been stored at 0 °C; judge its ability to withstand a process; a particular grade of a product; or it may foreshadow what the product is anticipated to be like when it is acquired, cooked and eaten by the consumer [20].

Although QIM is an important tool for the prediction of the commercial validity expiration or rejection time, the results should be supported by other assessment methods. Correlation between different techniques is becoming increasingly important in market development. Therefore, to properly validate a QIM scheme, it has been proposed that the method should be supported and correlated with other evaluation methods in a range of subjective (sensory), objective (non-sensory), and statistical methods, providing much more precise and realistic results [45,49]. Sensory methods such as the Torry method can be used to estimate the rejection point of QIM schemes [3,44]. However, for its application, the form of the final product should be taken into consideration. This method is mostly applied to fillets, or for the same fish sold as a whole product since the rejection point in this form occurs sooner than for fillets [49]. Objective methods such as chemical analysis (*e.g.*: Trimethylamine; TVB-N; K- value) are frequently used for the freshness assessment of transformed fish products [2]. Even though, for some species they might not be perfectly suited for freshness evaluation, due to low increase during the first days of storage, they can be correlated with spoilage progress. Such methods quantify the number of specific chemicals (*e.g.*: volatile amines and ammonia) that are originated during decomposition or that increase in the same period [50]. Other methods could be the quantification during storage, of microbial activity through TVC counts or specific spoilage organisms (SSO). Also, the measurement of dielectric properties of fish muscle during spoilage is applied using instruments like the Torrymeter [49].

2.3.2. Applications

The QIM-EUROFISH project (www-qim-eurofish.com) has been important for the method dissemination, with a manual containing 13 QIM schemes as an example, published in multiple languages [22]. There has been considerable publication effort demonstrating the continuous importance of QIM, with the development of schemes for new species and products from wild or farmed species, and the optimization of previously published schemes. Table 2.2 summarizes the QIM schemes created or improved, in the scientific literature from 2012 until 2019. For the period before 2012 information is present on Barbosa & Vaz-Pires, 2004; Bernardi, Mársico, & de Freitas, 2013; Sant'Ana, Soares, & Vaz-Pires, 2011 [7,49,51] and the QIM-EUROFISH database.

In this period QIM methodology has been routinely applied in most of the scientific works related to freshness or sensory analysis of seafood products. The works can be divided into three main study areas some examples are: first schemes for a specific species [45,52–55], the impact of storage conditions [56–60] and type/formulation of products [61–63]. Other areas are, the effect of rearing conditions and harvest methods [64–67], post-harvest procedures [68,69] and feed formulation studies [70–72]. Some examples can be found, in which QIM methodology was used as support for the development of new freshness sensors, as a control method to verify the sensors results [73,74]. Ice storage is still the most studied preservation method, probably resulting from the fact that it is the most used method for the product presentation to the consumer. This means that QIM is an increasingly important methodology for the determination of deterioration progress of fish sold as fresh. Other storage conditions and packaging techniques have also been studied, with an emphasis on modified atmosphere [75–77], vacuum [78,79], gels [80,81] or emulsions formulation [82–84].

Table 2.2 - Applications of QIM scheme reported in the literature between 2019 and 2012.

Species	**Product**	**Storage Conditions**	**Total demerit point**	**Shelf-life (days)**	**Country**	**Ref.**
Chelon subviridis	Fresh	4 ºC	34	12	Iran	[85]
Dicentrarchus labrax	Fillets & nanoemulsions	Vacuum Package (2ºC)	18	14-16	Turkey	[86]
Mugil cephalus	Eviscerated	Ice	25	15	Brazil	[87]
Trachinotus falcatus	Whole & Gutted	Ice	18&11	18	Vietnam	[88]
Octopus insularis	Whole & Eviscerated	2ºC	24	15 & 20	Brazil	[89]
Litopenaeus vannamei	Whole	Ice	36	9	Brazil	[90]
Gadus morhua *Salmo salar*	Whole & fillets	Ice (2ºC)	18 &15	8 10	Ireland	[91]
Lagocephalus guentheri	Fillets	2ºC	14	10	India	[92]
Seriola dumerili	Whole	Ice (0ºC)	25	12	Portugal	[45]
Metapenaeus affinis	Whole	Ice	18	9	Iran	[93]
Rhabdosargus sarba	Whole	Ice	34	9	Iran	[94]
Oncorhynchus mykiss	Whole & fillets	-18ºC	20	-	Turkey	[71]
Pagellus bogaraveo	Whole	Ice (4ºC)	30	-	Spain	[72]
Oncorhynchus mykiss	Whole Gutted	Ice (2ºC)	30 15	12 14	Turkey	[95]
Gadus morhua	Fillets	-28ºC	14	6	Norway	[69]
Pollachius virens	Fillets	2ºC	14	9-10	Iceland	[58]
Oncorhynchus mykiss	Fillets & nanoemulsion	Ice	16	17	Turkey	[96]
Rachycentron canadum	Whole	Ice (17ºC)	23	15	Brazil	[53]
Gadus morhua	Gutted	Ice	23	-	Spain	[74]
Oncorhynchus mykiss	Fillets & oil nanoemulsion	(4ºC)	9	15	Iran	[84]
Colossoma macropomum	Eviscerated	Ice (0ºC)	34	22	Brazil	[97]
Scophthalmus maximus	Whole	4 ºC	20	15	China	[98]
Penaeus monodon	Whole	Ice	21	10	Vietnam	[99]
Engraulis encrasicolus	Fillets & Plant Extracts	Vacuum Package (2ºC)	19	12	Turkey	[63]
Pseudoplatystoma corruscans	Whole	Ice	16	13	Brazil	[55]
Thunnus albacares	Frozen Steaks	4ºC	10	8	China	[100]
Lethrinus spp. *Lutjanus malabaricus* *Lutjanus erythropterus* *Lates calcarifer*	Fillets	Vacuum Package (4ºC)	10 11 11 10	5	Australia	[78]
Sparus aurata	Fillets & Limonene	Vacuum Package (2ºC)	8	15	Italy	[101]
Scophthalmus maximus	Whole, gutted	Vacuum Package (4ºC)	19	16	China	[79]
Dicentrarchus labrax	Fillets & nanoemulsion	(2ºC)	16	8-10	Turkey	[83]
Oncorhynchus mykiss	Whole	Ice	14	10	Portugal	[72]
Pseudoplatystoma fasciatum×Leiarius marmoratus	Eviscerated	Ice	18	12	Brazil	[54]
Cistopus indicus	Deskinned	Ice (5ºC)	16	12	India	[57]
Cyprinus carpio	Eviscerated	Ice	19	18	Argentina	[102]
Colossoma macropomum×Piaractus brachypomum	Eviscerated	Ice	18	10	Brazil	[103]
Salmo salar	Gutted	0ºC-15ºC	23	15-2	Australia	[68]
Panulirus argus	Whole	Ice	15	10	Brazil	[104]
Scomber scombrus	Whole	Irradiated UV; 4ºC	20	4-5	Croatia	[60]

(Table 2 continuation)

Merluccius merluccius	Gutted	3ºC	19	5-8	Spain	[105]
Sardina pilchardus	Fillets	Vacuum package	18	17	Algeria	[106]
Sparus aurata	Fillets	Ice	-	16-17	Spain	[82]
Anguilla anguilla	Fillets	Vacuum package & refrigerated	18	12	Turkey	[107]
Scophthalmus maximus	Raw Whole	Ice	30	16	Norway	[108]
Cyprinus carpio	Gutted & Ungutted	4ºC	33	8	Poland	[59]
Rhamdia quelen	Whole	Ice	28	35	Brazil	[65]
Lateolabrax japonicas	Fillets	Chitosan Coat	12	12	China	[81]
Colossoma macropomum×Piaractus mesopotamicus	Eviscerated	Ice	26	11	Brazil	[109]
Cynoscion acoupa	Eviscerated	Ice	23	8-9	Brazil	[52]
Engraulis encrasicolus	Fillets	Ice	19	12	Algeria	[110]
Piaractus mesopotamicus	Eviscerated	Ice	32	11	Brazil	[111]
Xiphopenaeus kroyeri	Whole	Ice	11	2	Turkey	[112]
Nephrops norvegicus	Gutted	MA-Packing	14	9	U.K.	[75]
Sepia officinalis	Whole	Ice	20	6	Italy	[64]
Rachycentron canadum	Gutted	Ice	25	>15	Norway	[113]
Ctenopharyngodon idellus	Fillets	Ice (2ºC)	14	12	China	[114]
Gadus morhua	Fillets	-	8	-	Norway	[67]
Oreochromis niloticus	Deskinned Fillets	1ºC -1ºC MA-packed	13	15 20 23	Iceland	[77]
Mullus surmuletus	Whole	Omap Map	13	15	Italy	[78]
Pseudosciaena crocea	Whole	Chitosan	33	19	China	[61]
Boops boops	Whole	Ice	20	12-17	Croatia	[48]
Carassius auratus	Whole	Ice (0ºC)	22	13-15	China	[80]
Gadus morhua	Fillets	Ice	13	5	Norway	[115]
Sardinella aurita	Fillets	Vaccum & zeolite	18	12	Turkey	[116]

MA – modified atmosphere packed

OMAP – ozone modified atmosphere packed

The type of product studied vary between whole fish, gutted/eviscerated, and fillets, reflecting the main method of presenting the product to the buyers, according to each region. In the early stages of the method, it was mostly applied in species widely consumed in Europe (*e.g.*: salmon, trout, seabream). Now the targeted species or type of product for QIM studies are the ones with increasing regional economic importance, the ones being studied for aquaculture diversification, or the ones that have the potential to be exported.

One of the main reasons to develop QIM studies is to establish an estimation for the product shelf-life based on human sensory perception (table 2.2). Its, ease of use and correlation with other methods (*e.g.*: microbial and chemical analysis) helps to be the complementary sensory method of choice for raw, whole, fresh fish analysis. The most common results for shelf-life estimation in ice storage vary between 8 and 17 days, with the lowest value referred to as 2 days [112] and the highest as 35 [65]. These estimations should be used with precautions, not only because of fish shelf-life being highly dependent on species but also due to uncertainty associated with the method (see section 2.3.3)

Since the EU recommendation is still the EU scheme a correlation table between QIM values and EU methodology was developed [24]. As it became accepted and established in European countries, it is possible to see that now its application is being adopted in different regions of the globe, reflecting the impact of quality control methodologies applied in Europe, one of the biggest fish consumer markets.

2.3.3 Appointed limits and pitfalls

As in any methodology, QIM has its drawbacks. Most of the common limitations are the ones attributed to general sensory analysis and among them are: costs, time-consuming, and the need for expert personnel [117]. All of them are true for the development of QIM schemes for new species or to new product conditions, as well as for other methods normally defined as traditional methods [16,45].

The most recurrent issue in the QIM literature that affects the method strength, is the confusion between the used terminology and the lack of compliance with the best practices for sensory evaluation. In the last case, the most common are the number of assessors used and the number of training sessions [118]. Other cases that arise from the search results about QIM publications are that some of them use the QIM terminology, but in the execution phase, it seems like a quality descriptive analysis (QDA). Most of them, state the development of a quality index scheme but the attributed values vary between 1-5 or 0-4, and in other cases, the higher scores are given to the fresher samples [90,119–124]. Even though some authors refer that the original method was altered, it is necessary to consider the correct usage of the terminology, to not hamper the implementation of QIM methodology or arise misconceptions about the method.

However, it is necessary to acknowledge other method limitations, for it to be efficiently applied and not undermine the benefits that are possible to be gained from its use. Along the following subsections, different limitations of the QIM methodology will be discussed

2.3.3.1 Quality specifications and tolerance limits

The utilization of QIM methodology requires a definition of what is the quality parameters that have to be assessed and their tolerance limits. Fish freshness characteristics are well studied and established throughout the supply chain [34]. Therefore, they are well established and accepted not only in sensory analysis but also in chemical and microbial methods [125]. However, is necessary some attention for the establishment of these limits, being necessary to consider the method applicability, the type of product, the company or client standards. The limit should not be arbitrarily applied or being simply inferred as the mean value of the QI values [118].

2.3.3.2. Assessors' variability

One of the principal limitations attributed to QIM is that being considered a rapid methodology, is possible to be used by factory personnel or semi-trained assessors, which challenges the reliability of the results, due to variability associated with each assessor [30,117,118]. Other sources of bias affecting sensory analysis panelists can be divided into psychological (*e.g.*: memory effect and concentration) and physiological (*e.g.*: age and nutritional health) effects [25].

Kilcast (2010) described some of the steps for panelist recruitment, training, and proficiency test, emphasizing the implementation of sensory quality control schemes in organizations [32]. Such approaches are relevant to decrease the probability of biased results from the sensory test outcomes. Lawless (2010) reviewed some of the methodologies for panel performance analysis, which can be univariate (*e.g.*: mean square errors (MSE)), multivariate (*e.g.*: principal component analysis (PCA)), or with the use of computational programs (*e.g.*: Panel Check) [30]. Variability measurement is important not only for panel control but also for shelf-life estimation as will be discussed in further sections.

2.3.3.3 Product variability

Product variability is normally attributed to the product's intrinsic conditions (*e.g.*: nutritional composition and autolysis processes) or external effects (*e.g.*: season, killing, handling) [74]. The measurement of these product variations is based on other methods referred to as traditional methods that encompass the chemical, physical and microbial analysis approach. Besides none of these approaches being a perfect choice, such measurements can provide valuable information about the fish freshness state [126].

Variation of results (QI values vs storage time), for the same species, can demonstrate the impact of storage conditions, product formulation, packaging, handling, and killing on the development of sensory attributes during the degradation process. It is relevant to state that normally QIM schemes are compared with other methods for results validation, such as chemical, microbial, textural, or sensory. Ndraha (2017) reviewed the correlation between sensory, microbial, and chemical analysis on fish degradation. All these methodologies have established their legal maximum limits, from which the products are considered unacceptable for consumption. When the results are compared between different methodologies, it is possible to see the influence of each biological process on product rejection. As a consequence, some parameters could deteriorate at different rates due to microbiological and/or chemical activity, being of importance the appropriate choice of methods to obtain more concise results [38].

The external effects have also been studied using the QIM methodology [77]. Taking the example of the washing effect on fish shelf-life there are different opinions, regarding whether the QIM approach is reliable enough to consider such impacts because they can lead to misinterpretations when used for predictive purposes, indicating longer or shorter shelf-life [74,127]. Therefore, it is not unusual for QIM schemes for the same species to present some variability in their results [128]. For some species the methods used for validation, for example, chemical analysis, may not be 100 % correlated with sensory analysis, due to specific endogenous characteristics of the fish species under study [38]. However, it is still important to perform such methodologies not only to determine for which species they are fitted but also to increment the knowledge about the occurrence of such differences.

2.3.3.4 Data analysis

The common procedures for QIM data analysis are regression analysis of the scores and storage time, followed by verification/validation through PCA and PLS analysis. The utilization of the statistical methods is dependent on the questions to be answered and how the data is structured. In the QIM case, the main objective is to verify the suitability of the selected attributes to describe the spoilage evolution, their correlation with storage time, and determine the efficiency of the prediction. The importance of statistical treatment, the conditions for their application, and associated limits have been extensively reviewed. Lawless & Heymann (2010) and O'Mahony (1986), give a very extensive and structured analysis of statistics applied to sensory analysis [30,129]. Galanakis (2019) and Kilcast (2010), approached a similar topic but under the quality control programs perspective [32,118], while Rossini (2012) and Pedro (2006) emphasize the application of PCA and PLS in sensory studies [130,131].

In terms of predictive model utilization of the QIM approach have some faults due to assumptions that are made. For example, the assumption that the zero-temperature is maintained throughout the storage does not correspond to a real-world condition, since it is known that temperature variations are common and have an impact on product quality [132,133]. Also, it is a punctual/ categorical count analysis carried out in a specific time, and the predictions based on these conditions are not the most reliable, since they occur in a pre-determinate experimental environment, resulting in static correlation result (most probable prediction), limiting its predictive capability [74,105,128]. Other common setbacks are related to the importance, or weightage, that is allocated to each evaluated attribute, that due to the summations of the scores gain some redundancy [128,134].

It is important to refer that the QIM method only does a prediction of the shelf-life under its specific conditions and is best suited for the industrial environment where quick decisions must be made. However, the outcome of QIM methodology has its importance on the predictive models for shelf-life determination and is frequently integrated into the model's equations. This specific area of shelf-life estimation, through mathematical models, solves some of the common hurdles associated with the QIM methodology [9,37,135].

2.4 Future improvements and trends

In the last years, a great effort has been made to spread the QIM methodology as a reliable and standardized method, to be adopted in the seafood chain. Different European projects were established to spread knowledge throughout the entire supply chain [136]. However, a new and recent study might be needed to determine at which level the method is in action and how the information is being used. In the investigation area, the method is well established, being also common in seafood quality courses [22].

The consumer QIM (C-QIM) scheme was also developed, not as an acceptance test, but as a decision tool to support the purchase of the product at fish markets [21,22]. The correlation of QIM knowledge and methodologies for consumer studies (*e.g.*: CATA) has been also studied, to determine the common ground between both approaches and reduce the gap between consumers opinions, researchers, and market players [87,137].

The creation of the app ‘’How fresh is your fish’’ in 2013 was an excellent initiative to reach the consumer (www.qim-eurofish.com). However, stagnation seems to exist in terms of the available species and integration of new ones, or the adaptation to more consumed species in each country. With the appearance of new QIMs and new partnerships, it might be possible to create a more robust database with the species organized by relevance for each country, markets demand, and other pertinent information [136]. More studies about the effective implementation of the method could also be important, as the one performed by Indian researchers, at the Veraval fish landing center, where it was followed the process of fish freshness evaluation and grading, using QIM and the Torrymeter [138].

The evolution of integrative systems for quality control led to the proposal of sensory analysis systems applied to the total fish supply chain. The main purpose is to spread the communication about sensory results between main players throughout the supply chain. QIM approach can be applied all over the supply chain and further contribute to quality improvement from catch to the final consumer [24,139]. In the case of E-commerce platforms and electronic auctions, QIM methodology will have an impact as an integrated part of the quality evaluation. The development of digital technologies (*e.g.*: blockchain methodologies) will allow the integration of information relative to several quality parameters throughout the supply chain [140]. The safety associated with the blockchain can reduce information errors and uncertainty between supply chain participants, making available information about the product that in other ways is difficult to access [10].

The recent developments achieved by the biosensors (*e.g.*: enzyme biosensor), sensory bionic technologies (*e.g.*: e-tongue), spectrometric techniques (*e.g.*: Vis/NIR), and time-temperature sensors (TTI), will facilitate the transference of information related with the fish freshness and spoilage, to on-line platforms. Also, it has the potential to surpass some of the limitations associated with the conventional approaches (*e.g.*: subjectivity and repeatability) and contributes to the future understanding of degradation processes and the impact on sensory evaluation [132,141,142].

A holistic overview of the influence between different factors could be obtained through more studies, following a metanalysis approach as performed by Batista, (2012) [143]. It would help to shed light on the possibility of integration of different QIM schemes under different categories of products or species, as proposed by Shabani and his coworkers. They suggest the utilization of a QIM scheme for salmonids. The hypothesis was tested through the application of a salmon (*Salmo salar*) QIM scheme in the rainbow trout (*Oncorhynchus mykiss*) shelf-life estimation. They concluded that a similar rejection time for trout was achieved with both schemes, therefore a QIM scheme for salmonids is possible to be developed [144].

New study areas for the QIM application will also be driven by the mathematical modeling of fish degradation. The first steps were given with the development of the Food Spoilage & Safety Prediction software (FSSP - (http://fssp.food.dtu.dk/), updated in 2014 [145]. The integration of several predicting models approaches will continue to contribute to QIM development, leading to new finds and a better understanding of the degradation process [21,22,146]. Also, it would help to overcome some of the associated limits of the method (section 2.3.3.), turning it into a more robust approach, not only for the fish sector but also for research purposes [18,74,126].

2.5. Conclusions

The application of food quality control systems, in the sensory analysis field, brings up questions related with the selection of properties or characteristics to be measured and the choice of methods. In this work, the merits and demerits of the QIM approach are reviewed. Most of the analyses performed under fish freshness analysis or spoilage determination are limited by the ‘‘one variable effect’’ (*e.g.*: chemical), lacking sometimes the consideration of the holistic view of the synergies between the other remaining variables (*e.g.*: autolysis, microbial, or sensory). In this case following the idea of the sequential effect, the sensory analysis could be the last step of the chain since it tries to evaluate the simultaneous effect of the other parameters, even though they might not have an ordered sequential evolution.

Progress has been made in identifying and measuring freshness or spoilage parameters and certain quality-related criteria, but the promise of novel, fast, and cost-effective methods, might be closer for the bigger players and research facilities, than for the small and medium enterprises, in which the use of the current approaches will remain the nearest possibility [23].

Even though, the common approach in the research field is to separate the methodologies according to their source (chemical, sensory, microbial), the rise of the chemometrics analysis, as well mathematical modeling, will improve the limitations between methodologies, allowing each approach to find its space at research and industrial level.

In the specific case of QIM methodology, once the most costly steps are surpassed (development and validation), it remains a suitable response, that allows achieving a compromise between the number of samples necessary and the list of characteristics, that allow simplicity into to check if the measured differences fulfill quality grade requirements, with enough precision for the industry where quick and cost-effective decisions have to be made [147].

The recognition of the method limitations will allow proper data analysis and avoid doubtful conclusions to be made. The proposed improvements on the method, based on the statistics and mathematical modeling will increase its reliability and predictive power, contributing to the standardization of procedures and seafood supply chain improvements.

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Chapter 3 - Freshness Assessment and Shelf-Life Prediction for *Seriola dumerili* from Aquaculture Based on the Quality Index Method

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Article

Freshness Assessment and Shelf-Life Prediction for *Seriola dumerili* from Aquaculture Based on the Quality Index Method

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Abstract

Fish and fish-based products are easily perishable foods due to different factors, including fragile organization, abundant endo-enzymes, psychrophilic bacteria, and impact of pre-harvest operations, that contribute to reducing its value. Therefore, a timely effective method for fish freshness and shelf-life evaluation is important. In this context, this study aimed to develop a sensory scheme based on the Quality Index Method (QIM) (sensory table and point system) for freshness monitorization and shelf-life prediction for *Seriola dumerili* from aquaculture in Madeira Island. Evaluation of appearance, texture, eyes, and gills was performed during 20 days of storage on ice (0 ± 1 °C). The shelf-life prediction was supported by the analysis of microorganisms (total viable colonies, TVC, counts), texture (Torrymeter), and production of trimethylamine (TMA), evaluated by HS-SPME–GC–MS and validated according to the Association of Official Analytical Chemists AOAC guidelines. The result is a QIM scheme with 25 demerit points, where zero indicates total freshness. From the integration of sensory analysis, microbial growth at the time of rejection (TVC, 10^8 cfu/cm^2 and H2S producers, 10^7 cfu/cm^2), texture (Torrymeter value <8), and TMA analyses (>12.5 mg/100 g), shelf-life was estimated as 12 days (± 0.5days). The obtained results show the high-throughput potential of the developed method for fish freshness assessment and shelf-life prediction. This QIM scheme is a secure way to measure quality and provide users with a reliable standardized fish freshness measure.

Keywords: Greater amberjack**;** *Seriola dumerili*; freshness; shelf-life; QIM; sensory analysis; physicochemical analysis; microbial analysis

3.1. Introduction

The European aquaculture sector is dominated by long-established species such as rainbow trout (*Oncorhynchus mykiss*), Atlantic salmon (*Salmo salar*), common carp (*Cyprinus carpio*), gilthead seabream (*Sparus aurata*), and European sea bass (*Dicentrarchus labrax*) [1]. To diversify the product offer, several attempts have been made to introduce new species at the pilot scale or production level in Mediterranean aquaculture [2]. One of the candidates for marine warm water cage culture is *Seriola dumerili* (Risso,1810), because of its high economic value and growth rate (6 kg in 2.5 years) and its diffusion in the circumglobal temperate area [3]. It is also known as greater amberjack (GrA) and by other common names in European territory (Table 3.1).

In the Mediterranean, GrA production in 2012 was approximately 2 tons, most from wild capture, with hatchery-produced individuals in Malta and some from production efforts in Greece, Spain, Italy, and Cyprus [4]. The conduction of several studies regarding the fatty acid composition of wild and reared fish [5], reproduction [6], feed formulation [7], rearing conditions [8], and handling effects [9] has led to important improvements to overcome some bottlenecks of the European aquaculture industry and GrA aquaculture development.

Table 3.1 - Common names for Seriola dumerili by country

Country	Common Name
Germany	Bernstein-Stachelmakrele; Bernsteinfisch; Grünel
Spain	Cèrvia; Pez de limón; Seriola; Serviola
UK	Amberjack; Greater amberjack
France	Ciriola; Seriole
Portugal	Charuteiro; Charuteiro-catarino; Írio; Lírio
Croatia	Bilizmuša; Felun; Gof; Orhan; Orva
Malta	Accola; Cervjola; Serjola; Serra
Denmark	Stor ravfisk
Sweden	Bärnstensfisk; Seriola
Serbia	Orfan; Orhan
Slovenia	Gof
Italy	Acciola; Alice grande; Alici; Alicosa; Aricciola; Cavagnola; Jarrupe; Lampuga; Lecc; Leccia; Lecciutte; Licciòla; Lissa; Lissa bastarda; Lupina; Occhio grasso; Ricciola; Sartaleone; Seriola; Seriola di Dumeril; Sirviola; Sumu
Greece	Magiatiko; Manali; Mayàtico; Mayatiko
Finland	Isopiikkimakrilli

Other studies were conducted in terms of consumer acceptability and sensory analysis [2]. Sensory characterization is an important food quality determinant, a driver for consumer acceptance, food choice, and market value. Also, the quality characterization of aquaculture products according to production and post-production factors is always of primordial importance [5,6]. Sensory analysis is adaptable to several points in the supply chain, being relevant to verify fish freshness at different transaction points. Sensory analysis applications will ultimately enhance the overall product perception in the modern demanding markets [7].

At the seafood industrial level, several methods can be used to evaluate the quality of the products to ensure that qualitative parameters are met by all agents in the value chain (producer to final consumer), including regulatory agencies, as well as in different stages of fish processing [8]. It is known that no single method is reliable enough to determine the freshness or quality of seafood products [6]. Recognition of the freshness, and acceptance or rejection, of fish on this basis may be all that industry needs.

On the other hand, different species and products spoil in different patterns, and the use of appropriate assessment methods is needed. Apart from sensory methodologies, physical, chemical, and microbial methods have been developed [8–10]. However, most of them have as fundamental base sensory knowledge to associate the analytical results with overall quality. Therefore, sensory evaluation is still considered one of the most effective techniques to satisfactorily assess freshness and grade fish or fish products [11]. Developed by the Tasmanian Food Research Unit (TFRU), the quality index method (QIM) is a fast, simple, nondestructive, and descriptive, grading system for seafood freshness evaluation. It integrates the differences between fish species through objective assessment of fish attributes (*e.g.*: gills odor) [12]. It also provides the user (producers, buyers, sellers, and resellers) with a reliable and standardized methodology that includes instructions and easily understandable illustrational material. It is well suited to teach inexperienced people and train or monitor panelists' performance [7]. Attributes are evaluated with a scoring system (0 to 3 demerit points), and as time progresses, higher punctuation is given [6]. However, it is common that one or more attributes do not possess the maximum demerit points because the changes during the storage period may not be significant to achieve such scores [13,14].

After adding up all scores, a quality index (QI) is obtained for shelf-life prediction. The prediction is possible if a linear correlation between QI and storage time is achieved. However, new QIM schemes are constructed with the support of other methods. The evaluation of fish muscle with the Torry Scale is common in regions where fish is mainly commercialized in fillets [9]. For fish sold as a whole, rejection occurs sooner; therefore, it is preferable to evaluate the external characteristics using different methods [13]. Other methods are the measurement of muscle dielectric properties (Torrymeter, TRM), the enumeration of specific spoilage organisms (SSO) [15], and chemical evaluation [9].

The QIM-EUROFISH project has been very important in the dissemination of the QI method, with the publication of the QIM manual in diverse languages, and with 13 QIM schemes as examples [12,16]. A considerable effort to publish and optimize the QIM schemes has been made, demonstrating the increasing importance of QIM for new species and products from wild or farmed species. Information can be found in Barbosa and Vaz-Pires, Bernardi et al., Sant′Ana, Soares, and Vaz-Pires, Ndraha [7,13,17,18], and in the Eurofish-QIM database. The objective of this study was to develop a sensorial scheme based on QIM (sensory table and corresponding point system) for freshness monitorization and shelf-life prediction of GrA, farmed in Madeira Island, through the evaluation of changes in general appearance (skin, anus, and odor), texture (firmness), eyes (color and shape), and gills (color, mucus, and odor) throughout 20 days of storage on ice (0 ± 1 °C). Microbiological analysis (total viable colonies counts, TVCc), physical (TRM) and chemical analyses (pH; and trimethylamine (TMA) quantification through HS-SPME–GC–MS) were also performed.

3.2. Materials and Methods

3.2.1. Samples

Fresh GrA specimens were acquired from Ilhapeixe SA (IPx) aquaculture facilities in Ribeira Brava, Madeira Island, Portugal. A total number of 30 specimens were captured between August and September 2018. The fishes were killed in a mix of ice and saltwater, placed on ice, and transported in polystyrene boxes to the company's refrigerated facilities. The time between capture and arrival at the facilities was between 3 and 4 h. The fishes were washed, placed in perforated polystyrene boxes, and conserved on crushed ice at 0 °C (±1 °C) for a maximum period of 20 days. Fresh ice was added when necessary. The samples had an average weight between 1.5 and 2.5 kg and a total length between 51 and 66 cm.

3.2.2. Sensory Analysis, QIM

The QIM protocol was carried out as reported by several authors and the method reference manual [7,13,16,22]. It consisted of three stages, two training sessions, and one validation step. To develop the quality index for ice-boxed GrA, six trained QIM assessors were selected among the staff of the fish factory IPx and the CQM-Centro de Química da Madeira, on the basis of previous experience with the fundamentals of whole-fish sensory analysis, no natural impediments (*e.g.*, allergies or low sensory sensibility), non-smoker status, vocabulary usage, and knowledge of the studied fish species. The first trial was necessary to familiarize the assessors with the degraded fish characteristics, find the parameters that change with time, and obtain a first draft of the QIM scheme. The QIM scheme for GrA was established considering attributes for appearance, texture, eyes, and gills and descriptions of how they change with storage time (0, 2, 4, 7, 9, 12, 15, and

20 days). Scores, ranging from 0 to 3, were given to each key attribute according to the descriptions, being 0 totally fresh fish, and 3 clearly spoiled fish. For attributes that do not present four clear degradation stages, the score was attributed according to the stage that was clearly present when the fish was rejected *(i.e.*, eyes having three clear alterations (0, 1, and 2) during the period of the trials. Since a fourth alteration was not detected, the score 3 was not attributed).

The second experiment was used to confirm the first trial impressions, clarify less clear points, and retrain the assessors with the obtained QIM scheme (1, 3, 6, 10, 14, 17, and 20 days). The panel performance was evaluated by obtaining the QI for each participant during these experiments. The parameters evaluated were the QI slope and r^2 values of the panelists.

The third experiment was performed using the developed QIM scheme, and the assessors were unaware of the storage time (0, 2, 4, 7, 9, 12, 15, 17, and 20 days). The samples of each batch were presented to the panel in random order. All observations of GrA were conducted under standardized conditions at room temperature. A minimum of three fishes per lot was evaluated to reduce natural variations effects. The panel performance was evaluated by obtaining the QI for each participant during the three experiments.

3.2.3. Microbiological Analysis

Microbiological analysis was performed according to Sant'Ana et al. [13]. Areas of 15 cm^2 of fish skin were sampled with sterile cotton swabs, and 1 ml of cooled ¼ Ringer solution (Oxoid, Basingstoke, Hampshire, UK) was used for microorganism transfer. After serial decimal dilutions, inoculation was made using the 20 µL drop method on iron agar (Ir), nutrient agar (Nt) (both from Oxoid, Basingstoke, Hampshire, United Kingdom), and MacConkey agar (McA) (PanReac AppliChem, Darmstadt, Germany). TVC, as well as selective counts of sulfide (H_2S)-producing bacteria, were performed after 48h of incubation at room temperature (23 °C). *Enterobacteriaceae* plates were grown at 30 °C and counted after 3 days of incubation. Duplicate counts were performed at day 1, 2, 3, 5, 7, 9, 10, 13, 14, 15 and expressed as log cfu/cm^2.

3.2.4. Physical Evaluation

The physical evaluation was executed with the TRM 295 (Distell, West Lothian, Scotland, UK) and performed according to the equipment user manual [24]. The fish anterior-dorsal area was chosen for measurements, both sides were analyzed, and residual ice was cleared from the surface. The electrodes were cleaned between measurements and placed on ice to maintain a temperature similar to that of the fish (around 0 °C). All fishes from the trials were tested at days 1, 2, 4, 5, 7, 9, 10, 11, 12, 13, 14, 15, 18, and 20 of storage.

3.2.5. pH Measurement

The pH measurements were carried out on a 5:1 ratio of distilled water/fish homogenate, using a glass electrode at 20 °C according to Kyrana et al. [32]. The pH of distilled water, controlled before use, was between 7–7.4. Analyses were performed at 0, 2, 5, 8, 12, 14, 15, 17, and 20 days.

3.2.6. TMA Analysis

3.2.6.1. HS-SPME Procedure

TMA was isolated through the HS-SPME method. Sample preparation was based on previous works, with minor alterations [33]. The fish flesh (10gr) was blended in trichloroacetic acid (TCA 7.5%) in a 1:2 (w/w) ratio. After centrifugation at 10,000× *g* for 10min at 4 °C, the supernatant was stored until analysis (−80 °C). The SPME procedure was carried out in accordance with the previously described high-throughput extraction protocol, with slight modifications [34]. Briefly, 0.5 mL of supernatant, 1 mL of a 15 M NaOH solution, and 1 mL of saturated NaCl solution (35%) were added to an 8 mL SPME glass vial. Propylamine was used as an internal standard. The vial was then placed in a 35 °C water bath, and a divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fiber was exposed into the headspace for 30 min. After this period, the fiber was withdrawn and injected into the injection port of the GC–MS equipment (Agilent Technologies 6890N; Palo Alto, California, USA) for analysis.

3.2.6.2. Method Validation

The following parameters were chosen for method validation: calibration function and linearity, precision, accuracy, limits of detection (LOD) and quantitation (LOQ), matrix effect according to Araujo, Silva et al., [28,35], and AOAC guidelines [29]. In brief, the calibration function was performed with six different TMA concentration points in the 0.1–15 µg/mL range. F-test was used to evaluate linearity, checking the suitability of the function model. The precision was determined by repeatability and intermediate precision, measuring inter- and intraday variation, respectively. The method of "standard additions" was used to evaluate the matrix effect. The LOD and LOQ determination was based on the standard deviation of the calibration curve interception and the slope of a regression curve.

3.2.6.3. GC–MS Conditions.

The GC–MS analysis was performed with an Agilent BP-20 (30 m × 0.25 mmi.d. × 0.25 µm film thickness) column. The inlet initial temperature for TMA desorption was 235 °C. The run conditions were: initial temperature 80 °C (2 min hold), ramped to 220 °C (50 °C min^{-1}), and kept for 5 min. The total run time was 9.80 min. Carrier gas flow (helium) was set at 1.0 mL min^{-1} under splitless mode. The electron impact (EI) mode was 70 eV. TMA analysis was performed through specific ion selection (*m*/*z* 58). TMA identification was accomplished through interpretation of spectra and matching using the Agilent MS ChemStation Software, equipped with a NIST05 mass spectral library, and by comparison with commercially standard.

3.2.7. Statistical Analysis

The data statistical treatment was performed using the software STATISTICA 10.0 (Stat Soft, Inc., Tulsa, OK, USA). Analysis of the association between sensory variables included in the QIM scheme was performed with PCA. Error Estimation of the QIM scheme was carried out by PLS regression.

3.3. Results and Discussion

3.3.1. QIM Development

The parameters that were selected and included in the preliminary QIM were based on the description of external characteristics (skin, texture, odor, appearance of eyes, gills, and anus), as they showed clear changes during the first storage observations (Figure 3.1). During the development of the preliminary QIM tables, successive modifications were made to obtain the highest possible number of descriptors.

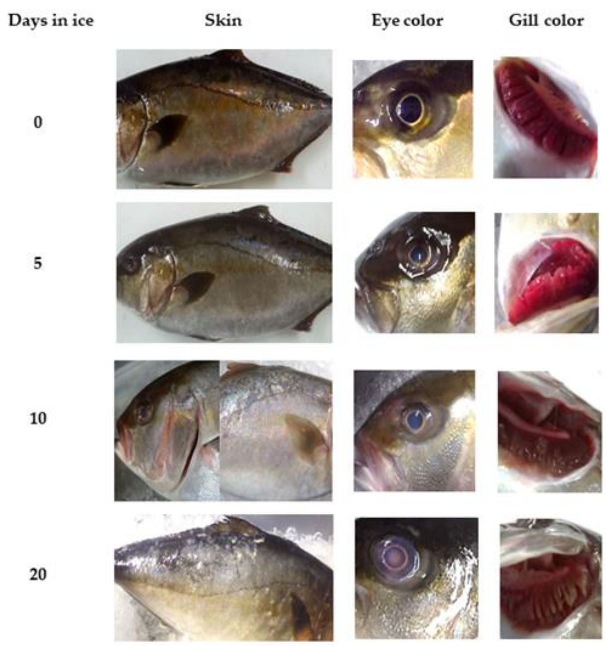

Figure 3.1 - Greater amberjack (GrA) main visual changes during storage at 0 °C on ice.

The panel performance evaluation was conducted by obtaining the QI for each specimen during the second experiment when the attributes for the evaluation were fixed. A significant correlation ($p < 0.05$) between all assessors' results was obtained. The slope and r^2 for each panelist (P) were: P1 (0.9781; 0.8945), P2 (1.0083; 0.9481), P3 (1.0706; 0.9445), P4 (1.0840; 0.8510), P5 (1.0754; 0.8443), P6 (1.1283; 0.8381).

The final scheme suggested in this work includes 25 demerit points, describing four quality attributes with 9 sensory attributes (Table 3.2).

Figure 3.2 shows the average points registered for each individual attribute as a function of the storage days on ice. It is possible to see that all quality attributes present an increasing trend during storage. QI is the sum of demerit points of the different quality attributes (Figure 2b). The QI for GrA showed a significant linear increase during storage on ice ($p < 0.01$), with a high coefficient of determination ($r^2 = 0.9771$) ranging from 0.4 to 25 from day 0 to 17, respectively.

Principal component analysis (PCA) analysis indicated that the proportion of variance for the first two principal components was 96.2%, PC1-93.8%, and PC2-2.4% (Figure 3.3a). All variables presented a high positive correlation with the first component; therefore, they contributed with the same approximate weight to QI. Analysis of variable importance confirmed each attribute's importance. For this scheme, values above 0.8 were considered relevant for the QIM protocol [19]. Thus, the variable importance for each attribute was, in decreasing order: odor–0.959; gill odor–0.958; eye color–0.950; eye shape–0.947; gill mucus–0.946; gill color–0.939; skin–0.919; firmness–0.905, and anus–0.815. However, the correlation results for QI values showed a higher correlation (0.991) than for single attributes. This indicates that the proposed QIM scoring scheme is suitable to follow the deterioration progress and correlate it with time, which is in accordance with other developed QIM methods [20,21].

Table 3.2 - Quality index method (QIM) scheme proposed for *S. dumerili* containing a description for each parameter and the attributed scores (from 0 to 3).

Attribute	Parameter	Description	
Appearance	**Skin**	Very bright and iridescent; Side brownish (>50% covered).	0
		Bright; side brownish (<50% covered).	1
		Pale and dull; Side greyish; mouth or operculum reddish pink.	2
		Marked; Superficial mucus; Blood on operculum and mouth.	3
	Anus	Clean and closed.	0
		Abundant yellow feces.	1
		Dry light brown feces and open.	2
	Odor	Sea, seaweed.	0
		Neutral.	1
		Rancid; Metallic.	2
		Putrid.	3
Texture	**Firmness**	In rigor mortis.	0
		Firm, recovers shape fast (≤2 sec).	1
		Softness; recovers shape slow (>2 sec).	2
		Clearly marked and spongy.	3
Eyes	**Color**	Black; shinny.	0
		Black opaque/slightly milky.	1
		Greyish/milky.	2
		White/grey in partial or total eye.	3
	Shape	Convex.	0
		Flat.	1
		Concave.	2
Gills	**Color**	Dark red.	0
		Light red.	1
		Brownish red	2
		Brown, grey, or discolored; Bacteria present.	3
	Mucus	Absent.	0
		Present and colorless.	1
		Whitish/cream.	2
		Abundant, brown, or yellowish.	3
	Odor	Sea or seaweed.	0
		Neutral.	1
		Rancid; metallic.	2
		Putrid.	3
		Total Demerit Points	**0–25**

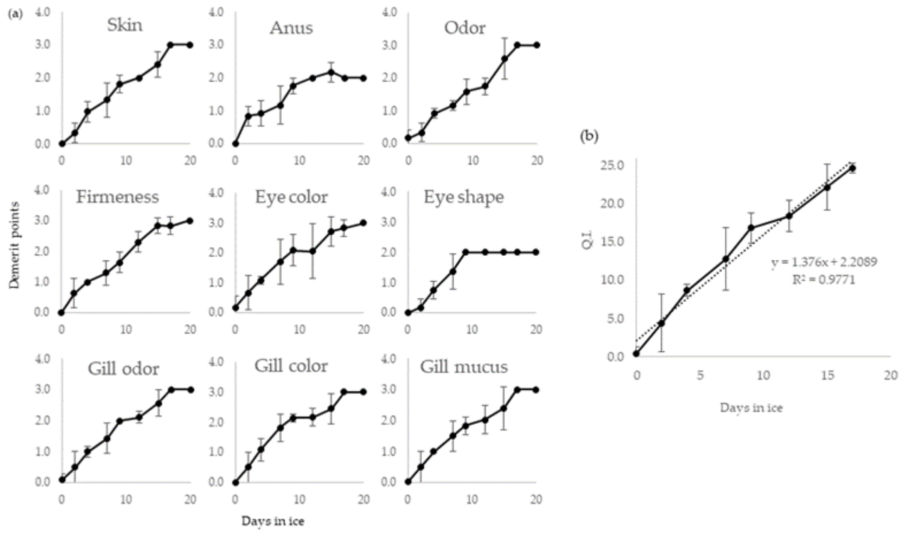

Figure 3.2 - (a) Evolution of different QIM parameters for GrA (mean values of the six assessors). (b) QIM calibration curve (QI versus days on ice) for GrA during ice storage (0 ± 1 °C). Points that seem without error bars have in fact error bars smaller than the symbol size.

Principal component analysis (PCA) analysis indicated that the proportion of variance for the first two principal components was 96.2%, PC1-93.8%, and PC2-2.4%.

For the GrA sensory scheme, the odor was one of the main contributors to the determination of deterioration progress. At the start of the trial, the skin and gill odors were described as seaweed or fresh and, from day 3, became neutral. The perception of the off-odors associated with degradation occurred on day 11, but the intensity was only considered sufficient for rejection on day 12. The QI average equations of observed and predicted values were subjected to the partial least-squares (PLS) regression model. The results showed that the obtained linear regression model had an estimation error of 0.54 days and a suitable linear regression (r^2 = 0.995) (Figure 3.3b). The predicted and measured values were statistically similar ($p < 0.05$).

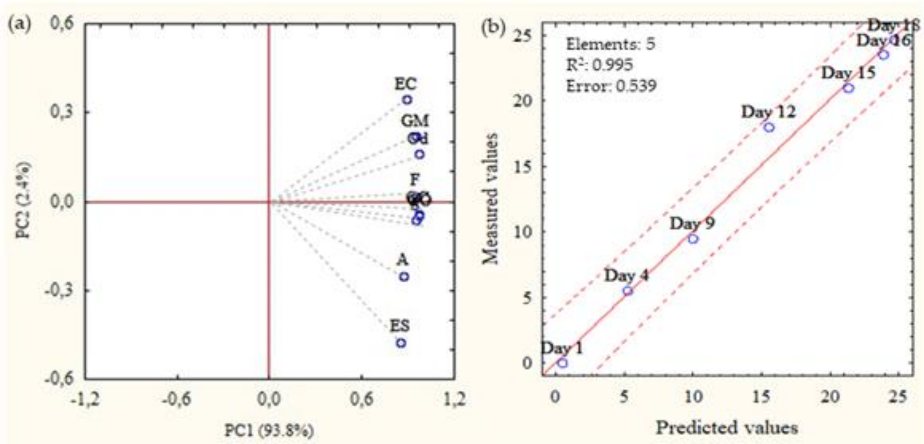

Figure 3.3 - (a) Principal component analysis (PCA) correlation plot describing QI values and individual sensory parameters: skin appearance (S), Odor (O), firmness (F), gill color (GC), gill odor (GO), gill mucus (GM), eye color (EC), eye shape (ES), anus (A) of all GrA samples; (b) partial least-squares (PLS) regression model of the QI scores of GrA samples versus the predicted values. Trace lines represent the confidence limits of regression (95%).

The developed QIM scheme successfully described different freshness levels of GrA and sustained individual panelists variation. Taking into consideration all sensory data, the rejection was estimated to occur between days 11 and 12, mainly due to the presence of significant off-odors on the skin and gills (rancid odors).

3.3.2. Microbiological Analysis

Microorganisms associated with aquatic species reflect the bacterial population of the surrounding environment and the conditions during manipulation or processing. In newly processed seafood, SSO exists in low numbers and constitute a small fraction of fish microflora. During storage under particular conditions (*e.g.*, specific temperature), SSO grows faster than the remaining microflora, produces metabolites responsible for off-flavors, and finally causes sensory rejection of a product [9,22]. The most common SSO associated with marine species from temperate waters, stored on ice (0 °C) under aerobic conditions, are *Shewanella* spp. and *Pseudomonas* spp. They are associated with ammoniacal spoilt and hydrogen sulfide-type odors [13]. The TVC after the catch varies between 10^1 and 10^4 cfu/g or cm^2, while at times of rejection, the values vary between 10^7 and 10^9 cfu/g, according to fish species or fish product [13,14].

The microbiological analysis results are presented in Figure 3.4a. All data shown represent the mean of two different samplings from all individuals tested ($p < 0.01$). Microbiological analysis of the skin showed an initial TVC of 10^3 cfu/cm^2, which increased gradually until day 7 (10^6 cfu/cm^2). The H_2S-producing microorganisms (that appear as black colonies owing to the precipitation of iron sulfide) presented lower TVC than in Nt and Ir during the first 9 days (10^3–10^5 cfu/cm^2). However, after day 9, the plate counts achieved values close to the TVC in Nt and Ir. Using the fitted regressions, the recommended limit values for bacterial counts (10^7–10^8 cfu/cm^2) was surpassed after 10 to 11 days, with values of 10^8 cfu/cm^2 for TVC on Nt and Ir, while for H_2S producers, the plate count was 10^7 cfu/cm^2. Counts of *Enterobacteriaceae* on McA were very low, never exceeding 10^2 cfu/cm^2 or being negligible. The general hygiene condition of fish samples depends on water quality and handling, which starts on board and seemed to be acceptable for the samples studied, taking into account the results obtained in this work.

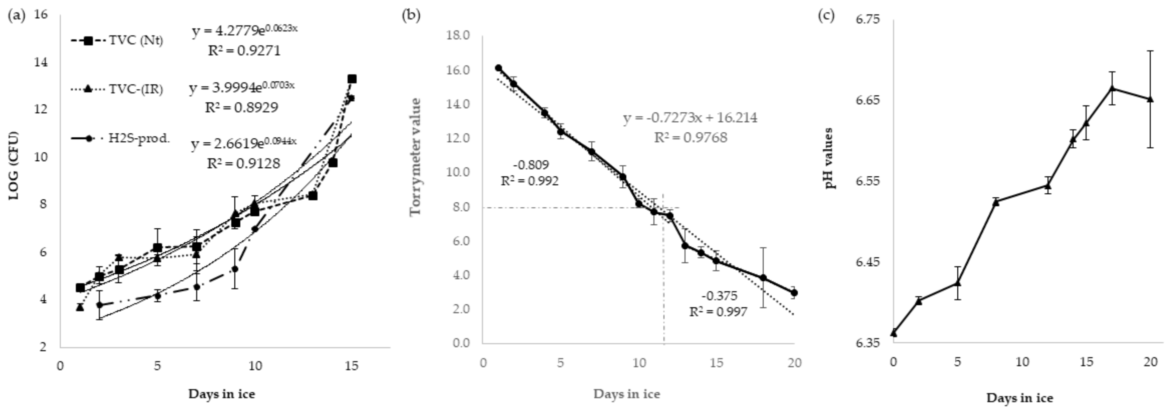

Figure 3.4 - (a) Bacterial growth results, shown as total viable counts (TVC) in nutrient agar (Nt) (■); (TVC) in iron agar solid medium (Ir) (▲), and H2S-reducing bacteria (●) in ice-stored GrA for 16 days (CFU: colony-forming units). Each point is a mean value of two analyses, with vertical bars indicating standard deviation; (b) Torrymeter values of whole GrA during ice storage; (c) pH values of GrA samples against storage time on ice. Each point represents the mean values of three analyses, with vertical bars indicating standard deviation.

3.3.3. Physical Evaluation

The base for electrical measurements on fish is the fact that the cell membranes in fish muscle tissue are progressively disrupted by autolytic enzymatic degradation and, later, by microbial action, leading to a decrease of electrical resistance and capacity. Therefore, as time progresses, the amount of electric impulse detected will be lower [22]. During the three storage experiments, all fishes were analyzed during harvesting, handling, and storage in order to reduce the variability of equipment readings due to differences in biological conditions and physical damage to the tissues. They could present lower or more variable dielectric properties, indicating apparently advanced spoilage [20].

The changes in dielectric properties during storage on ice of the GrA samples are shown in Figure 3.4b, which shows the mean of all fishes from the three trials. At the beginning of storage, the TRM means values (>16) of GrA samples indicated "very fresh fish", and a relatively high negative correlation with storage time was observed (r^2 = 0.9768).

The mean TRM values decreased almost linearly in the course of the first 11 days of the trial (16.0–8.0). Between 12 and 20 days, TRM values varied less (from 8.0 to 3.0), creating two distinct regions with different slopes. The first region showed a slope value of −0.809 (r^2 = 0.992), and the second region presented a value of −0.375 (r^2 = 0.997). The change in slope is a consequence of the two-phase quality-loss phenomenon explained by two different mechanisms of degradation, *i.e.*, enzymatic autolysis and microbial action [23]. The change of the trend of the slope could indicate the mean value for consumption acceptability [23]. In this study, the slope change occurred between the mean values of 7 and 8, corresponding to 11–12 days of storage. The value of 8 as the limit for consumption is also used for other species [24,25].

The proposed value for consumption acceptability is also in agreement with the QIM values, which set sensory rejection between 11 and 12 days of storage. The TRM values were interpreted as follows: absolutely fresh fish was given a score of 16; fish in the commercially useful storage period received a score of 8 or more; scores below 7.5 would be indicative of low-quality products for consumption. The results presented here show that this instrument is useful for quality evaluation during the first 11–12 days of storage.

3.3.4. pH Analysis

The determination of the concentration of hydrogen ions (pH measurement) in fish products can be used to assess the degradation progress [19]. The typical pH of live fish varies between 7.0 and 7.3. After death, the pH is generally between 6.0 and 6.8 and in some species, is below 6.0, because of high glycogen concentrations. The results of the pH measurements during spoilage showed that after rigor mortis, the pH increased from 6.4 to 6.7 (Figure 3.4c), throughout the experiment duration. However, pH measurements were not statistically significant (p = 0.21) and may not be suitable for GrA, in contrast to other species, because of individual initial differences, fluctuations of the values, and the low correlation with the sensory results [21,22].

3.3.5. TMA Analysis

3.3.5.1 HS-SPME–GC–MS Method Validation

HS-SPME, developed for the analysis of volatile compounds, has demonstrated a unique capability of incorporating extraction and concentration in one single step as well as compatibility with GC–MS [26,27]. The validation of the analytical methodology for TMA analysis is presented in Table 3.3.

Calibration curve linearity was calculated as suggested by Araujo, 2009 [28]. The experimental Fisher value was compared to the critical theoretical Fisher value at 95%, with 12 and 4 freedom degrees (3.26).

Table 3.3 - Summary of trimethylamine (TMA) method validation parameters.

Validation Parameters[1]	
Concentration range (µg/mL)	2.0–15
Regression equation	$y = -0.0108x^2 + 1.080x + 0.1102$
R^2	0.9994
Linearity test (F_{theo}/F_{exp})	2.51[2]
LOD (µg/ml)	0.6
LOQ (µg/ml)	1.9
Matrix effect (%)	106
Recovery (%)	93.3 LL[3]; 98.0 ML[4]; 99.0 HL[5]
Intermediate precision (% RSD)	8.4 LL; 7.1 ML; 9.7 HL
Repeatability (% RSD)	8.2 LL; 6.0 ML; 9.6 HL

[1] For method validation, triplicate measurements were taken; [2] *p*-value calculated experimentally through *t*-Student test between slopes obtained from the calibration curves for TMA in 7.5% TCA and TMA in fish samples. If p-value ≥ 0.05, no significant matrix effect was observed. [3] LL: low-level concentration of TMA; [4] ML: medium-level concentration of TMA; [5] HL: high-level concentration of TMA.LOD: Limit of detection; LOQ: Limit of quantification; RSD: Relative standard deviation.

If the experimental dataset describes a proposed function calibration of the given form, then the condition (Ftheo. > Fexp.) must be fulfilled. The best result was achieved for the presented 2nd degree polynomial equation. The results obtained for the remaining evaluated parameters were in agreement with the reference values stipulated by the AOAC guidelines [29]. For the matrix effect, the reference is between 70 and 125%; the intermediate precision and repeatability references are below 15% RSD, and recovery is 80–115%.

3.3.5.2. Method Application for TMA Quantification in GrA

TMA is a volatile amine, commonly associated with the typical “fishy” smell during fish degradation. It can be produced by the bacterial reduction of trimethylamine oxide (TMAO) in marine fish species [30]. A small production of volatile nitrogen components could indicate limited growth of bacteria that produce TMA, such as *Shewanella putrefaciens* [21]. In Europe, the limit established for TMA, according to Directive

91/493/EEC, is 12 mg/100 g [31]. HS-SPME developed for the analysis of volatile compounds, has demonstrated the unique capability of performing extraction and concentration in one single step and compatibility with GC–MS [26]. The results obtained from the application of HS-SPME/GC-Ms are presented in Figures 3.5a,b.

The obtained data indicated a gradual slow increase of TMA content during the first 7 days (5.6–9.0 mg/100mg) (Figure 3.5c). Between 12 and 15 days, the TMA content increased much faster, since the values went from 11.1 to 20.7 mg/100 mg during this period. This is in accordance with the microbiological results obtained in this work. Using the fitted model for TMA data, the 12 mg/100 mg criteria could be reached between 12 and 13 days.

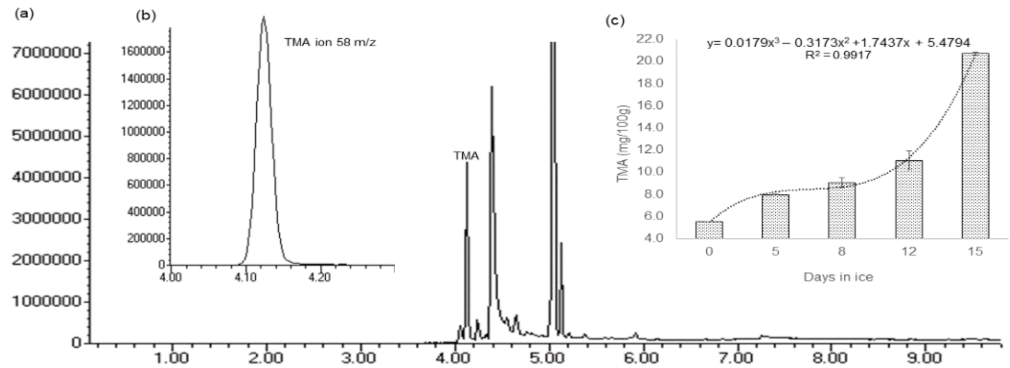

Figure 3.5. (**a**) Total ion chromatogram of a GrA sample obtained through the HS-SPME/GC–MS method. (**b**) TMA selected-ion chromatogram *m*/*z* 58, from the GrA sample. (**c**) Results of TMA quantification with the HS-SPME/GC–MS method, during 15 storage days. Each bar represents the mean of three analyses, with vertical bars denoting standard deviation. Points that seem without error bars have in fact, error bars smaller than the symbol size.

Since the fish deterioration process involves several simultaneous changes, Table 3.4 summarizes the results for sensory, physicochemical, and microbiological evaluation, corresponding to the criteria used to predict and estimate GrA shelf-life.

Table 3.4 -Rejection values and estimated rejection day according to each assay.

Method	Rejection Criteria	Value at Rejection	Estimated Rejection Day
QIM	Off-odors	17.5	11–12
Torrymeter	Slope change	8	11–12
Microbiology (TVC)	Log (cfu/cm^2) = 7–9	8.0	10–11
Microbiology (H_2S)	Log (cfu/cm^2) = 7–9	7.5	10–11
Trimethylamine (TMA)	12 mg/100g	12.5	12–13

3.4. Conclusions

QIM methodologies are being widely applied to various fish species [36–39] and products [40,41], reflecting the importance of the methodology for fish freshness evaluation and shelf-life prediction. Besides the expected differences between different species, it is also possible to obtain variations in the values of the evaluation parameters for the same fish species, mainly due to storage conditions or type of product [42–45], reflecting the expected variance that other methods do not incorporate (*i.e.*, EU scheme).

To the authors' knowledge, this is the first QIM scheme developed for GrA. A quality index with a maximum of 25 demerit points and 9 descriptive parameters (skin and anus appearance; fish odor; firmness; eye color and shape; gills mucus, color, and odor) is proposed. By applying this QIM scheme on whole, ungutted GrA, it was possible to estimate a shelf-life of 12 days (±0.5) on ice.

As stipulated in the QIM methodology, the results were compared with those of other freshness-evaluation methods, such as dielectric properties determined by TRM, TMA quantification, and microbiological analysis. From these comparisons, it was possible to confirm the shelf-life prediction. The results of TRM reflected the textural alterations of GrA during the storage period, starting with a maximum value of 16 and a rejection value of 8 between days 11 and 12 when off-odors were also detected. In other studies, the value 8 was also reached when rejection was determined [24,25]. The microbiological analysis also showed a significant increase of bacteria during ice storage. TVC values reached the rejection limit (between 10^7 and 10^8 cfu/cm^2), after 10–11 days. The results are similar to those of other studies that also correlate microbial rejection values with sensory rejection time [13,17,21,46]. TMA quantification through HS-SPME–GC–MS was compared with quantification by reference methods, such as total volatile base nitrogen (TVBN) quantification, and good correlations were achieved [33,34,47]. In the present work, the TMA rejection value (12 mg/100 g) was reached between 12 and 13 days. TMA production is also related to the increase of microbial load on fish, so it is common that the TMA limit is achieved when SSO are present above the rejection limits [30].

Even though newly developed methods for identifying and measuring spoilage parameters have been developed [8], they are used so far almost exclusively in research laboratories or, to some extent, in projects in cooperation with the industry [22]. Both classical and instrumental verification of freshness assurance require fast inexpensive procedures, and their results must correlate with those of sensory analysis to be useful in quality control. The methods used in daily practice in small and medium enterprises (the majority of seafood enterprises, for example, IPx) are those which have been used for decades (TVB-N, pH, sensory inspection) and do not require extensive economic effort to be implemented or maintained [22]. The developed QIM scheme offers detailed information about sensory quality, fulfilling, primary producer's knowledge, and requirements to integrate traceability systems, without the need for higher investments. Further validation of the scheme, under different seasons, storage conditions, or handling procedures may be required for the future application of the proposed scheme. However, the strengths of QIM indicate that it has a high potential to become a reference method in Europe.

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investigation, J.F.; resources, P.V.-P., and J.S.C.; data curation, J.F.; writing—original draft preparation, J.F.; writing—review and editing, J.F., P.V.-P., and J.S.C.; visualization, J.F., P.V.-P., and J.S.C.; supervision, P.V.-P. and J.S.C.; project administration, J.S.C.; funding acquisition, J.S.C.

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Chapter 4 - A Systematic AQbD Approach for Optimization of the Most Influential Experimental Parameters on Analysis of Fish Spoilage-Related Volatile Amines

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Article

A Systematic AQbD Approach for Optimization of the Most Influential Experimental Parameters on Analysis of Fish Spoilage-Related Volatile Amines

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Abstract

The volatile amines trimethylamine (TMA) and dimethylamine (DMA) could be used as important spoilage indices for seafood products, assisting in the determination of the rejection period. In the present study, a systematic analytical duality-by-design (AQbD) approach was used as a powerful strategy to optimize the most important experimental parameters of headspace solid-phase microextraction (HS-SPME) and gas chromatography-mass spectrometry (GC-MS) conditions for the quantification of TMA and DMA in *Sparus aurata*. This optimization enabled the selection of the best points in the method operable design region for HS-SPME extraction (30 min; 35 °C; NaOH 15 M and NaCl 35%, *w/v*) and GC-MS analysis (80 °C; gradient 50 °C/min; flow rate 1 mL/min and splitless mode). The rejection day, estimated through the TMA concentration (>12 mg/100 g, at days 9–10), was compared with sensory (quality index method: day 7–8), physical (Torrymeter: day 8–9), and microbial (day 9–10) analysis, corroborating the suitability of the proposed approach for estimating the period for which they will retain an acceptable level of eating quality from safety and sensory perspective.

Keywords: *Sparus aurata*; trimethylamine; dimethylamine; freshness; HS-SPME; GC-MS; microbiology; dielectric properties; sensory analysis

4.1. Introduction

Fish constitute a complex system in which enzymatic, microbial, and physicochemical interactions occur simultaneously, which has an impact on flavor, texture, and shelf-life (the time a product remains acceptable for consumption). Understanding the shelf-life of foods from animal or vegetable origin is of primordial importance in designing appropriate preservation strategies that extend their shelf-life [1,2]. After fish death and rigor mortis resolution, a succession of reactions takes place, which are of great importance for fish freshness and shelf-life. These reactions are influenced by fish species, their physiological and environmental conditions, (*e.g.*, water temperature), and will impact fish freshness and spoilage progress. Additionally, catching and harvesting methods will have a major influence on fish deterioration. Such alterations can arise from: (i) Chemical deterioration that can be non-enzymatic, enzymatic, or rancidity (oxidative or hydrolytic); (ii) physical changes that can have several forms, such as moisture loss; and (iii) microbiological changes by bacteria, fungi, viruses, and parasites [2,3]. The most common consequences of these processes are pH and physical alterations as well as the production of slime, off-odors, and off-flavors attributed to volatile compounds, mainly amines and short-chain alcohols. Among the amines, the volatile amines trimethylamine (TMA) and dimethylamine (DMA) can be used as potential markers to monitor the spoilage state of fish, assisting in the determination of the time in which the fish will remain acceptable for consumption, retaining its sensory and eating qualities [4–6]. The impact of secondary amines on fish products is relevant because of the involvement of amines as probable precursors of carcinogenic compounds.

The most common methods to quantify volatile amines in fish include total volatile base-nitrogen determination (TVB-N) and TMA quantification [7]. The TVB-N measures the ammonia (intrinsic and formed during analysis), TMA, and DMA. The increment in these components, in combination or alone, will increase TVB-N amounts [4,8]. The ammonia present in the immediate postmortem flesh (after death during harvesting or after a few hours in ice storage) has its origin in the inosine monophosphate formed through adenine nucleotide deamination [2,4]. Additionally, trimethylamine oxide demethylase (TMAOase), a molybdoenzyme present in the periplasm of specific spoilage organisms, catalyzes the cleavage of trimethylamine oxide (TMAO) into DMA and formaldehyde (FA). However, several days (±10 days) after the catch, bacterial reduction (genus *Shewanella*, if fish are from cold water, or *Pseudomonad* if fish are from warm water) of TMAO to TMA is the dominant process. During storage, the increment in TMA levels is characterized by a fast increase in the first 5–7 days, followed by an apparent stable concentration. DMA formation, besides not being present in all species, is more relevant during frozen storage even though in small amounts [4,8].

In Europe, chemical analysis of fish samples follows Regulation (EC) No. 2074/2005, which determines the quantification of TVB-N through steam distillation and establishes acceptable amounts according to fish species [9–11]. In the case of the determination of TMA concentration, the official recommended method is based on Dyer's method [10]. Other common methodologies to quantify the TVB-N, ammonia, TMA, and DMA include steam distillation and titration of the amines, using muscle samples or extracts from the muscle, as described in Table 1 SM (Supplementary Material - Annexes). Capillary electrophoresis (CE), high-performance liquid chromatography (HPLC), colorimetry, gas chromatography, flow injection analysis using potentiometric detection, and electronic nose have also been used [8,12].

Even with the automatization and efficiency of recent equipment, several problems are still prevalent and hinder data quality. Recurrent issues, such as decomposition during distillation, incomplete recovery of the amines, interference of the reagents, among others, contribute to bias between results obtained by the different methods, and overestimation of the measured compounds [2,4]. The comprehensive and versatile headspace solid-phase microextraction (HS-SPME), a solvent-free extraction technique that incorporates extraction and concentration in one single step, has demonstrated unique capabilities for the quantitative analysis of volatile components in a wide variety of food samples [13–15]. However, as with other methods, there is still a lack of standardization in the extraction protocol, fiber selection, and conditions for GC-MS analysis (Table 2 SM, Supplementary Material - Annexes).

The analytical quality-by-design (AQbD) approach has been recognized as a reliable methodology for the development of analytical measuring systems and promoted by several international regulators, such as the International Conference on Harmonization [16]. The AQbD principals started on risk management, which initiates with the understanding of the control of the system process and product, through predefined objectives to promote the overall quality [17–19]. The application to the analytical methods is supported by the following four key steps: (i) Selection of the analytical target profile (ATP), (ii) definition of a method operable design region (MODR), (iii) integration of risk assessment, and (iv) multivariate statistical analysis. All of them contribute to the overall knowledge of the method and quality verification [17–19]. The AQbD concept is commonly applied to pharmaceutical development and was recently introduced in the analysis of foods, reinforcing its contribution to the methods harmonization [20–22].

The purpose of this study was to use the AQbD approach as a powerful strategy to determine the optimal analytical conditions for the quantification of TMA and DMA levels in *Sparus aurata* (gilthead seabream, GSB) with origin from aquaculture, by HS-SPME/GC-MS methodology. The optimization approach will allow the selection of the best points in the MODR for HS-SPME and GC-MS analysis. The HS-SPME/GC-MS results will be compared with data from physical, microbial, and sensory analyses. Additionally, the rejection day for the gilthead seabream will be estimated based on the data results from all the analyses.

4.2. Materials and Methods

4.2.1. Reagents and Materials

The amines TMA, DMA, and the internal standard, n-propylamine hydrochlorides, were acquired from BDH and Merck. Sodium chloride (NaCl, 99.5%) and sodium hydroxide (NaOH) were obtained from PanReac (Barcelona, Spain). Ultra-pure water was obtained from a Milli-Q® system (Millipore, Burlington, MA, USA), and helium of purity 5.0 was used as the GC carrier gas, supplied by Air Liquide (Alges, Portugal). The SPME holder for the manual sampling of SPME fiber and the respective fibers, namely the divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS), with a 50/30 mm film thickness, carboxen/polydimethylsiloxane (CAR/PDMS) with a 75 mm film thickness, and polydimethylsiloxane/divinylbenzene (PDMS/DVB) with a 65 mm film thickness, were purchased from Supelco (Bellefonte, PA, USA). Ringer solution (Oxoid, Basingstoke, Hampshire, UK) was used for microorganism transference. Inoculations were made in iron agar, nutrient agar (Oxoid, Basingstoke, Hampshire, UK), and MacConkey agar (PanReac AppliChem, Darmstadt, Germany). For physical evaluation, a Torrymeter 295 (Distell, West Lothian, Scotland, UK) was used.

4.2.2. Degradation Trial

Fresh GSB specimens were kindly given by Ilhapeixe SA facilities, Madeira Island, Portugal. Forty-five specimens were killed in a mix of saltwater and ice using the current company practice. The fish were transported to the company refrigerated facilities 2–3 h after capture. Before storage, the fish were washed with fresh water, then placed in perforated plastic boxes with crushed ice and stored at 0 °C (±1 °C) for 20 days as a maximum. When necessary, ice was added. The sampling periodicity for all experiments were: amines analysis: 0, 4, 8, 12, and 15 days; physical properties: 0–6, 8–10, 12–15, 17, and 20 days; sensory analysis: 0, 1, 3, 5, 7, 9, 12, and 15 days; and microbial analysis: 0, 2, 3, 5, 6, 7, 9, 10, and 15 days.

4.2.3. Sample Preparation for Amine Analysis

Stock solutions of TMA and DMA (1000 µg/mL) were prepared by dissolving quantitative amounts of neat standards in trichloroacetic acid solution (TCA 7.5%). Dilutions of the stock were made to six concentration levels between 2 and 20 µg/mL. Six-point (including blank) calibration curves were established.

Sample preparation for TMA and DMA extraction was based on previous studies [12,23,24]. The fish were filleted, and 10 g of flesh were blended in a solution of TCA 7.5%, with a proportion of 1:2 (*w/w*). The blended fillets were centrifuged at 10,000× *g* for 10 min at 4 °C, and the supernatant was collected and stored at −80 °C until analysis.

4.2.4. HS-SPME Procedure

The HS-SPME procedure was carried out according to Freitas et al. (2019) [24]. In brief, in an 8-mL glass vial, an aliquot of 0.5 mL of the sample supernatant, 1 mL of 15 M NaOH, and 1 mL of NaCl solution (35%, *w/v*) were added. The DVB/CAR/PDMS fiber was placed into the vial headspace at 35 °C (thermostatic bath) for 30 min. After the extraction, the fiber was withdrawn and introduced into the GC injector port at 235 °C for 6 min for the thermal desorption of the analytes. All assays were carried out in triplicates.

4.2.5. GC–MS Conditions

Chromatographic separation was performed using an Agilent 6890N (Palo Alto, CA, USA) gas chromatography system combined with an Agilent 5975 quadrupole mass selective detector and splitless injector equipped with a BP-20 (30 m × 0.25 mm i.d. ×

0.25 μm film thickness) fused silica capillary column supplied provided by SGE (Darmstadt, Germany) with helium (Helium N60, Air Liquid, Alges, Portugal) as the carrier gas at a flow rate of 1 mL/min (column-head pressure: 13 psi). An insert of 0.75 mm i.d. was used and the injector temperature was fixed at 235 °C. The GC oven temperature was held for 1 min at 80 °C and ramped to 220 °C at 50 °C/min (held for 5 min). The manifold, GC-MS interface, and quadrupole temperatures were held at 180, 220, and 180 °C, respectively. In addition, MS detection was performed in full scan, the ion energy used for the electron impact (EI) was 70 eV, and the source temperature was 180 °C. The electron multiplier was set to the auto-tune procedure. The mass acquisition range, made in full scan mode, was m/z 30–300, 1.9 spectra. The TMA and DMA identification was carried out by comparison with the mass spectra and retention times of pure standards using the same instrumental conditions, and by matching the mass spectra with the data system library (NIST05).

4.2.6. Method Validation

The developed and optimized HS-SPME method was properly validated in terms of linearity, detection and quantification limits, intraday and interday precision, accuracy, and matrix effects (ME), according to the Association of Official Analytical Chemists (AOAC) and International Union of Pure and Applied Chemistry (IUPAC) guidelines [25,26]. Matrix-matched calibration curves were obtained for each compound to evaluate the linearity of the proposed method, using six concentration levels for TMA (2, 5, 8, 10, 15, and 20 μg/mL) and seven for DMA (2, 5, 7, 8, 10, 15 and 20 μg/mL). The calibration curves were constructed by plotting the peak area of TMA and DMA against the analyte concentration and were fitted by linear regression analysis. The ME was evaluated by comparing the slopes of the matrix-matched and solvent-based standard calibration curves. Intermediate precision and repeatability were calculated by the standard addition method at a low (3 μg/mL, LL), medium (9 μg/mL, ML), and high concentration level

(15 µg/mL, HL) through inter and intraday variation measurements. Recovery was also calculated by spiking a known concentration sample with the previous LL, ML, and HL concentrations. The method limit of detection (LOD) and quantification (LOQ) for each analyte was determined using the residual standard deviation (*Sy/x*) of the corresponding calibration curve. The LOD and LOQ were calculated as 3.3 × ((*Sy/x*)/b) and 10 × ((*Sy/x*)/b), respectively, where (*Sy/x*) represents the standard deviation of the ordinate at origin and *b* represents the slope of the regression line [27].

4.2.7. Shelf-Life Estimation

The results were compared with other methods commonly used for shelf-life estimation studies [28–31]. The rejection day obtained with the proposed chromatographic method was compared with results obtained from the sensory analysis by the quality index method (QIM) approach, Torrymeter evaluation for dielectric property measurement, and microbial growth analysis. The microbial experiment was performed as described previously [32]. In summary, the fish skin was swabbed with sterile cotton swabs and the microorganisms were transferred to 1 mL of ringer solution for serial dilutions. Plate inoculation was executed with the 20-µL drop method. Total viable colonies (TVC) were performed after 48 h at room temperature, in the plates of nutrient agar. Iron agar was used for counting H_2S-producing bacteria after 48 h at room temperature. *Enterobacteriaceae* counts were grown on MacConkey agar and counted after 72 h at 30 °C. In all cases, the values were expressed as colony forming units (cfu). The physical analysis was made using a Torrymeter [24] and measurements were taken on the fish dorsal region as recommended [33]. Sensory evaluation was performed following the QIM protocol, as previously described [34]. In brief, a total of 3 sessions were performed, with two training sessions before to the final experimental session. Six trained assessors participated in the trials and their performance was evaluated following the r^2 values and the slopes of the quality index (QI) values for each one [24]. The values obtained by each assessor were averaged and summed to express the overall QI. The utilized table for sensory evaluation is presented in Table 3 SM (Supplementary Material - Annexes).

4.2.8. Statistical Analysis

Statistical analysis was carried out using the software STATISTICA 10.0 (Stat Soft, Inc., Tulsa, OK, USA), and the significance was set at a probability of p-values < 0.05.

4.3. Results and Discussion

4.3.1. Establishing the Quality by Design Bases for Analysis

4.3.1.1. Defining the Analytical Target Profile and Method Scouting

A relevant step in the AQbD approach is the definition of the analytical target profile (ATP), defined as the separation, identification, and quantification of TMA/DMA in fish muscle extract using HS-SPME/GC-MS analytical methodology.

The scouting of the analytical method, which implies the study of the relevant parameters for the chosen methodology and the target analyte characteristics, was based on previously developed studies for HS-SPME/GC-MS [4,8,12,23,27,35–38]. The sample preparation was based on the work of Dehaut (2016) to facilitate comparison with other quantification methods [23]. The knowledge from these studies also helped to define the critical quality attributes, critical process parameters, and quality risk assessment were also defined.

4.3.1.2. Method Critical Quality Attribute Definition Methods

For the selection of HS-SPME critical method attributes (CMAs), the efficiency of the extraction method was taken into consideration. Therefore, the attributes selected as being important for extraction efficiency include the number of extracted amines, total peak area (TPA), and intermediate precision (IP) of the analytes. TPA, IP, peak resolution

(PR), tailing factor (TF), and symmetry factor (SF) of the analytes were the attributes considered for GC-MS analysis.

The requirements for each CMAs were defined as: The amines under analysis must be identified (TMA and DMA); the TPA value will be selected by the maximum that can be reached; and PR values should be greater than 1.5, TF should be lower than 1.5, while SF should be lower than 2.5. For IP, the residual standard deviation percentage values (%RSD) should be as low as possible and are considered acceptable when they are not higher than 15% [25,26].

4.3.1.3. Critical Method Parameters and Quality Risk Assessment

The selection of the critical method parameters (CMPs) was based on the extraction efficiency of SPME fiber and the GC-MS analytical performance. The selected parameters for quality risk assessment (QRA) are presented through the Ishikawa diagram (Figure S1, Supplementary Material - Annexes) and were based on scouting phase analysis. For HS-SPME, the chosen CMPs were fiber type, extraction time, extraction temperature, pH (alkalizing agent), and ionic strength (salt concentration, NaCl). The main parameter regarding HS-SPME effectiveness is the fiber type, which is greatly influenced by the selectivity of the polymer that forms the fiber and its interaction with the target analytes [39].

In GC methods, the type of column is a relevant parameter that influences the separation performance. Optimization is achieved by selecting the column constituents according to the functional groups, bonding type, and chemistry of the stationary phase. The other parameters that can be adjusted are gas flow, initial oven temperature, ramp temperature, split method, eluent composition, and EI mode [37,38]. For GC-MS methodology analysis, the chosen critical method parameters were gas flow, initial oven temperature, and ramp temperature. The parameters that were not chosen for investigation were fixed as in previous works [27].

4.3.2. Method Knowledge of Space

4.3.2.1. HS-SPME Fibers

The HS-SPME/GC-MS methods previously described by our and other research groups provided the necessary knowledge for defining the CMPs range, before the investigation of the knowledge space (KS) [12,23,24]. Taking into account the obtained results, a fractional factorial design (FFD) based on a 4^{3-1}-level model was applied as the design of the experiment (DoE) for HS-SPME-KS screening, being evaluated by the following parameters: NaOH concentration (5, 15, and 25 M), extraction temperature (25, 35, and 45 °C), extraction time (5, 25, and 45 min), and NaCl percentage (10%, 30%, and 50%). DoE was used for each of the studied fiber types (DVB/CAR/PDMS, CAR/PDMS, and PDMS/DVB). The results are presented in Table 4 SM (Supplementary Material - Annexes).

The TPA and IP results for each fiber under the optimal extraction conditions are shown in Figures 4.1a and b, respectively. According to the results, all fibers were capable of extracting TMA and DMA, which is in agreement with the results obtained by Chan (2006) [12]. However, they showed variable extraction efficiency. The efficiency of the fibers for TMA and DMA extraction followed the order DVB/CAR/PDMS > CAR/PDMS > PDMS/DVB.

From the obtained data, CAR/PDMS and PDMS/DVB fibers showed better results when the extraction parameters were in their higher range conditions corresponding to runs 24 and 22, respectively. The DVB/CAR/PDMS fiber presented the best results under milder extraction conditions (run 13), mainly in the extraction time, which was half of the time necessary for the other fibers.

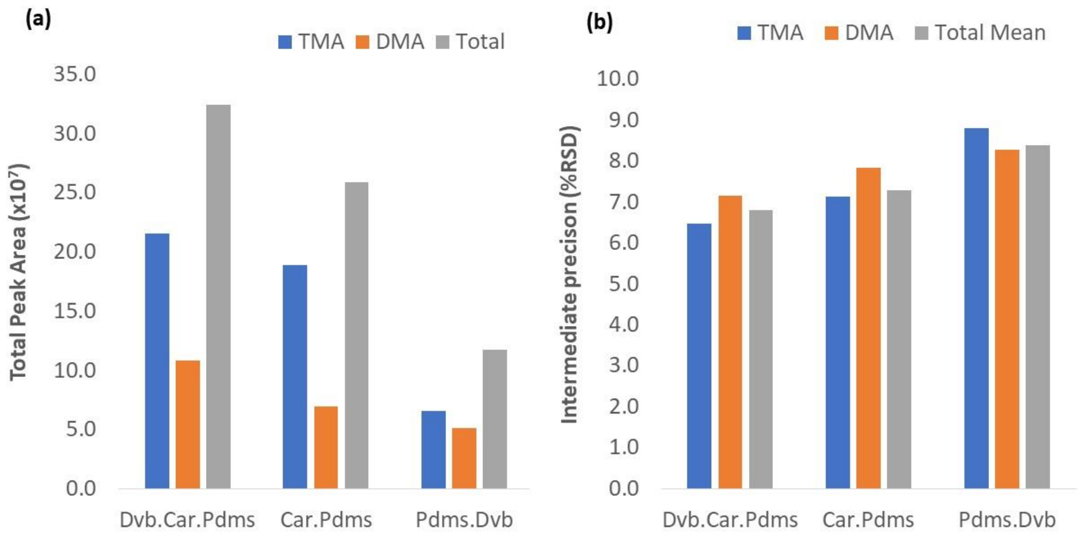

Figure 4.1 - Results from the HS-SPME knowledge space of extraction conditions (a) Total peak area analysis for all fibers; (b) Intermediate precision for all assayed fibers.

Table 4.1 - Some characteristics of SPME fibers and targeted volatile amines.

Parameters	SPME Fibers			Amines	
	DVB/CAR/PDMS	**CAR/PDMS**	**PDMS/DVB**	**TMA**	**DMA**
pKa	-	-	-	9.80	10.37
Molecular Weight	40–275	30–225	50–300	59.11	45.08
Polarity	Bipolar	Bipolar	Bipolar	Polar	Polar
Thickness (µm)	30–55	75	65	-	-
Core type	Stabflex	Fused silica	Fused silica	-	-
Operating temperature	230–270	250–320	200–270	-	-
Extraction mechanism	Adsorption	Adsorption	Adsorption	-	-

At their highest TPA values, the percentage of difference concerning DVB/CAR/PDMS fiber is 20% for CAR/PDMS and 64% for PDMS/DVB. For IP (%RSD), the total means for each fiber performed below the maximum acceptable value of 15% RSD, as presented in Table 4 SM (Supplementary Material). Only two conditions registered values in the limits of the maximum acceptable value, 15.0% for the DMA value with DVB/CAR/PDMS (run 18), and 15.4% for the TMA value with PDMS/DVB (run 16). These results reflect the fiber properties and amine characteristics as summarized in Table 4.1.

Four parameters must be taken into consideration for fiber selection:

1. Molecular weight and analyte size are related to analyte speed; the smaller the analytes, the faster it moves, and the retention might be harder. Highly cross-linked fibers could better retain fast-moving analytes.
2. Analyte polarity is defined by the type of coating and its ability to retain analytes according to polarity. However, each fiber has a certain capacity that might be better suited for one than others.
3. Analyte concentration level and range-thicker fibers might require higher concentrations of an analyte to be more efficient.
4. The complexity of the sample is related to the analyte displacement by the other sample components with higher affinity or concentration.

Considering the properties of the fiber, the thicker ones will require more time to reach the equilibrium and a high analyte concentration present in the headspace, and consequently harder extraction conditions (*i.e.*, temperature, extraction time, alkaline conditions, and ionic strength (salt addition). Pore size also influences the compound molecular weight range and its capability to be retained. Thicker coatings tend to be more effective in the quantity of extracted analytes; however, longer times are required to achieve equilibrium and to analyte desorption. On the other hand, the extraction efficiency of the fiber increases with the decrease in film thickness [37–39]. PDMS/DVB showed the lowest extraction efficiency, expressed by the lowest amount of extracted TMA and DMA. Additionally, its lower cross-linked structure decreases the extraction efficiency of low molecular and highly volatile analytes, such as TMA and DMA [37,39]. Even though the CAR/PDMS fiber has a wider range of MW, its higher thickness requires a longer extraction time to reach equilibrium and a higher desorption time owing to the coating properties and configuration. Higher temperatures are also required due to their thickness as well as a higher analyte concentration to improve efficiency [37,39]. DVB/CAR/PDMS fiber has a highly cross-linked structure, an intermediate polarity strength, and a thinner coating layer when compared with PDMS/DVB and CAR/PDMS [37]. Considering the sample complexity, DVB/CAR/PDMS is the most suitable for complex samples, and a low concentration of the target compounds, owing to its mixed layer properties. The largest compounds are retained in the first layer (DVB), maintaining free coating to retain the lowest MW compounds. Therefore, for the next steps, the DVB/CAR/PDMS fiber was chosen because of its overall performance and acceptable values.

4.4.2.2. GC-MS Conditions.

In the GC methodology, some parameters, including the column, splitless mode, flow rate of the carrier gas, and temperature gradient, must be taken into consideration [37,38]. There are several columns for GC analysis and all of them can be suited for numerous analyses; however, it is necessary to evaluate the operational range of the parameters that originate the best possible results. There are few studies on the optimization of GC-MS conditions for volatile amines. The obtained results are presented in Supplementary Material Table 2 SM (annexes). Chan (2006) presented a more complete study but with a high emphasis on the SPME fiber selection using a unidimensional approach [12]. On the other hand, Dehaute (2012) [23] studied the impact of some GC-MS conditions on TMA and DMA analysis, such as the injector temperature, oven temperature, and split ratio. In the present study, and according to the obtained results, it was decided to proceed the investigation with a BP-20 (polar) capillary column, an oven temperature ranging between 35 and 85 °C, ramp speed 40–60 °C/min, and a flowrate between 0.8 and 1.3 L/min. The fixed parameters are described in Section 4.3.5, where the GC-MS conditions are detailed.

4.3.3. Determination of Method Operable Design Region

A MODR was defined for HS-SPME and GC-MS to define the space where the CMPs will fit the purpose of promoting the CMAs responses.

4.3.3.1. HS-SPME Extraction.

The definition of MODR for HS-SPME$_{(DVB/CAR/PDMS)}$ was performed based on CMAs responses (TPA and IP) from interactions between extracted amines. The CMPs analyzed included NaOH concentration, extraction temperature, extraction time, and ionic strength (salt percentage). Figure S2 (Supplementary Material-annexes) shows the Pareto chart analysis of the effects between the studied CMPs on the TPA and IP responses. According to the results, the principal factor affecting TPA is the NaOH concentration, while for IP, it is mainly affected by temperature. Alkalinization of the sample will increase the amines volatility and therefore their concentration in the headspace. This is influenced by the pKa value of the amines (Table 4.1). The NaOH concentration should raise the pH two units above the pka of the analyte [37]. This effect was confirmed by Dehaut (2016), who verified the appearance of amines after the addition of an alkaline agent [23]. The time of extraction and temperature effects have a role in the development of the equilibrium between the gas phase and the sample. Temperature influences the mass transference kinetics between the sample, headspace, and fiber, and consequently the extraction efficiency and precision [38]. In the case of extraction time, its role is to assure that the amount of the extracted analyte is proportional to the concentration present in the sample [38].

The effects of all CMPs on TPA and IP, respectively, through the desirability surface contour method, are shown in Figure S3a,b (Supplementary Material-annexes). The values chosen for TPA to perform this analysis were defined as the highest desirable (3.24×10^8), acceptable mean (1.83×10^8), and lowest unacceptable (4.23×10^7). In the case of IP, the desirability parameters were defined as the highest desirable (1.5), acceptable mean (6.5), and unacceptable (12). Here, it is possible to see that the regions with higher desirable TPA values are between 10 and 24 M for the NaOH concentration, 20 and 50 min for the extraction time, extraction temperature between 25 and 40 °C, and NaCl percentage ranging from 15–45%. In the case of IP, for the same interactions, the lowest desirable RSD values are between 1 and 30 min for the extraction time, 6 and 20 M for the NaOH concentration, 25 and 35 °C for the extraction temperature, and 45% and 55% for the NaCl percentage. Overlaying the CMAs responses (Figure 4.2a), the desirability plots show a mix between TPA and IP results, resulting in an extended range red area, mostly for the interaction between the NaOH concentration and extraction time. The highest desirability index (dark red zone) is achieved between all CMPs in the following ranges: 10–18 M NaOH concentration; 20–40 min extraction time; 25–35 °C extraction temperature; and 35–55% NaCl percentage.

Data verification was performed through the analysis of the agreement between the predicted and the observed values at the optimal condition range, which are described in Supplementary Material Table 5 SM (annexes). According to the results, it was found that all the values observed in the experiments were close to the predicted values. However, the results showed slight differences between the observed and predicted values in CMA responses. Such differences might be due to the extended range values for the CMPs and small variations on the optimum point, which produce variability in the CMA responses. Therefore, the parameters at the optimal point were studied through a robustness test (see Section 4.3.4).

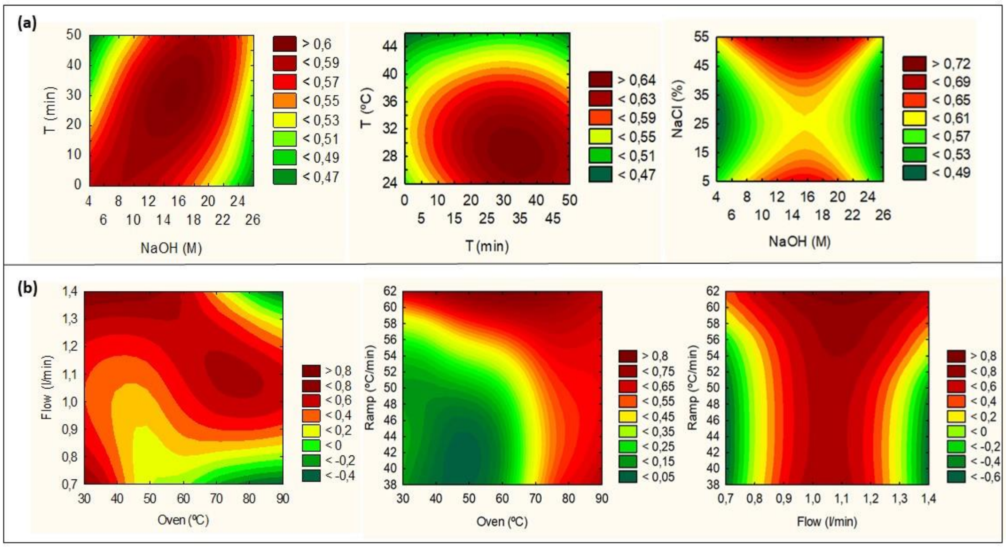

Figure 4.2 - The effects of all CMPs through the desirability surface contour method. (a) The desirability plots for HS-SPME(DVB/CAR/PDMS), and simultaneous analysis of the TPA and IP results. (b) The desirability plots for CG-MS(BP20), and simultaneous analysis of the TPA, IP, PR, TF, and SF results.

4.3.3.2. Chromatographic Conditions

The CG-MS(BP20)-MODR definition was performed based on CMA responses from the interactions between the oven temperature (35, 60, and 85 °C), ramp speed (40, 50, and 60 °C/min), and flow rate (0.8, 1.0, and 1.3 L/min). A 3^{3-1} level FFD was used to perform this study (Supplementary Material Table 6 SM - Annexes). The Pareto ranking analysis for TPA, IP, PR, TF, and SF is shown in Figure 4 SM (Supplementary Material - Annexes), respectively. None of the CMPs had a significant influence on the CMA response. A desirability plot was performed overlaying all CMAs, with the following values being defined for each one (respectively TPA, RSD, PR, TF, and SF): desirable (3.68×10^8, 1.0, 10, 1.0, and 1.0), acceptable (2.0×10^8, 10.0, 5.0, 0.9, and 0.9), and unacceptable (3.20×10^7, 14.0, 2.0, 0.5, and 0.5). The desirability plots of the interaction between the change level of continuous CMPs (flow, ramp, and oven temperature) on

CMA responses are shown in Figure 2b, respectively. From the results, it was possible to verify that the higher CMA values were influenced by the flow rate and oven temperature, with two clear desirable areas. One in the lower temperature range (30–40 °C) is associated with higher flow rates (1.2–1.4 L/min), and the other at a higher temperature range (70–80 °C) is associated with mild flow rates (1.0–1.1 L/min).

Ramp temperature did not influence the CMAs much. The ramp temperature vs. flow rate plot shows a clear area between 1.0 and 1.1 L/min, throughout the ramp temperature range. Similar results are presented in the ramp vs. oven temperatures, with the highest desirability between 75 and 90 °C, throughout the ramp temperature range. Taking into consideration the equipment and material consumption, the optimum point for GS-MS(BP20)-MODR was defined with an oven temperature of 80 °C, a flow rate of 1.0 L/min, and a ramp temperature of 50 °C/min. Data verification was performed through analysis of the agreement between the predicted and observed values, which are described in Table 7 SM (Supplementary Material-Annexes). According to the results, it was found that all the values observed in the experiments were close to the predicted values. However, the results showed slight differences between the observed and the predicted values in the CMA responses. Such differences might be due to the extended range values for the CMPs and small variations on the optimum point, which produced variability in the CMA responses. Therefore, the parameters at the optimal point were studied through a robustness test (see Section 4.3.4.).

4.3.4. Robustness and Method Control

Robustness was evaluated at the optimal conditions in the MODR using a new experimental design with CMP range values around the determined optimal value. The results for the CMA and CMP response are described in Table 8 SM and Table 9 SM (Supplementary Material-Annexes) for HS-SPME and GC-MS, respectively.

4.3.4.1. Amines Extraction

The FFD for the robustness screening was based on the 3^{3-1}-level factors model, with the temperature (33, 35, and 37 °C), NaOH solution (14, 15, and 16 M), and time (28, 30, and 32 min) being evaluated. The selection of the desirability values was based on the TPA and IP (%RSD) results at the optimum point; therefore, 1% was defined as desirable (7.04×10^8 and 1.68), 0.5% as acceptable (6.12×10^8 and 6.32), and 0% as unacceptable (5.20×10^8 and 10.97). Figure 4.3a presents the desirability plot for the evaluated conditions. The highest desirability index was reached within the studied range. Table 10 SM (Supplementary Material - Annexes) describes the analysis of the agreement between the observed and the predicted values for robustness verification. According to the results, all the observed values are within the range of the predicted values at the 95% confidence interval.

4.3.4.2. Chromatographic Analysis

The FFD for GC-MS robustness screening was based on the 3^{3-1}-level factors model, around the optimal point, with the gas flow rate (0.8, 1.0, and 1.2 L/min), oven temperature (75, 80, and 85 °C), and ramp gradient (45, 50, and 55 °C/min) being evaluated (Table 9 SM, Supplementary Material - Annexes). A desirability plot was performed by overlaying all CMPs. For the selection of values to be used in the desirability analysis, the TPA, RSD, PR, TF, and SF data were considered at the optimum point, where 1% was defined as desirable (7.5×10^8, 2.0, 3.0, 1.0, 1.0), 0.5% as acceptable (6.0×10^8, 7.0, 2.0, 0.6, 2.3), and 0% as unacceptable (5.2×10^8, 10.0, 2, 0.5, 2.9). The desirability plot is shown in Figure 3b. The highest desirability index was reached with flow rates between 0.9 and 1.1 L/min, oven temperatures of 75–80 °C, and ramp temperature of 50–55 °C/min. In Table 11 SM (Supplementary Material-Annexes), the agreement analysis between the predicted and the observed values for robustness verification is shown.

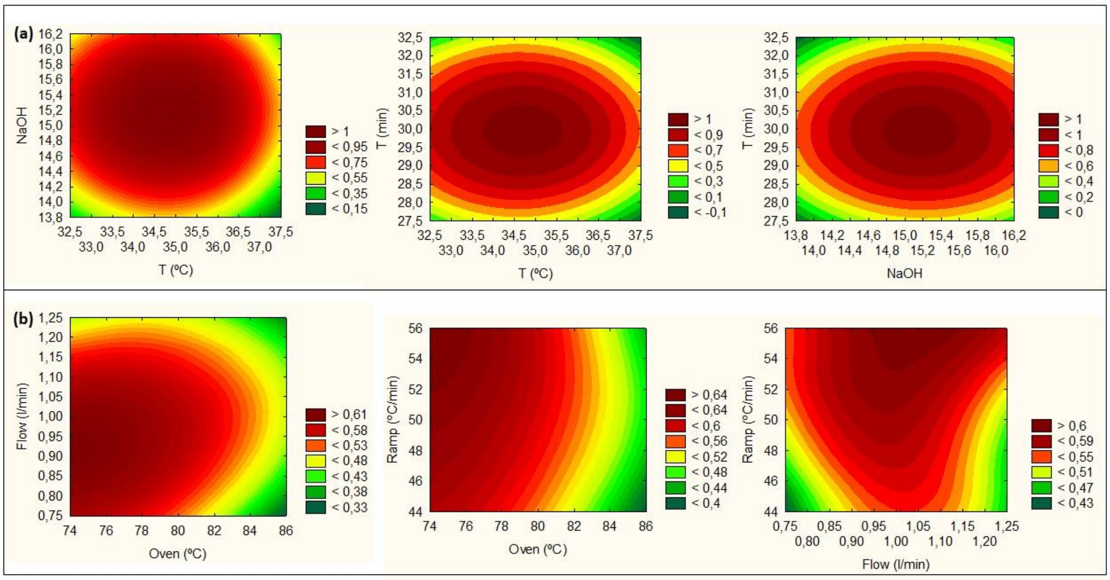

Figure 4.3 - Robustness analysis results of all CMPs through the desirability plots. (a) Desirability analysis results for HS-SPME(DVB/CAR/PDMS), simultaneous analysis of TPA, and IP robustness results. (b) The desirability analysis for GC-MS(BP20), simultaneous for TPA, IP, PR, TF, and SF robustness results.

From the results, it is possible to verify that all the observed values are within the range of the predicted values, within a confidence interval of 95%, confirming the good robustness of the method. However, the sensitivity of the CMPs influences the robustness analysis, thus it is necessary to establish the system suitability limits (see Section 4.3.4.3).

4.3.4.3. Method Control

The method control was performed by generating a large amount of data (100 cases) through Monte Carlo simulation at the CMP range with the highest desirability index. Then, the capability analysis process was applied to the estimated residual errors from all CMA responses. The established limits and results of the capability analysis are listed in Table 4.2 and presented in Figure 4.4 for HS-SPME and for GC-MS.

Table 4.2 - Method control results

Method	CMP [1]	LSL [2]	USL [3]	CpK [4]
HS-SPME	TPA [5]	0.05	0.17	1.49
	IP [6]	0.03	0.18	1.47
GC-MS	TPA	0.03	0.18	1.47
	IP	0.02	0.20	1.49
	Resolution	0.04	0.17	1.38
	TF [7]	0.02	0.19	1.36
	SF [8]	0.05	0.17	1.37

CMP-critical method parameters. [2] LSL: Lower Specification Limit. [3] USL: Upper Specification Limit. [4] CpK: Process capability Index. [5] TPA: Total Peak Area. [6] IP: Intermediate Precision. [7] TF: Tailing Factor. [8] SF: Symmetry Factor.

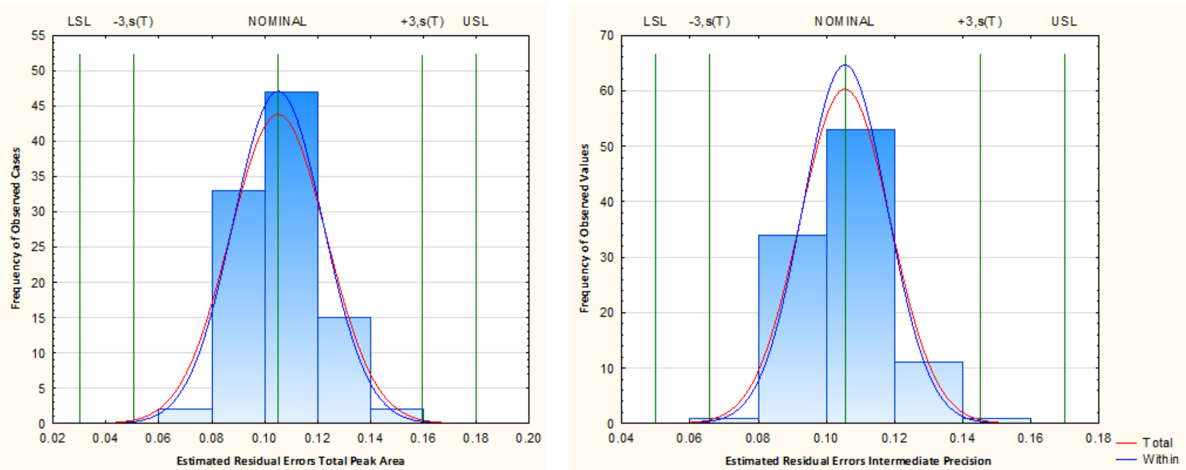

Figure 4.4 - Established limits and results of the capability analysis, for total peak area and intermediate precision.

The process capability index (Cpk) values varied between 1.36 to 1.49. From the results, it can be concluded with a high level of confidence that the proposed analytical method is robust, since all CpK values were above the minimal reference value (1.33), for a method to be considered robust [16].

4.3.5. Analytical Method Validation.

The method was validated in terms of the linearity of the calibration function, accuracy, precision, limits of detection (LOD), limits of quantitation (LOQ), and ME. Briefly, six TMA and DMA concentrations points were used (2–20 µg/mL range). Table 4.3 summarizes the obtained results for all the evaluated parameters and confirms that the results are under the stipulated values of the guidelines (ME: 70–125%; intermediate precision and repeatability under 15% RSD; recovery: 80–115%) [25,26].

Linearity was evaluated by the suitability of the function model, comparing the experimental and theoretical Fisher values at 95%. If the dataset expressed a linear function, the condition $F_{theo} > F_{exp}$ is fulfilled [40]. In this work, the described condition was achieved for both amines with a polynomial equation of second degree. The ME was studied using the standard additions method. Repeatability and intermediated precision were used for precision determination, through inter-and intraday variation measurements. LOQ and LOD were calculated through the regression curve slope and standard deviation of the interception Figure 4.5 shows the chromatographic profile of targeted amines obtained with the optimal method conditions.

Table 4.3 - Parameters results of the HS-SPME/GC-MS method for quantification of TMA and DMA in fish.

	TMA	DMA
Ion (m/z)	58	44
Retention time (min)	4.12	4.23
Concentration range (µg/mL)	2-20	2-20
Regression equation	$Y = 11143x^2 + 2\times10^7x - 1\times10^7$	$Y = 11153x^2 + 2\times10^7x - 3\times10^7$
R^2	0.9504	0.9989
Linearity test (F_{theo}/F_{exp})	3.0 [1]	15.4 [1]
LOD (µg/mL) [5]	0.4	0.6
LOQ (µg/mL) [6]	1.3	2.0
Matrix effect (%)	105	109
Repeatability (%RSD [7])	8.2 [2] 6.0 [3] 9.6 [4]	8.3 [2] 11.2 [3] 7.4 [3]
Intermediate Precision (%RSD [8])	8.4 [2] 7.1 [3] 9.7 [4]	9.6 [2] 13.6 [3] 8.8 [4]
Recovery (%)	93 [2] 98 [3] 99 [4]	90 [3] 93 [2] 95 [4]

[1] *p*-value calculated experimentally through t-Student test between slopes obtained from the calibration curves for TMA in 7.5% TCA and TMA in fish samples. If *p*-value ≥ 0.05, no significant matrix effect was observed. [2] LL: low-level concentration (3 µg/mL). [3] ML: medium-level concentration (9 µg/mL). [4] HL: high-level concentration (15 µg/mL). [5] LOD: Limit of detection; [6] LOQ: Limit of quantification; [7] RSD: Relative standard deviation.

4.3.6. Method Application and Shelf-Life Estimation.

To confirm the applicability of the developed method, a degradation experiment with GSB specimens was performed. The deterioration process of fish includes several simultaneous alterations; therefore, different evaluation methods were applied. Besides the proposed chemical analysis, the following methods were applied: dielectric properties with the Torrymeter; sensory analysis following the QIM method; and microbiological analysis through TVC.

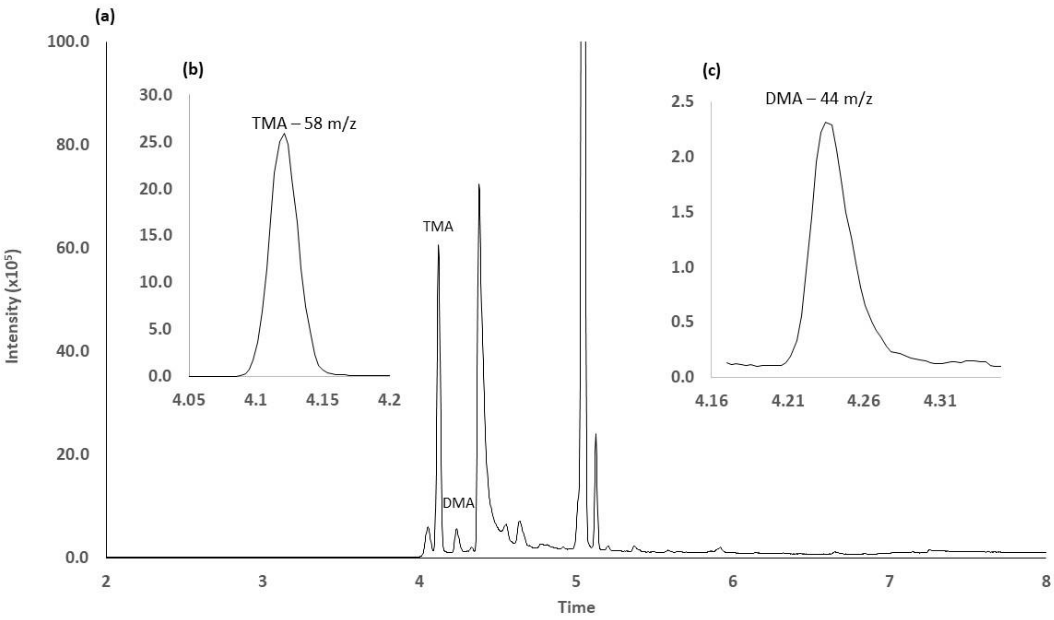

Figure 4.5 - Chromatographic profile of targeted amines with the optimal method conditions (a) total ion chromatogram results; (b) TMA ion selection m/z 58. (c) DMA ion selection m/z 44.

The results obtained with each method are presented in Figure 4.6. With the Torrymeter, the alterations of the muscle texture of GSB were registered throughout the storage experiment. The values started at a maximum value of 15, representing the fresh fish condition. With the progression of the days, the registered data gradually decreased until the value of 8, between 8 and 9 days, at the same time that off-odors were detected (Figure 4.6a). Other works also propose that when a value of 8 is achieved, the product reaches the borderline for consumption acceptability [24,41,42].

The common initial values for TVC vary between 10^1 and 10^4 (cfu), at first days of storage. At the rejection time, the TVC values could be between 10^7 and 10^9 (cfu), depending on fish species [32,43], as in this case, as the TVC limit was achieved at 8–9 days (Figure 4.6b). For *Enterobactereacea* counts, the values throughout the experiment never exceeded 10^2 (cfu), indicating appropriate hygiene and handling conditions of the product. Similar results were also described in other works [44].

Application of the QIM methodology allowed to determine altered GSB freshness levels. Through the analysis of QIM data (Figure 4.6c), the odor was a lead contributor to evaluate the degradation progress. During the three first days, gills and skin odor were described as being similar to seaweed or sea breeze. From the third day, the neutral descriptor was used to describe the condition in which the specimens had no clear distinctive odor. Degradation began to be perceived on days 5–6, with off-odor emergence but was only considered significant for rejection from day 7–8, associated with rancid odors (Table 12 SM, Supplementary Material -Annexes).

According to the European Directive 91/493/EEC, TMA presence in samples is limited to 12 mg/100 g [45]. From the chromatographic results (Figure 4.6d), it is seen that after the slow increase of the TMA, the limit is achieved between 8 and 12 days. Applying the fitted model, the TMA limit could be achieved between 9 and 10 days. Regarding the DMA analysis, even though the methodology allows simultaneous extraction and quantification, in the present study, the DMA was detected, but its values during the storage remained residual in comparison with TMA. Table 4.4 compiles the information obtained from the different methods, showing the association between the different used methods to predict and estimate GSB shelf-life.

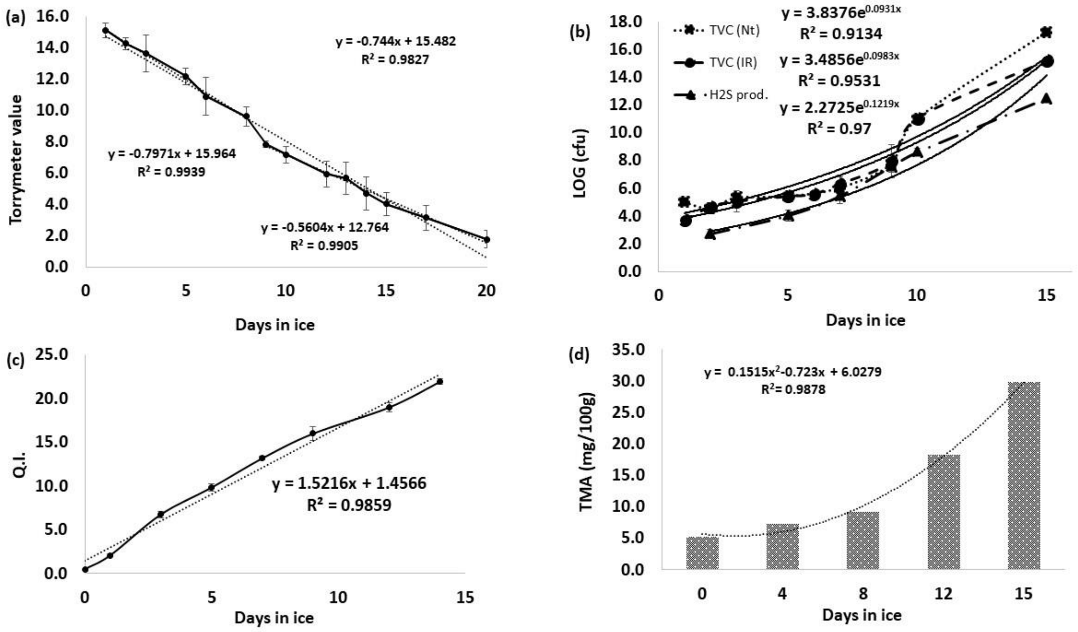

Figure 4.6 - Degradation results in GSB specimens. (a) Torrymeter analysis; (b) microbial analysis; (c) Quality index method analysis; (d) TMA chemical analysis with the proposed HS-SPME (DVB/CAR/PDMS)/CG-MS (BP20) method.

Table 4.4 - Estimated rejection day for different assayed methods.

Method	Rejection Criteria	Value at Rejection	Estimated Rejection Day
QIM	Off odors	13	7–8
Torrymeter	Off odors Slope change	8	8–9
Microbiology (TVC)	Log cfu/cm^2 = 7–9	8.0	8–9
Microbiology (H_2S)	Log cfu/cm^2 = 7–9	7.5	9–10
TMA analysis	12 mg/100 gr	12.5	9–10

4.4 Conclusions

A systematic AQbD approach was used to improve the knowledge around the HS-SPME/GC-MS method for TMA and DMA analysis in fish samples. After the extraction optimization, the proposed conditions for its implementation were based on the use of DVB/CAR/PDMS fiber, with 0.5 mL of the sample, 1 mL of the NaOH solution (15 M), and 1 mL of the NaCl solution (35%), in a water bath at 35 °C for 30 min. For GC-MS analysis, the best instrumental conditions achieved with a BP-20 column were an oven temperature of 80 °C, ramp speed of 50 °C/min to a maximum of 220 °C, and gas flow rate (helium) of 1.0 mL/min. The method robustness was demonstrated through the Monte Carlo simulation and capability analysis process (1.36–1.49 Cpk). The method validation revealed the satisfactory figures of merit, demonstrating its suitability for the target analysis (Table 4.3). The method was tested on GSB specimens through the simulation of a degradation experiment, using complementary methodologies to confirm the shelf-life results, such as microbial analysis, sensory analysis, and physical analysis. The outcome allows the proposal that the criteria of rejection through TMA analysis (12 mg/100g), was reached between 9 and 10 days. Additionally, analyzing all the results, it is possible to suggest a shelf-life period of 9 days (±1 day) for GSB, under the specific conditions of the present study. Besides, not all fish species generate TMA during degradation, and the proposed HS-SPME/GC-MS methodology revealed a reliable alternative to the traditionally used distillation techniques.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure 1 SM: Ishikawa diagram for quality risk assessment; Figure 2 SM: Pareto chart analysis for HS-SPME and GC-MS; Figure 3 SM: Desirability surface contour method analysis for the effects of all CMPs; Figure 4 SM: The pareto ranking analysis for CG-MS(BP20); Table S1: Chemical methods used for fish freshness determination; Table 2 SM: Gas chromatography works related with TMA and/or DMA extraction; Table 3 SM - Scheme of the Quality Index Method (QIM) proposed for *Sparus aurata* (GSB); Table 4 SM: Results from DoE 3^{4-1} level, for HS-SPME Knowledge of space, for all tested fibres; Table 5 SM:The HS-SPME/MODR results for the observed vs. predicted values agreement; Table 6 SM: GC-MS MODR results, from DOE fractional factorial design 3^{3-1} level; Table 7 SM: GC-MS/MODR observed vs. predicted values analysis; Table 8 SM: Fractional Factorial Design 3^{3-1} level, for HS-SPME(DVB/CAR/PDMS) robustness analysis; Table 9 SM: GC-MS Robustness results, from DOE fractional factorial design 3^{3-1} level; Table 10 SM: Analysis of agreement between observed and predicted values for HS-SPME robustness; Table 11 SM: GC-MS robustness evaluation by, observed vs. predicted values analysis; Table 12 SM: Attributes evaluation for QIM analysis.

Author Contributions: Conceptualization: J.F., P.S., P.V.-P. and J.S.C.; methodology; J.F., P.S., P.V.-P. and J.S.C.; software: J.F. and P.S.; validation: J.F. and P.S.; formal analysis: J.F.; investigation: J.F.; resources: J.F., P.V.-P. and J.S.C.; data curation: J.F.; writing—original draft preparation: J.F.; writing—review and editing: J.F., P.S., P.V.-P. and J.S.C.; visualization: J.F.; supervision: P.V.-P. and J.S.C.; project administration: J.S.C.; funding acquisition: J.S.C. All authors have read and agreed to the published version of the manuscript.

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Chapter 5 - Improved approach based on MALDI-TOF MS for establishment of the fish mucus protein pattern for geographic discrimination of *Sparus aurata*.

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Improved approach based on MALDI-TOFMS for establishment of the fish mucus protein pattern for geographic discrimination of *Sparus aurata*

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Abstract

Food fraud is still a recurrent practice throughout food supply chains. In the case of seafood, misidentification of species and products repackaging constitute the most common frauds. Therefore, the development of appropriate analytical approaches to be used against food fraud is necessary. The present study goal is to explore for the first time, the possibility to differentiate between *Sparus aurata* from two different mariculture farms located in Madeira Island (Caniçal and Ribeira Brava), using the mass fingerprint of fish mucus obtained from MALDI-TOF MS and analyzed using Mass-UP software for multivariate statistical analysis and biomarker identification. It was possible to establish from the mucus protein fraction, a set of potential biomarkers for each location in a total of 35 peaks, being 17 peaks specific to Caniçal located farm and 18 to Ribeira Brava. The proposed analytical approach revealed a useful strategy providing accurate and fast results for fish geographical origin discrimination.

Keywords (6): fish mucus; MALDI-TOF MS; Mass-UP; PCA; Authenticity; Traceability.

5.1. Introduction

Food frauds constitute a global concern not only at the economic level but can also have severe adverse health, societal, and sustainability effects. Even though considered illegal and several regulatory guidelines are in practice to enforce food protection, is still a recurrent action towards the increase of financial profits, in violation of legal rules. Throughout history, there are registers of several events, with different impacts, from loss of human life to economic damage, some examples are the "rapeseed oil" fraud (1981), milk adulterated with melamine in China (2008), and more recently the horsemeat scandal (2013) [1]. All of them illustrate the negative impact of food fraud, including both authenticity and adulteration [2]. This type of incident affects the confidence in the food system with a strong impact on the internal and external market, leading the consumers to lose trust in foods and food chain, the companies to lose money as well credibility, and the authorities to lose trustworthiness. Different types of food fraud had evolved with the improvements in the production and detection processes, from formulation to procedures or data documentation. The lack of control or proper monitorization, due to a shortage in human resources or inappropriate technical support, makes such activity still prevalent and economically feasible [3].

In the case of fisheries and aquaculture products, the most common frauds are related with masking poor product quality, replacing of product constituents, species substitution, geographical provenance, mislabeling, repackaging of products, and tax evasion [2,4,5]. The complex structure of the seafood chain also contributes to fraudulent activities, being the main nodes reviewed by Fox and coworkers (2018) [6]. These practices increased the pressure on food laboratories to develop fast and reliable screening methods to fight food fraud [7]. Anatomical and morphological analysis for fish identification is limited, not only due to similarities between some species but also due to the loss of physical traits during seafood processing [8]. Even though European Union presented several directives and regulations for the labeling and commercialization of fishery and aquaculture products, having also in mind to guarantee food safety, the control of such measures is limited by the capacity to analyze the product with proper methodologies to confirm the authenticity [9] or traceability of the information [10].

The development of "foodomics" platforms contributed for the knowledge of several food properties that can be used as different layers of control to improve food quality and confirm the authenticity of the claims made throughout the supply chain. The approach is similar to metabolomics, which is based on the analysis of organism molecules (or metabolites), providing information about it at a certain time and specific conditions [11].

Fish mucus is a complex matrix with variable composition as a response to biotic (*e.g.*: growth stage, species, pathogens) and abiotic factors (*e.g.*: exposure to toxic elements; temperature), for example, incrementing the number of proteins related with immune response or reproduction [12]. It is a thin viscous, colloid layer, generated by the goblet cells which produce mucous granules that migrate to the cell surface and spread their content, covering the fish epithelial surface. The continuous superposition of mucous layers leads to the formation of a dynamic coating that allows the first interactions between the fish and the environment [13]. Therefore, a diverse range of molecules can be found: fatty acids, lysozyme, immunoglobulin, histones, ribosomal proteins, and antimicrobial peptides [14].

In the specific case of *Sparus aurata*, most of the mucus based works have as objective the identification of proteins for welfare status determination [15–18] or use two-dimensional electrophoreses coupled with spectrometric techniques for proteins identifications [14,19]. In order to study complex metabolomic matrices, as in the case of fish mucus, mass spectrometry has arisen as one of the most relevant high-resolution techniques. Mass spectrometry allows the identification of different compounds through the measurement mass-to-charge ratio of ions resulting in fragmentation patterns that can be used to identify different compounds [20]. The most emergent methodology is the Matrix-Assisted Laser Desorption Ionization- Time-of-Flight Mass Spectrometry (MALDI-TOF MS). Through this method it is possible to determine the species authenticity [20,21], the origin of the product [22], and the identification of food allergens and other hazards [23], also fulfilling other pre-requisites such as short time of analysis, flexibility, and reliability [24].

Since environmental factors can affect the proteins that are present in the fish mucus, this work aims to define the mass pattern from the protein fraction of mucus complex matrice to determine the fish geographical origin, from two different mariculture farms, using the MALDI-TOF MS method based on untargeted Mass Fingerprint analysis.

5.2 Material and methods

5.2.1 Sample collection and geographical areas

The samples were collected from two farms, one located at Baía d'Abra, in Caniçal (CN), Madeira (32°44'31.4"N 016°41'26.7"W); and the other located at Campanário, in Ribeira Brava (RB), Madeira (32°39'38.1"N 017°03'22.1"W) (Figure 5.1). Four sampling groups were formed according to the time of arrival to the sea farm cages. Due to operational constraints (*e.g.*: weather, capture schedules, available time frame, company covid restrictions) and alternated production cycles between farms, the number of lots to be sampled was restricted and it was not possible to occur with the predicted frequency. Therefore, the number of new lots studied for CN was one, while for RB was three. The new arrived lots were sampled from the arrival moment, after 3 months (3M), and up to 6 months (6M).

Figure 5.1 - Geographical indication of the sea-farm locations.

The CN lot, upon arrival (August 2020) was divided into two cages, as a company standard procedure due to cage size constraints and for better management of the fish. These two cages (a and b) were equally sampled, at arrival, after 3 months (3M), and after 6 months (6M). The RB new four lots (a, b, c, d), with arrival in June 2020 were not divided being directly transferred to the cages. Due to company schedules and COVID-19 operational constraints, it was not possible to continuously follow the four lots from arrival to 6M. To surpass these constraints the samples were analyzed not individually but under the group's categorization.

The group samples with approximately 12 months (12M), were composed by ready to capture fishes (around 300-400g or higher) from different pre-existing lots. The time needed to be ready for capture could vary between 10 and 13 months, influenced by the environmental conditions, specifically water temperature. These harvestings were taken in 5 different moments. At RB location the samples were collected in October 2019, February 2020, May 2020, August 2020, and October 2020. At the CN location, the samples were taken in October 2019 and from June 2020 (a and c are different cages) to July 2020 (b and d are different cages).

Between 5-10 ml of mucus was collected at each sampling moment. The mucus was gently scraped from the fish dorsal and lateral area above the pectoral fin. The samples were stored in ice until the laboratory arrival and then at -20 ºC with a solution of 35% glycerol for preservation until the analysis. All mucus collected for the arrival, 3M and 6M groups, were from fishes sampled during control routines. Due to fish size and the number used for the control routine (approximately 60-80 fishes), an anesthetic solution was used, to reduce fish stress and possible injuries. Due to the size of the fish, a number between 40-55 fishes were necessary to collect 5-10ml of mucus. The 12M group samples were collected during the fish capture for commercialization. A mixture of ice and water was used for the capture. The fishes that were in the ice water mixture for more than 5min were not sampled. A number of 20-25 fishes were used for mucus collection.

5.2.2 Sample preparation for MALDI-TOF MS analysis

Samples were cleaned using precipitation with chloroform/methanol to remove detergents, salts, and protein concentration. Briefly, to 100 µL of mucus sample, 400 µL of methanol were added and vortexed. 100 µL of chloroform were added to the previous solution and vortexed, followed by the addition of 300 µL of water. After mixing in the vortex, the solution was centrifuged at 14,000 *g* for 1 min. The top aqueous layer was removed, the remaining pellet was vortexed, stored (-20 ºC), and used for analysis on MALDI-TOF MS analysis.

5.2.3 MALDI Sample Preparation and spectra acquisition

For dried droplet crystallization, 1 µL of sample extract and 1 µL of HCCA matrix (10 mg/mL in MeCN:H_2O:TFA, 50:45:5 v/v) were premixed. Then 0.5 µL of this mixture was deposited on a ground steel target plate and dried at room temperature (20 ± 1 ºC) for 30 min. Calibration for MALDI-TOF MS analysis was carried out using insulin ([M +H]$^+$, m/z 5734.51), ubiquitin I ([M+H]$^+$, m/z 8565.76), cytochrome C ([M+H]$^+$, m/z 12360.97; [M+H]$^{2+}$, m/z 6180.99), and myoglobin ([M+H]$^+$, m/z 16952.30; [M+H]$^{2+}$, m/z 8476.65) from the protein calibration standard I kit (Bruker Daltonics, Germany). A model Autofex maX device MALDI-TOF MS from Bruker Daltonics with flexControl 3.4 and flexAnalysis 3.4 software was used. This instrument is equipped with the Smartbeam-II™ laser emitting at 355 nm. MS spectra were acquired in the m/z range of 2-20 KDa in a positive linear mode, using 60% laser intensity and 1000 laser shots with random walk mode of the shooting position pattern at a laser frequency of 200 Hz.

5.2.4 Statistical analysis

The free statistical program Mass-UP (Mass-Up, Vigo, Spain), was used for pre-treatment of the MALDI-TOF spectrums, following the respective program workflow [25]. The spectrums were exported from flexAnalysis 3.4 software as .XML files (raw data files). The 5 replicates for each sample were filed under the appropriate sample coding.

The raw data files were uploaded to Mass-UP software, as labeled files. The following pre-process options were chosen: Intensity transformation – square root; Smoothing – Savitzky Golay; Baseline correction – Snip; Standardization - none. For peak detection, the MALDI quant package with minimal intensity established at 0.0 value was used [26]. For all samples labeled peak list was generated. The peak matching process between peak lists was performed with the Maldiquant package with a tolerance of 0.002, for intra-sample matching and inter-sample matching. The option of generating a consensus spectrum was not selected. The generated Labeled matched peak lists sets were then used for Principal Component Analysis (PCA) and Biomarker discovery analysis (BMD). BMD approach was used to reduce the number of peaks to be analyzed, and generate a discriminant peak list, allowing to determine which peaks were present or absent in the different groups of samples [25]. The obtained discriminant peak lists were used for Hierarchy Clustering Analysis (HCA). The following parameters were chosen: Cluster reference – Farthest; Distance function – Euclidean; Conversion values – Percentage of presence; Intra-sample minimum presence – 0.33.

5.3. Results and discussion

An example of the spectrums obtained from the pre-treatment and peak alignment procedures following the Mass-UP options is presented in Figure 5.2a and Figure 5.2b, respectively. This pre-process and alignment was necessary in order to clean the large data set from signal noise and identify true signals, following the software workflow [25]. With this pre-treatment it was reduced the m/z range from 20 KDa to 9 KDa, reducing the number of peaks to be analyzed from the 40000 in the raw spectra to 100-150 of the pre-processed data.

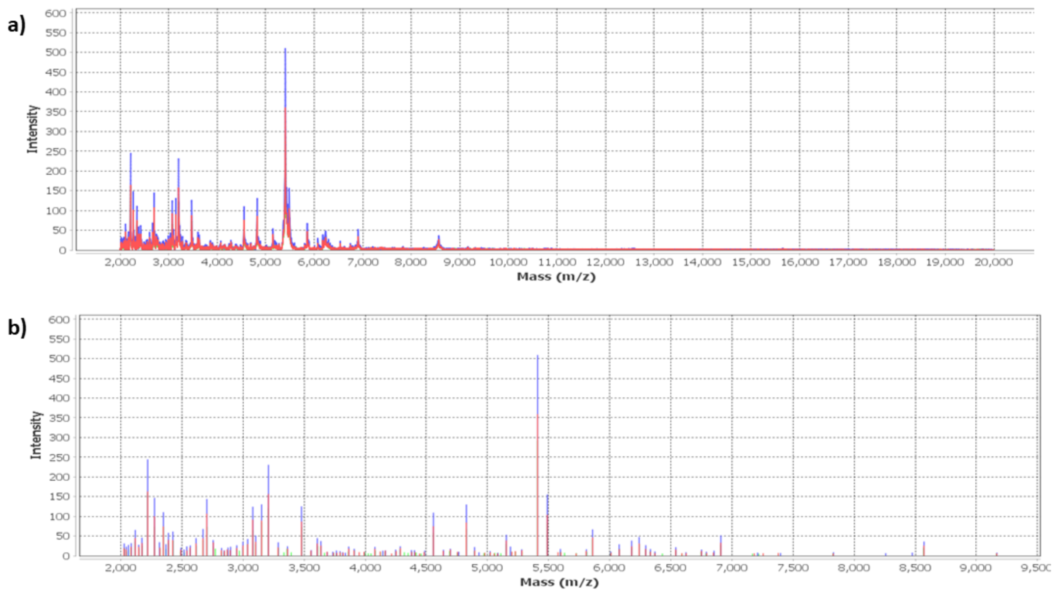

Figure 5.2 - Example of MALDI-TOF MS spectra on the Mass-UP software. a) after the pré-process treatment; b) after peak detection and peak alignment.

5.3.1 Multivariate analysis according to time of arrival

The alteration on fish mucus was evaluated to determine if the fish mucus suffered significant alterations during the permanence in the sea cages. Taking into consideration the Madeira island specificities (*e.g.*: warmer waters throughout the year), the time needed to achieve a commercial size is less (10-16 months) when compared with farms located at higher latitudes that require extended periods [27]. Therefore, sample groups were formed having in consideration both locations (CN and RB) as following: at the arrival moment (red); 3 months after arrival (3M - green); 6 months after arrival (6M - blue) and captures from lots with more than 12 months (12M - yellow).

5.3.1.1. Principal Component analysis

To gain a better perception between the similarities and differences among the groups, a multivariate approach PCA was used. To obtain better visualization between the correlations, 3D representation was performed (PC0 vs PC1 vs PC2). Figure 5.3 represents the PCA results for RB (Figure 5.3a) and CN (Figure 5.3b). For both locations, the projection of the sample forms distinct clusters. Supplementary Table 1 (annexes) shows the results for each sample and principal component associated with the RB location and supplementary Table 2 (annexes) for CN location.

In the case of RB, the variance associated with each component is PC0 – 33%, PC1 – 11%, PC2 – 10%, representing 54% of the data. PC0 separates the arrival samples (red) from all the others, with values between 8-15. For the other groups, the variations were between -1 to 1 for the 3M, 6M, and 12M, (green, blue, and yellow, respectively). The PC1 presents better separation for the 3M, 6M and the 12M samples, corresponding to values between -4 to -3 (green), 0.3 – 0.95 (blue) and -9.0 and -7.0 (yellow) respectively.

For the CN samples, cluster formation was also achieved, when the 3 components variance is analyzed, corresponding to PC0 – 25%, PC1 – 20%, and PC2 – 15% with a cumulative value of 60%.

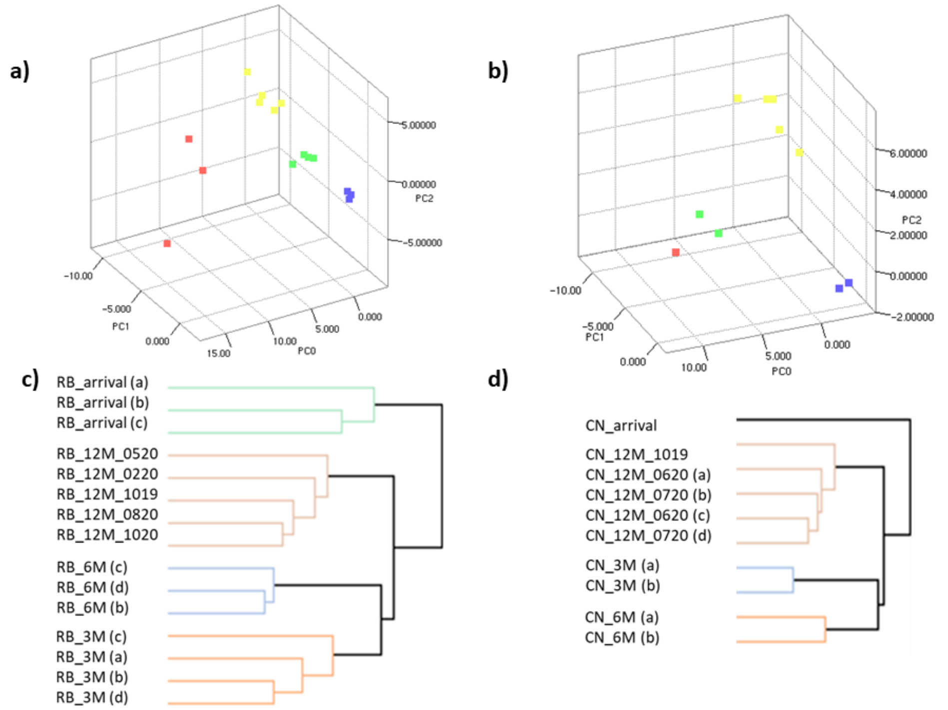

Figure 5.3 - 3D representation of the PCA results. a) Ribeira Brava (RB); b) Caniçal (CN). Red – arrival samples; green - 3M samples; blue - 6M samples; yellow - 12M samples. Hierarchy cluster analysis results for each group according to the location. c) Ribeira Brava (RB); d) Caniçal (CN).

In this case, the PC0 separates between all the different clusters, each one with a distinct quadrant. The projection of arrival samples formed a cluster in 10 to 5 quadrant (red), 3M samples in the quadrant 5 to 0 (green), 6M samples between -3 to -2, and 12M samples between 0 and -2. PC1 improved the resolution between the 6M, 3M, and 12M clusters.

These results demonstrate the evolution of the mass fingerprint of fish mucus, influenced by the new environmental conditions in which they are inserted [12]. The separations between the arrival samples, mainly in the RB group samples, could reflect the different origins of the fish lots that are frequently imported from Spanish or French suppliers.

5.3.1.2 Hierarchy clustering analysis

HCA was used to obtain other different representations from the data projection, establishing relations between the cluster samples. The aligned peaks list used for PCA analysis was subject to HCA analysis. Figure 5.3c and Figure 5.3d show the cluster analysis for each group of samples according to the location, RB and CN respectively. It is possible to see that, for both locations, the arrival cluster forms a distinguished cluster from the other ones. For the 3M, 6M, and 12M samples, for both locations, they form clusters with a closer association between them, having the 3M and 6M samples a closer relation than with the 12M.

These results could be an indication that each moment of capture reflects a specific environmental condition, with an impact on the mass fingerprint [13,25]. A closer analysis of the 12M samples from RB (Figure 5.4c), reveals that having into consideration the month of the capture, the samples associated with captures from August and October have proximate relations than the ones from May or February, even if they were done in different years. However, confirmation of such relations requires bigger data set for pattern verification.

The 12M cluster formation associated with the CN location (Figure 5.3d) is influenced by fish lot distribution in two different cages, due to fish density constraints. This procedure could explain the closer relations between the samples, nevertheless, as in the previous case, more fish lots need to be followed to increase the data set for verification of this pattern.

5.3.2 Multivariate analysis according to farm location

Even considering the analysis of the samples based on the time of arrival revealed promising results, the main objective is to access the feasibility of the approach in determining a specific group of mass peaks, to be used as biomarkers for each location (RB and CN). For this part, only the samples taken at captures moment of lots with commercialization size were taken into consideration (300-400 g or higher).

5.3.2.1. – Principal component analysis and Biomarker discovery

Figure 4a presents the PCA results for the capture samples from CN and RB. The variance results to the three principal components are PC0 – 51%, PC1 – 8%, and PC2 – 7%, representing a cumulative value of 66%. Without the influence of the other samples (arrival, 3M and 12M), the component PC0 clear projects the samples in distinct clusters, the CN cluster (blue) being placed between the quadrants -9 to -6 and the RB cluster between 10 and 9. Supplementary table 3 summarizes the values for all samples and principal components results.

For the determination of peaks responsible for clusters discrimination, the same dataset was used for biomarkers discovery analysis in the Mass-Up software (BMD). First, the data set was processed to obtain a list of peaks intersection between the samples (option BMD-intersample). For exclusion of insignificant peaks, the presence thresholds parameters were set to 35% as maximum absence and 50% as minimum presence in the samples. The used percentages are associated with the peak occurrence in the samples. Also, the algorithm calculates, for each peak, the p-value based on Fisher's exact test of independence and the q-value using the Benjamini Hochberg FDR [25]. A list with 772 peaks was generated and the p-values variated between 0.008-1.0, while for the q-values the variance was between 0.12-1.0. Supplementary table 4 presents the peaks with p-value <0.05 and q-value <0.4, corresponding to 161 peaks.

Reduction of peak number for analyses was processed under BMD-intrasample analysis. In this case, the RB group samples, and CN group were uploaded simultaneously.

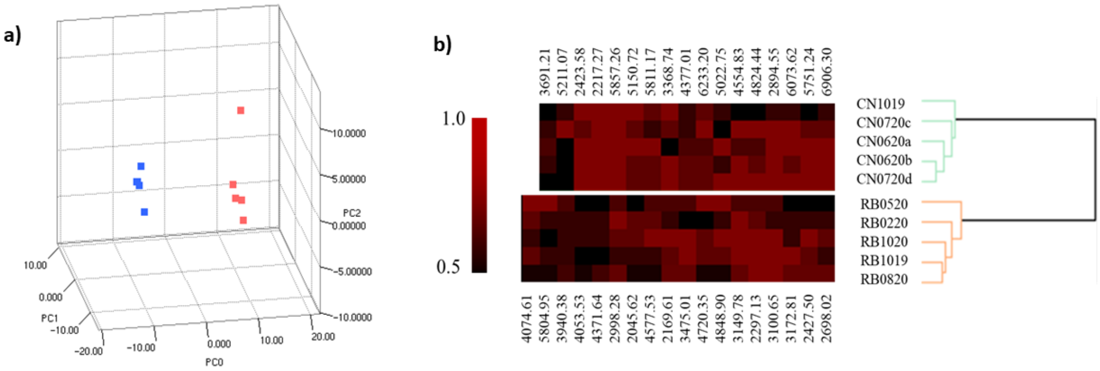

Figure 5.4 - a) PCA results of the capture samples (12M group) from Caniçal (blue) and Ribeira Brava (red). b) HCA dendrogram and the correspondent heat map for the 35 potential biomarkers. Ribeira Brava (RB -red); Caniçal (CN - blue). Black color corresponds to 0.5% of frequency between samples and red color to 1%.

This allowed refining the peaks search analysis, through comparison between groups, and determine the ones that are present or absent simultaneously. The threshold conditions were set as previously (35% maximum absence and 50% minimum presence). The resultant analysis allowed the creation of a smaller discriminant peak list of 43 peaks, 22 relatively to RB and 21 to CN, with the following p-values < 0.008 and q-value < 0.10. The selected peaks correspond to the first 43 peaks in supplementary table 4 (Annexes).

5.4.2.2 – Hierarchy clustering analysis

For HCA analysis the data set that was uploaded and the reduced discriminant peak list was used to restrain the analysis only to those peaks. The resultant dendrogram, the peaks used, and the correspondent heat map is presented in Supplementary Figure 1 (Annexes). HCA analysis results in the formation of two separated clusters, one correspondent to RB and the other to CN. However, a closer analysis of the selected peaks shows that there were 4 similar peaks between the groups, that vary less than m/z 2.0

units, the m/z 2346.77-2346.59, m/z 3629.30-3631.57, m/z 3604.54-3605.88, and m/z 5405-5407. Due to their similarities, the peaks were removed from the list and the HCA was repeated, given the results present in Figure 5.4b. The alteration on the peak list did not result in significant alterations on the HCA results, confirming that they were not significant peaks for the HCA discrimination. Therefore, the final set of potential biomarkers consists of 17 biomarkers for CN location and 18 for RB location.

5.4 Conclusions

Even though fish mucus is an interesting target of study, its variable composition due to environmental and fish physiological influence creates several challenges on the analysis of its constituents. Also, the scarce number of studies on fish mucus metabolomics and dedicated databases contribute to challenges in mucus studies. However, the incorporation of omics technologies provides new prospects to uncover new knowledge that otherwise would remain unnoticed.

In this work, fish mucus was analyzed with the MALDI-TOF MS method, employed as a mass-fingerprint technique for the origin discrimination of aquaculture fish from two different mariculture farms, Caniçal - Madeira and Ribeira Brava - Madeira. The Mass-Up software was used for multivariate analysis and potential biomarkers discovery.

As result it was possible to discriminate between samples with different origins, resulting in a list of 35 potential biomarkers, being 17 biomarkers specific for Caniçal and 18 for Ribeira Brava. Also, the mass-fingerprint approach allowed to differentiate between samples with different residence times in the sea cages, confirming the alterations that fish mucus undergoes from the moment of arrival to the moment of capture. The results also confirm the potential of MALDI-TOF MS to be applied for origin discrimination studies of aquaculture fish from different farms and as a fast, reliable approach.

CRediT authorship contribution statement

Jorge Freitas: Conceptualization; Methodology; Validation; Formal analysis; Investigation; Resources; Data curation; Writing-Original Draft; Writing – Review & Editing; Visualization; Pedro Silva: Conceptualization; Methodology; Validation; Writing – Review & Editing; Visualization; Rosa Perestrelo: Methodology; Validation; Investigation; Resources; Data curation; Writing – Review & Editing; Paulo Vaz-Pires: Conceptualization; Writing – Review & Editing; Supervision; Project administration; José Sousa Câmara: Conceptualization; Resources; Writing – Review & Editing; Visualization; Supervision; Project administration; Funding acquisition.

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Chapter 6 - Bacterial diversity analysis of coastal superficial seawaters near aquaculture facilities, using MALDI-TOF approach and Ribopeaks database.

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Bacterial diversity analysis of coastal superficial seawaters near aquaculture facilities, using MALDI-TOF approach and Ribopeaks database.

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Abstract

The upcoming environmental regulations when applied to the aquaculture sector will further require that the industry adopt routine control methodologies and results registration, to support that its activity is respecting the adjacent environment and to sustain its sustainability claims. This work aimed to determine the capability of microbial analysis with the MALDI-TOF approach and Ribopeaks database, to establish the microbial community diversity of coastal superficial seawaters, near aquaculture facilities and the possible influence of anthropogenic pressure. The main conclusions are the capability of the Ribopeaks database to be used for the analysis of environmental bacterial samples since it was able to identify a much higher diversity of marine bacteria when compared with the MBT Compass database. The MALDI-TOF-Ribopeaks approach has a clear potential for fast and cheaper routine analysis of the seawater microbial community, which is becoming more relevant in the progress of quality control procedures in the aquaculture industry. The present study also reveals the need for more studies on the interactions between anthropogenic influence in coastal waters and the effect it might have on aquaculture sustainable management.

Keywords (6): Environment; Sustainability; Monitoring; Coastal Waters; Anthropogenic impact

6.1 Introduction

Coastal areas are important zones, either due to favorable habitational conditions or economic relevance for the development of several activities (*i.e.:* tourism). However, the combination of a growing population and economic development, increases the threats on the marine ecosystems, due to nutrient inputs from anthropogenic activities on the coast or land-based activities [1]. Nutrient inputs can have different origins such as insufficient treatment of wastewater (*e.g.*: primary treatment only), domestic wastes, industrial wastes, agricultural runoffs, etc. All of these are sources of organic carbons and minerals, that once reach the marine coastal areas, can lead to eutrophication affecting the ecosystem balance [2]. Also, marine activities developed near the coast can impact the quality of the proximate waters.

Aquaculture activities are one of the most scrutinized sectors, mostly their influence on the adjacent environment [3]. The new environmental legislation will require that the industry adopt additional routine control methodologies and results registration, to support that its activity is respecting the surrounding environment and to withstand its sustainability claims [4]. In European Union (EU) the aquaculture exploration is regulated under nine major legal requirements, that cover all the steps from the project phase, implementation, and operation, to prevent conflict of interest between all active users of coastal areas and habitat protection. The major policies are Marine Strategy Framework Directive; The Water Framework Directive; Birds and Habitats Directives; Natura 2007 regulations; Regulation on the use of alien and locally absent species in aquaculture; EU Regulation on the prevention and management of the introduction and spread of invasive alien species; Environmental Impact Assessment; Strategic Environmental Assessment and Directive on Maritime Spatial Planning [5].

Since aquaculture exploration and land-based anthropogenic activities are both major sources of nutrients inputs, it appears that they have direct interactions. The overflow to coastal waters, of anthropogenic run-offs, rich in nutrients or pollutants (*e.g.*: storm waters, sewage), can affect aquaculture management increasing fish mortalities, diseases outbreaks, or product contamination [6]. In the case of aquaculture, negligent practices could result in environmental degradation (*e.g.*: anoxic sediments). Nevertheless, aquaculture can also have a beneficial effect on the surrounding environment, providing shelter [7] and nutritional input on oligotrophic waters [8].

Besides the influence on the aquatic fauna and flora, independently from the nutrient sources, one of the major impacts will be on the microbial diversity on coastal marine waters [9]. It is known that microbial metabolism has ecological importance on the balance of matter (organic and inorganic), energy and carbon cycle on the marine ecosystem dynamics. Microbial diversity and structure, on the water column, are shaped by the prevailing conditions at a specific time, being the most common, seasons influence, inland discharges, anthropogenic pressures, or ecological limitations (*e.g.*: oxygen accessibility and temperature) [10].

Consequently, knowing the marine microbial community diversity and how interacts with water quality monitoring, and the influence on the pathogens life cycle, is of utmost importance. Usually, the identification is made complementing morphological (*e.g.* cell shape), phenotypic (*e.g.* Gram staining), and sequencing techniques (*e.g.* polymerase chain reaction (PCR)) [11,12]. Sequencing techniques have proven to be very important providing new insights and understandings of marine microbial structure throughout the globe, remaining an essential instrument in this field [13]. Though, a high level of expertise and cost undermines the implementation as routine analysis techniques [14].

Also, some limitations are attributed to the choice of the primer that could leave some taxonomic groups out of the analysis [15].

An alternative method for microorganism identification is based on the acquisition of protein fingerprint through MALDI-TOF MS and comparing it to reference profiles on a database or analyzing the unknown profile against known profiles of bacteria. Also, its capability to provide fast, high throughput analysis at a lower cost, as well the reduced amount of sample preparation, catapulted the technique as an important advance in the microbiology field [14].

On the other end, the main drawbacks are associated with the capability to use only culturable bacteria as well as the quality of the database. Even though current cultivation techniques are limited in the number of individuals from a phylum that is possible to isolate, it still represents a very valuable technique, to study microorganism characteristics that are difficult to deduce from genetic data (*e.g.*: growth characteristics, metabolism, or physiology) [15]. The databases used on MALDI-TOF protocols, strongly influence the method accuracy and efficiency of identification. Currently, MALDI BioTyper™ (Bruker Daltonics, Inc.) and VITEK® MS Plus (bioMérieux) are the most used databases. They differ in the used algorithms for identification, as well in the procedures of how the spectra are obtained, Main Spectrum analysis, and SuperSpectrum approach, respectively [16]. However, results provided by databases are biased towards clinical and food isolates since it is the most common application for MALDI-TOF analysis. Therefore, identification of environmental microorganisms may not be accurate as for clinical or food specimens [17].

In an attempt to overcome such limitations, Tomachewski and coworkers developed Ribopeaks (RBP), an open-access database, which is centered on the creation of spectral information from ribosomal proteins data stored at GenBank and using it for bacterial taxonomic classification, through artificial intelligence algorithms. The GenBank offers access to the sequences of 2 807 341 amino acids belonging to 57 different r-protein families [18].

On the geographical region of this study (Madeira Island, Portugal), microbial studies on marine coastal areas are scarce when compared with marine macro, microfauna or flora. From the analysis of literature published between 2015-2021, from the marine research center Oceanic Observatory of Madeira (OOM), only two are relative to microbial community analysis, one associated with aquaculture nets [19] and the other to top shell community of *Phorcus sauciatus* [20]. Other existing studies are related to the analysis of sand and seawater quality control which are focused on Coliforms, *Escherichia coli,* and *Enterococcus* spp. [21,22].

This work aims to determine if the MALDI-TOF methodology associated with RBP database analysis, is capable to establish the diversity of microbial communities and distinguish seasonal variability, through the qualitative analysis of the identified microorganisms.

6.2 Material and methods

6.2.1 Sample collection and geographical area

The samples collection was performed at three locations, presented in Figure 6.1. Two associated with fish farm facilities, one located at Campanário, in Ribeira Brava (RB), Madeira (32°39'38.1"N 017°03'22.1"W) and the other in Baía d'Abra, Caniçal (CNL), Madeira (32°44'31.4"N 016°41'26.7"W). The third place was chosen between farms locations at Caniço, Santa Cruz (STCZ), Madeira (32°38'42.8"N 016°49'31.4"W).

The samples were taken in three different periods, September 2020, February 2021, and between May-June 2021. The sampled volume was 3L of superficial seawater at each location, in sterile dark glass bottles. The samples were stored in hermetical sealed containers until the laboratory arrival and processed upon arrival.

Figure 6.1 - Location of the sampling areas. Ribeira Brava (RB); Santa Cruz (STCZ); Caniçal (CNL).

6.2.2 Microbial cultivation

The cultivation strategy was based on the work of Sanz-Saez [13]. In brief, heterotrophic bacteria that grow easily under laboratorial cultures were retrieved using Marine Agar 2216 (Difco ^{TM}), to recover comparable portions between different oceanographic conditions or future studies. The objective was not to increase the novelty of the isolates but to access the capability of the approach to correlate with the ones already known.

One litter of seawater samples was filtered through 0.2 µm membrane filters (Whatman ^{TM}). The filters were placed on 10 ml of Ringer solution (Oxoid ^{TM}) and serial dilutions were performed. Colonies isolates were obtained through the addition of 100µl (10x diluted and undiluted) on marine agar plates, incubated at 22ºC for 72h.

6.2.3 MALDI Sample Preparation

The Brucker Bacterial Test Standard (BTS) was prepared in 50 µL of standard solvent (acetonitrile 50%, water 47.5 %, and Trifluoroacetic acid 2.5%). Aliquots 5 µL of BTS solution were stored at -80 ºC into screw cap microtubes. The matrix used was α-

Cyano-4-hydroxycinnamic acid (HCCA) in a concentration of 1 mg/mL, dissolved in a standard solvent.

For MALDI analysis, BTS solution (1 µL) was placed on the MALDI target and dried at room temperature. Immediately after drying 1 µL of HCCA solution was added. The single colonies were extracted from the agar plate with a 1µL loop and directly spread on the MALDI target plate. The same BTS procedure was repeated for each colony in triplicate.

6.2.4 MALDI spectra acquisition and MBT analysis

Autoflex maX device MALDI-TOF MS (Bruker Daltonic, Bremen, Germany) and the software MALDI Biotyper (MBT) version 3.4 (Bruker Daltonic, Bremen, Germany), was used for single colonies identification. The AutoXecute acquisition control software parameters were 240 laser shots in 40 shot steps. Each spot was measured twice automatically. The instrument was calibrated using a BTS (Bruker Daltonic, Bremen, Germany).

6.2.5 Ribopeaks software and data analysis

The extraction of the peak lists was performed on the Flex Control 3.4 software. Peak lists were extracted as mzmxl files. The extracted peaks were placed on the RBP software (http://www.ribopeaks.com) [18].

The chosen criteria to run the program were: 30S and 50S option was selected since the algorithm was able to search in the complete genome database or the 30S/50S database. The other option was the species artificial intelligence protocol since one of the main objectives was to establish the possible environmental origin of the microorganisms.

The retrieved information was: all ten microorganisms identification results (mID), the number of similar peaks, and total parity percentage for each mID. The results were transferred to Excel software for the determination of the percentage of occurrence of the mID.

The information regarding the environment of origin of each microorganism was retrieved from NCBI Taxonomy Browser. Statistical analysis was performed on software STATISTICA 10.0 (Stat Soft, Inc., Tulsa, OK, USA).

6.3 Results and Discussion

6.3.1 Ribopeaks vs MBT Compass

For each peak list inserted on Ribopeaks software, the best first 10 results were exhibited and registered. The information retrieved from Ribopeaks was mID, the number of similar peaks, and the total parity percentage for each result.

The results from the MBT analysis were automatically provided by the software as a PDF file, with the rank quality, matched pattern (microorganism ID), score value, and the NCBI identifier.

In both cases, all the best 10 results that are provided were analyzed, independently of the score value or the parity percentage. To facilitate the analysis from both software's, each mID result was attributed to one of the following groups, according to the main environment from which was first sampled (according to NCBI Taxonomy database): *Aquatic Environment* – encompassing results from marine and freshwater environment, aquatic fauna and flora; *Human associated* – related with sewage, clinical or commensal do human body; *Animal associated* – mostly terrestrial animals and birds; *Soil/Plant* – results associated with rhizosphere, plant structures; and *Others* - included microorganisms with origins other than the previous groups. The information about the possible environment of origin was retrieved from the NCBI taxonomy database. This grouping structure was used for all sampling sites comparison.

For the comparison between RBP and MBT compass results, the summer data were chosen in all the sampling locations and are presented in Table 6.1.

Table 6.1 - Comparison between databases results, for the samples taken in the summer.

Database	Location	Aquatic	Human	Animal	Plant/soil	Other
Ribopeaks	CN	43.0	18.7	11.8	21.6	5.0
	RB	33.5	25.1	9.8	28.8	2.8
	STCZ	37.0	23.3	15.4	24.2	8.7
MBT	CN	28.3	25.4	11.5	22.0	12.9
	RB	16.3	33.1	15.6	18.1	16.9
	STCZ	21.3	27.5	11.3	22.5	17.5
p-values		0.024	0.105	0.841	0.187	0.010

From the results is possible to confirm the existence of significant statistical differences ($p < 0.05$) between the "*Aquatic*" and the "*Other*" groups. The analysis done with the RBP database was able to indicate a much more variety of mID related to the aquatic environment other than the MBT compass analysis. Also, RBP provided fewer results with unrelated origins (*e.g.*: engineered origin, food fermentation, or laboratory environment) knowing that the inputs are from an environmental sample. On the other hand, the MBT database provided similar results ($p>0.05$) to RBP for the remaining groups *"Human"*, *"Animal"*, *"Plant/soil"*.

These results support the idea that MBT compass analysis, presents some bias toward the analysis of clinical samples [17], while RBP shows greater potential for the analysis of environmental samples. In the case of RBP, the use of the GenBank database allows access to a higher number of protein amino acid sequences with diverse origins from which was predicted the mass spectra of each protein[18], while in the case of MBT compass the database was created from the direct MALDI-TOF analysis of the collected specimens [14,16]. Since in this case, the samples have a clear environmental origin (marine environment), the RBP data analysis was chosen to proceed with the study.

6.3.2 Genus diversity analysis

All the identified genus, for each location and different seasons, are presented in supplementary table 1. The values used are from the analysis of the genus frequency of the occurrence of the mID and the total number of mID. Figure 6.2 is a representation of the genera with the highest frequency of occurrence, for better visualization of the variability between seasons and locations. By decreasing order, the selected genera were: *Vibrio*> *Pseudoalteromonas*> *Xanthomonas*> *Alteromonas*> *Staphylococcus*> *Escherichia*> *Pseudomonas*> *Photobacterium*> *Acinetobacter*. In the following subsections, each genera occurrence will be analyzed, having into consideration the season and the location.

6.3.2.1 Vibrio

Vibrio is widely distributed in marine environments and is an important element of the aquatic ecosystem due to its role in the biogeochemical cycle [23]. The abundance and community composition of *Vibrio* is a complex dynamic affected by environmental factors (*e.g.*: temperature, salinity, or dissolved oxygen), chemical (*e.g.*: inorganic and organic nutrients), and biological (*e.g.*: protozoa, marine animals, and algae) [24]. In the case of temperate regions, due to low variance of temperature, the concentrations of organic and inorganic nutrients, as well phytoplankton communities, could have a significant role on *Vibrio* diversity. Also, *Vibrio* can enter in a viable but non-culturable state under adverse environmental conditions (*e.g.*: oligotrophic or sunlight radiation) but maintaining a quick growth response when the conditions become favorable (*e.g.*: nutrient concentration), transitioning from a small part of the community to a dominant bacterium in response to environmental and climate variances [25].

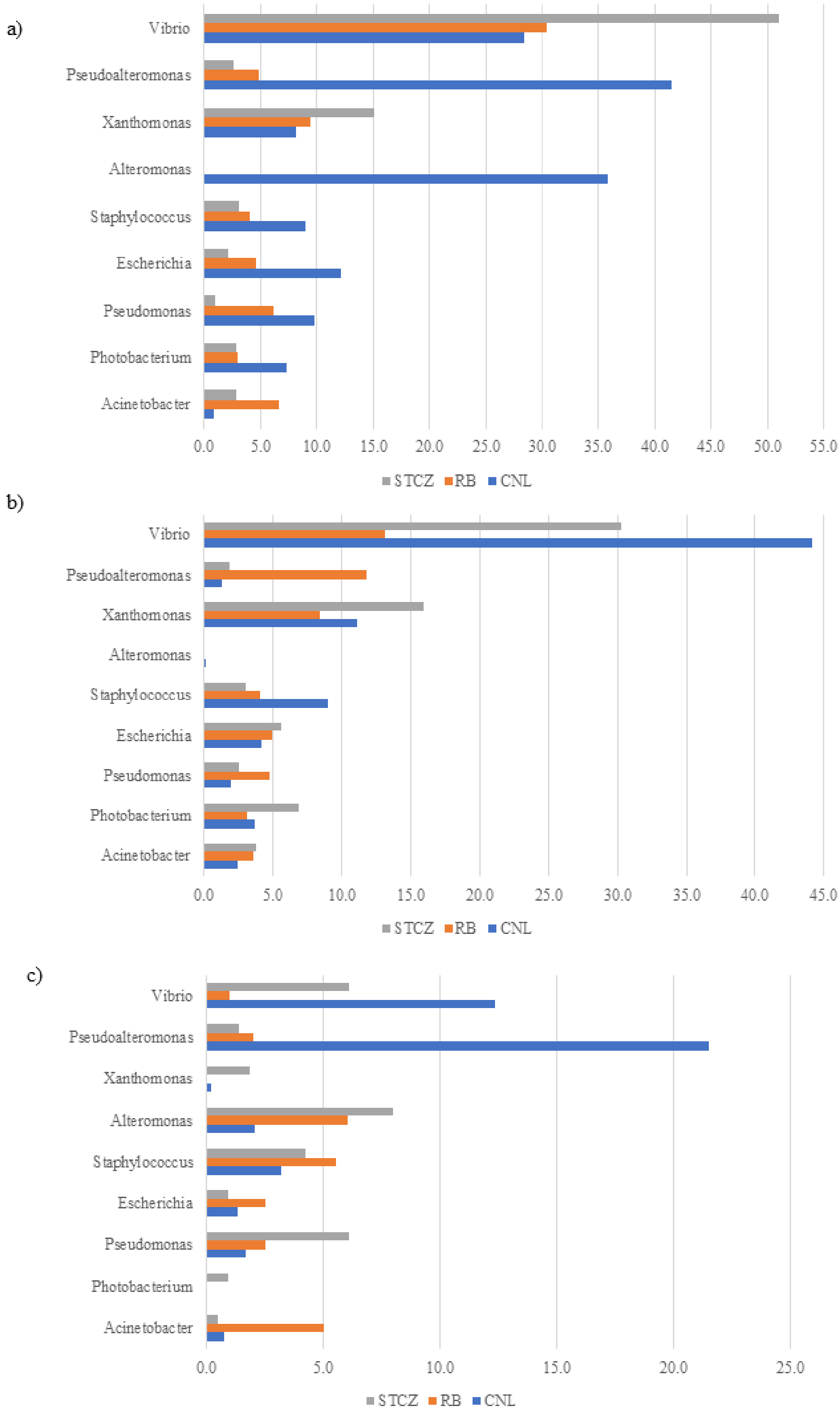

Figure 6.2 - Most relevant bacteria genus. a) Winter; b) Spring; c) Summer. Ribeira Brava (RB); Santa Cruz (STCZ); Caniçal (CNL). The values are in percentage of occurrence.

Therefore, recognizing the dynamic alterations of the *Vibrio* community is of great importance for the sustainable improvement of aquaculture and public health control.

From figure 6.2, is possible to see that summer is the period with lower frequency (CNL 12%, RB 1%, STCZ 6%) comparing with the other seasons. Its presence throughout the year could be related to the temperate conditions associated with Madeira Island. The increase in the spring (CNL 44%, RB 13%, STCZ 30%) and winter (CNL 28%, RB 30%, STCZ 51%), is probably due to high nutrient inputs during the more frequent raining periods. The synergetic effect of other parameters could also impact on its occurrence during the summer, such as the example of oxygen, salinity, and sunlight radiation.

6.3.2.2. Pseudoalteromonas

Pseudoalteromonas are Gram-negative, aerobic, and heterotrophic bacteria that are ubiquitous obligatory marine bacteria. The strains affiliated to this genus are often described as fast-growing bacteria, able to efficiently convert dispersed organic matter into growth substrates [26]. It is present in all marine habitats, such as coastal, open, and deep-sea waters, sediments, is also frequently associated with healthy algae, invertebrates, and marine animals[27]. Few strains have been described as pathogenic or opportunistic being some examples of *P. piscicida*, *P. agarivorans,* and *P. undina* [28–30].

Therefore, having the respective precautions, this genus could be an indicator of the relatively good status of the marine environment. In this case is necessary to highlight the relative occurrence in the CNL sampling zone, which is the one with a significantly higher percentage during winter (42%) and the summer (21%).

6.3.2.3 Xanthomonas

Xanthomonas spp. is a Gram-negative bacterial genus in the class Gammaproteobacteria, mostly associated with plants diseases [31]. Therefore, its relative abundance in the results could most probably originate from strong runoffs originated from inland during rainy periods. This could be verified when compared the relative abundance during the winter (CNL 8%, RB 9.5%, STCZ 15%) and spring (CNL 11%, RB 8.4%, STCZ 16%) while in the summer season its presence is basically in the STCZ (<2%) sampling site, which is closer to a water stream. This location also presented a higher occurrence during the rainy seasons.

6.3.2.4 Alteromonas

The genus *Alteromonas* incorporates Gram-negative, aerobic, motile bacteria living in marine tropical and temperate waters, in coastal, open waters, or deep sea [32,33]. This genus may be dominant in heterotrophic conditions since is generally described as a copiotroph organism, which means it grows very rapidly when organic nutrients are available in an oligotrophic marine environment [34]. Therefore, it has an important active role in the circulation of biogenic mater, energy flow in the ocean and as a growth facilitator for other species [35,36].

From the present data in figure 6.2, is possible to see a specific event in winter at CNL location, where the *Alteromonas* occurrence was 36%, which is probably not due to aquaculture operation since in the spring season the occurrence is zero in all locations. However, in the summer the *Altermonas* occur (CNL 2%, RB 6%, STCZ 8%), which may be due to higher temperatures and the increase in the nutrient's availability, but not as significant as in the wintertime at CNL location. Also, interesting is the increase in the frequency in the summer at the locations associated with recreational zones.

6.3.2.5 *Staphylococcus*

Staphylococcus spp. is considered a commensal genus from human skin, but also to domestic animals and wildlife [37,38]. The genus comprises several opportunistic pathogens, that have been gathering attention due to their relevant presence on clinical samples and association to diverse infections (*e.g.*: *S. aureaus*) [39]. Therefore, its occurrence in the marine environment is normally linked with recreational bath areas as well with urban or rural runoffs, in high anthropogenically influenced zones [40].

The occurrence of *Staphylococcus* spp. in the sampling point is relatively constant throughout the sampling time (winter - CNL 9%, RB 4%, STCZ 3%; spring - CNL 4%, RB 3%, STCZ 4%; summer - CNL 3%, RB 6%, STCZ 4%). In the case of CNL and RB, other than runoffs sources, the presence of aquaculture workers throughout the year could be a reason for the relative constant presence of the genus in the marine water samples.

6.3.2.6 *Escherichia*

The genus *Escherichia* and the species *E. coli* is one of the most studied model microorganisms since it is referred to as one of the most important indicators of water quality (fecal indicator) and an important pathogenic agent [41,42].

Escherichia coli is mainly associated with the intestinal tract of homeothermic organisms (*e.g.*: humans, other mammals, and birds) reaching the environment through feces or wastewater treatment plants [43,44].

In this study its relative abundance was higher in the winter (CNL 12%, RB5%, STCZ 2%) and spring (CNL 4%, RB 5%, STCZ 6%) and, lower in the summer period (CNL 1%, RB3%, STCZ 1%). The presence of this genus on the samples throughout the sampling time is not unexpected since in aquaculture facilities, is common the presence of seabirds. However, is not possible to dismiss the occurrence of episodic wastewater discharges as a major source for *Escherichia* occurrence peaks, due to the proximity of treatment plants.

6.3.2.7 Pseudomonas

The *Pseudomonas* genus is one of the most ubiquitous genera, with species isolated from very diverse ecological niches (*e.g.*: water, soil, plants, animals, humans, tropics, and Antarctica environments) [45,46]. Its metabolic and physiological versatility gives them tools that promote their persistence in diverse environments [47].

Therefore, this is one of the genera that are not easy to determine the most probable origin, without a specific species analysis. Its presence in the sample's locations is: CNL 10%, RB 6%, STCZ 1%, for the winter; CNL 2%, RB5%, STCZ 3% in the spring; and CNL 2%, RB 3%, STCZ 6% in the summer.

6.3.2.8 Photobacterium

The genus *Photobacterium* is very prosperous in worldwide oceans and has considerable ecophysiological variety. The environments and isolation sources of these species comprise coastal, open-ocean, deep-sea, sea sediments, and saline lake waters. They can also be found in a variety of marine organisms with which is established distinct relationships, from symbiotic to pathogenic [48]. Members of this genus are also effective saprotrophs in marine habitats [49], and more recently have been identified in meat products, increasing its environmental influence [50].

In this study its relative abundance is higher in the winter (CNL 7%, RB3%, STCZ 3%) and spring (CNL 4%, RB3%, STCZ 7%) and, and virtually inexistent in the summer period (CNL 0%, RB0%, STCZ 1%).

6.3.2.9 Acinetobacter

Acinetobacter is a bacterial genus of Gram-negative, non-motile and aerobic microorganisms that are also referred to as commensals. Most of the *Acinetobacter* spp. are commonly found in the natural environment where they act as cleaning agents for hydrocarbons, heavy metal contamination, and phosphorus removal [51]. Common environments are wastewater, sewage, healthy humans, animals, water bodies, soil, and food [52,53]. Some species have been also identified as pathogens in clinical environments [54].

In this study, its relative abundance is higher in the winter and spring (CNL 2%, RB 4%, STCZ 4%), while relatively low for winter (CNL 1%, RB 7%, STCZ 3%) and summer (CNL 1%, RB 5%, STCZ 1%) with exception of RB location, with low variability throughout the seasons. Since RB is the site with the higher number of cages, one possible reason for this occurrence could be due to the dominant presence of the microorganism in the fish gut, associated with resistance to unfavorable conditions of the environment, and the capacity to produce antibiotic compounds against other microorganisms [55].

6.3.3 Sources of inputs pressure

Madeira waters are characterized as oligotrophic, temperate temperatures and a small island platform with great depths [56]. Also, oceanographic conditions have a role in the dispersal of nutrients, temperature, salinity throughout the water column, and impact microbial diversity. However, this is not the subject of this work, more information about the oceanographic conditions around Madeira Island can be found in other works [57–59].

The orography of Madeira Island is steep, favoring the natural creation of streams with different sizes and flow intensities. In Figures 6.3b, 3c, and 3d it is represented the main (blue lines) and secondary (light blue lines) water streams near the sampling areas. The presence of these streams represents one of the major sources of inputs related with soil, plants, agriculture, or sewages. It is also indicated in the same figures the location of wastewater treatment plants, harbor zones, and recreational areas.

RB (Figure 6.3b) is the sampling site under the major influence of freshwater streams. Seven main streams being one of them among the majors in hydrographic basin area, located between the recreational zone at the west and the harbor zone at the east [60]. Also, the nearby harbor, besides given support to the aquaculture facilities, is used by smaller fishing vessels to re-supplies and bait preparation. The aquaculture facility is constituted of 16 cages of 25 meters in diameter and is the largest in this study.

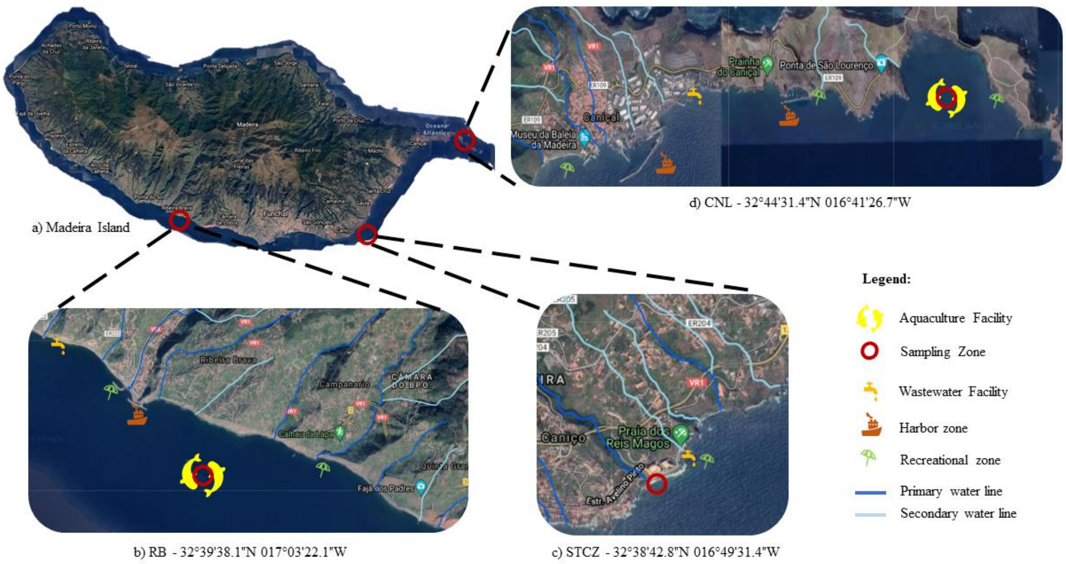

Figure 6.3 - Location of the sampling areas and possible sources of nutrient input. a) Madeira Island. b) Ribeira Brava (RB) – sampling site location and nearby sources of nutrient inputs. c) Santa Cruz (STCZ) – sampling site location and nearby sources of nutrient inputs. d) Caniçal (CNL) – sampling site location and nearby sources of nutrient inputs.

In CNL (Figure 6.3d), the sampling area near the aquaculture facility is the most isolated in terms of anthropogenic and freshwater streams influence. The point with most pressure near the aquaculture facility is a small pear that supports the touristic point of

‘*Cais do Sardinha*’, and Madeira natural park rangers (http://www.visitmadeira.pt/en-gb/what-to-do/activities/search/pr8-vereda-da-ponta-de-sao-lourenco). Is often used by the tourists as a bathing zone. On the other hand, is closer to the island's main port for cargo ships and fishing vessels. The aquaculture infrastructure is formed by 8 cages of medium size, 2.800 m^3 each, currently being the smallest cage structure on the island.

STCZ sampling zone (Figure 6.3d) is characterized for being a high-frequency recreational zone and near a wastewater treatment plant, with a history of occasional emergency flushes. However, is necessary to refer that the main recreational zones in STCZ and RB are regularly examined for water quality and frequently attributed with the Blue Flag Award (https://www.blueflag.global/; https://bandeiraazul.abae.pt/).

In the case of the aquaculture facilities, the position of the cages respects the recommendation of the Marine Space Planning from the Madeiran Government [56,61]. The structures are positioned between the bathymetric depth of 20-80m, being exposed to marine currents that guarantee the proper water flow between the structures and effective dispersion of the nutrients.

The variety of input sources in the coastal area, plus the complexity of the interactions between environmental conditions, increases the challenge of the microbial community analysis. This work opens the possibility to infer the influence of future large-scale, land nutrient inputs (episodic or seasonal) on bacterial communities, like the ones studied by Abreu (2016) and Pereira (2013) [21,22]. On the other hand, due to the oceanographic conditions characteristic to the island, long-term effects of the nutrients inputs are not expected [61]. However, for aquaculture activities it might have drastic impacts with an increment of mortality events, emphasizing the need for monitorization.

6.4 Conclusion

This work aimed to determine the capability of microbial analysis with the MALDI-TOF approach, to establish the microbial community diversity of coastal superficial seawaters, near aquaculture facilities and the influence of anthropogenic pressure.

The choice of using a standard marine medium was intending to reach the most common heterotrophic bacterial fraction. Even though the use of standard culture media will leave out many other relevant microorganisms, it will facilitate the comparison with future studies and between marine environments. Also, the identification of new species was not the objective of this study, so the purification of bacterial isolates was not performed [13].

The main conclusions of this work are the capability of the RBP database to be used for environmental analysis since it was able to identify a much higher diversity of marine bacteria when compared with the MBT Compass database. These results are corroborated by other works that state the limitations of some current databases for microorganism identification from environmental samples [17].

Furthermore, the present results demonstrate another possible utilization of the MALDI-TOF method, as a method for the determination of the overall bacterial community in superficial waters. The utilization of MBT Compass protocol and the software automatic analysis, reduce some of the variabilities associated with the cultivation and instrumental parameter [14]. In this case, the RBP database, has an important role, since it allows access to much higher variability of proteins mass spectrums to support the analysis [18]. The utilization of AI methodologies for the creation of databases from already existent data, reveals a very promising future in the improvement of bacterial databases. Improvements on the software, or the development of new ones, might expand the information output, making it closer to the information analysis associated with the analysis of sequencing methodologies.

Further studies are necessary to confirm the present results, as the comparison with quantitative methodologies in order to determine the relevance of the percentages of frequency of the identified microorganism. Since the MALDI-TOF analysis presents some variability in its results, studies for comparison with the DNA sequences might be necessary. The present study allowed to elucidate the need for more studies on the interactions between aquaculture production and the anthropogenic influence in coastal waters, and the impact it might have on aquaculture environmental control parameters.

As an overall conclusion, the studied approach has a clear potential for fast and cheaper routine analysis of seawater microbial community, which is becoming more relevant on the development of quality control protocols in the aquaculture industry.

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https://bandeiraazul.abae.pt/ last access 8/6/2021

General Conclusions

General Conclusions

Consumer oriented demand for sustainability or quality is an important driver for changes in governance policies and industry performance. However, consumers are only one part of the food sector chain and without the convergence of governments and industry, the needed improvements on the sector can become short in meaningful and last longing advancements. The resolution of issues related with sustainability and food safety by the industry requires transformation and adaptation to new monitoring/evaluation procedures, to assure consumers about the proclaimed quality. The aquaculture sector is one of the most questioned by this new paradigm. As the sector expands and grows, incorporation in regional or global markets becomes more challenging due to issues related to fraud, pollution, or food safety.

In this work three main areas were studied to demonstrate overall quality: freshness, traceability, and safety.

Product freshness was studied to improve knowledge about spoilage evolution of the fish species produced in the aquaculture facilities. At the time the species available were greater amberjack (*Seriola dumerili*) and gilthead seabream (*Sparus aurata*). During this study it was possible to develop a sensory approach based on QIM methodology for data collection and treatment. It was possible to establish a timeline for the product spoilage, specific for each species accompanied by the photo registration of the main physical alterations during spoilage evolution. For gilthead seabream, it was determined a 9 days (±1 day) period and for greater amberjack 12 days (±1 day). Is necessary to highlight that the study was conducted as close as possible to the company daily working conditions. This work also allows the improvement of the company knowledge on the freshness monitoring procedures, on the product stock control, captures programming and increment the value of the information passed to clients. Future work on this area could be focused on new fish species, products, or the improvement of the correlation between the studied parameters (*e.g.*: microbial, chemical, physical, or sensory).

Product traceability studies were focused on the development of a methodology to determine the fish origin. The method proposed was based on the protein mass fingerprint of the fish mucus, acquired using MALDI-TOF equipment. Using this technique and multivariate statistical treatment was possible to differentiate between the fishes that originated from two different aquaculture facilities. MALDI-TOF is a very versatile and powerful equipment, that allows data gathering in cost effective manner. Even though is not suggested the equipment acquisition by the company (due to high costs) continuation of similar studies is highly recommended. Future work could be done on the follow up of the product until customer acquisition, to improve company product control policies and management of authenticity/quality issues.

Safety is a diverse field, that can encompass different areas, for example, product safety, facilities management, or personal hazards. The area identified as the one raising immediate concerns was in the environmental management, more specifically the impact of microorganisms on the production cages. Since knowledge about the marine water bacterial community is scarce in Madeira Island, it was necessary to improve the company knowledge in this area. It was necessary to develop an approach that could be done in a routine, fast and costly manner without needing to export samples, and easily accessed in the future.

The capabilities of the MALDI-TOF equipment to accomplish such requirements were studied. The method is being exponentially recognized as capable of microorganisms' identification. The method relies upon the quality of the databases, that are constantly being improved. Nevertheless, most of the databases are biased to clinical or food samples, while information on environmental microorganisms is lacking.

Therefore, Ribopeaks a newly developed database based on artificial intelligence was evaluated to conduct this work.

As main result, it was confirmed the possibility to use the Ribopeaks database to assess the bacterial community diversity around sea cages, and how it could vary between seasons. Furthermore, the present results demonstrate another possible utilization of the MALDI-TOF method, as a method for the determination of the overall bacterial community in superficial waters. The overall bacterial community was qualitatively accessed and the most common were in decreasing order: *Vibrio*> *Pseudoalteromonas*> *Xanthomonas*> *Alteromonas*> *Staphylococcus*> *Escherichia*> *Pseudomonas*> *Photobacterium*> *Acinetobacter*.

Also, the proximity to anthropogenic sources of nutritional inputs (*e.g.*: streams and wastewater treatment plants) could alter the community diversity leading to constraints in cages management. However, the good positioning of the structures and the exposure to regular sea currents, rapidly disperse the excess nutrients, decreasing the frequency of episodic occurrences.

Due to the MALDI-TOF approach, applied to bacterial community analysis being a recent application of the methodology, future work will be needed to confirm these results with quantitative analysis or DNA sequencing. On the other hand, MALDI-TOF analysis has a clear potential to correspond to the requirements stipulated at the beginning of the study, cost effective, fast, and suitable for routine analysis of seawater bacterial community. These types of analysis are becoming more relevant to the development of environmental safety protocols in the aquaculture industry.

In conclusion, the integrated multi-disciplinary approach used in this project was very useful in the improvement of the company knowledge about its products and management procedures. It also contributes with indications on how to address relevant issues on the aquaculture sector, such as traceability, safety, and freshness, from the methodologies that can be used to how to gather and treat the data. It highlights the importance of demonstration of compliance and validity, as a strategy to become more prepared for the adaptations needed, from the upcoming EU and governmental regulations.

Annexes

These annexes contain the supplementary material, referred in this thesis. They are part of the published material, as external links in the journal web pages.

Analytical Quality by Design approach for determination of fish spoilage related volatile amines

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SUPPLEMENTARY MATERIAL

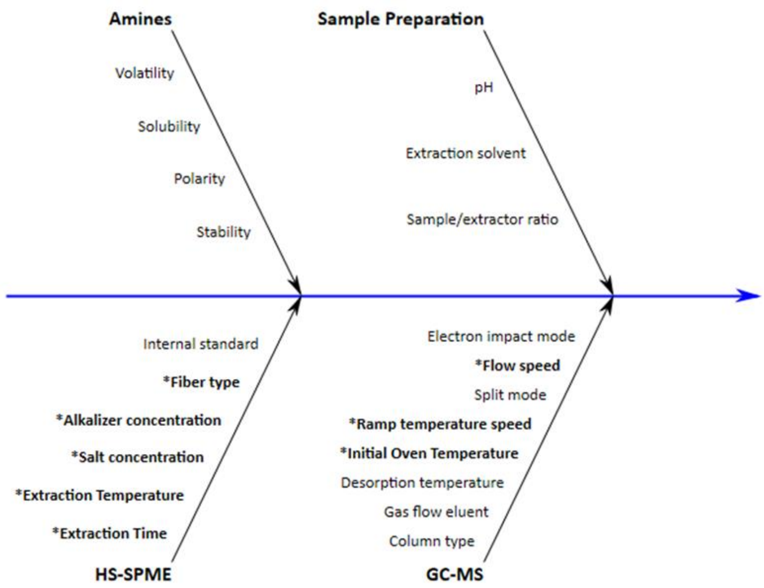

Fig.1 SM - Ishikawa diagram of the selected parameters for quality risk assessment (QRA).

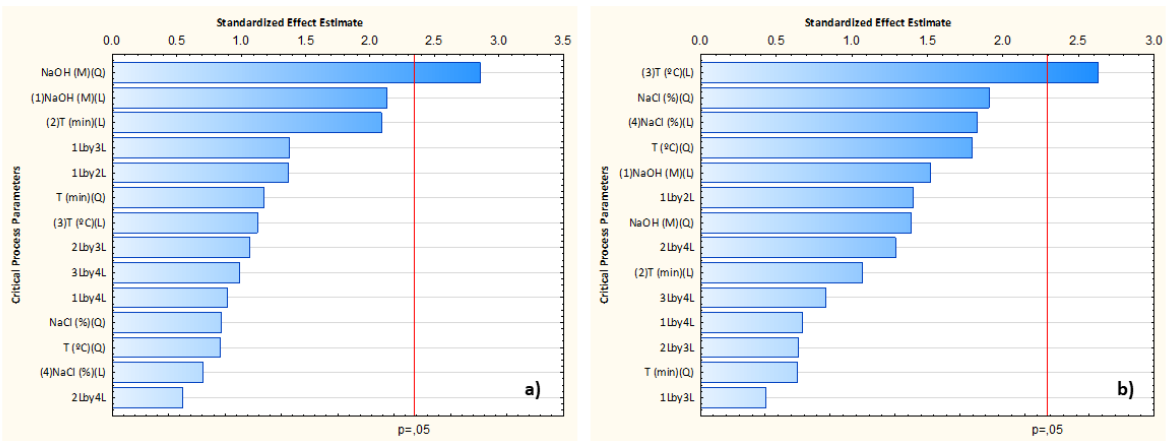

Fig. 2 SM - Pareto chart analysis of the effects between the studied CMPs on the TPA and IP response, for HS-SPME$_{(DVB/CAR/PDMS)}$.

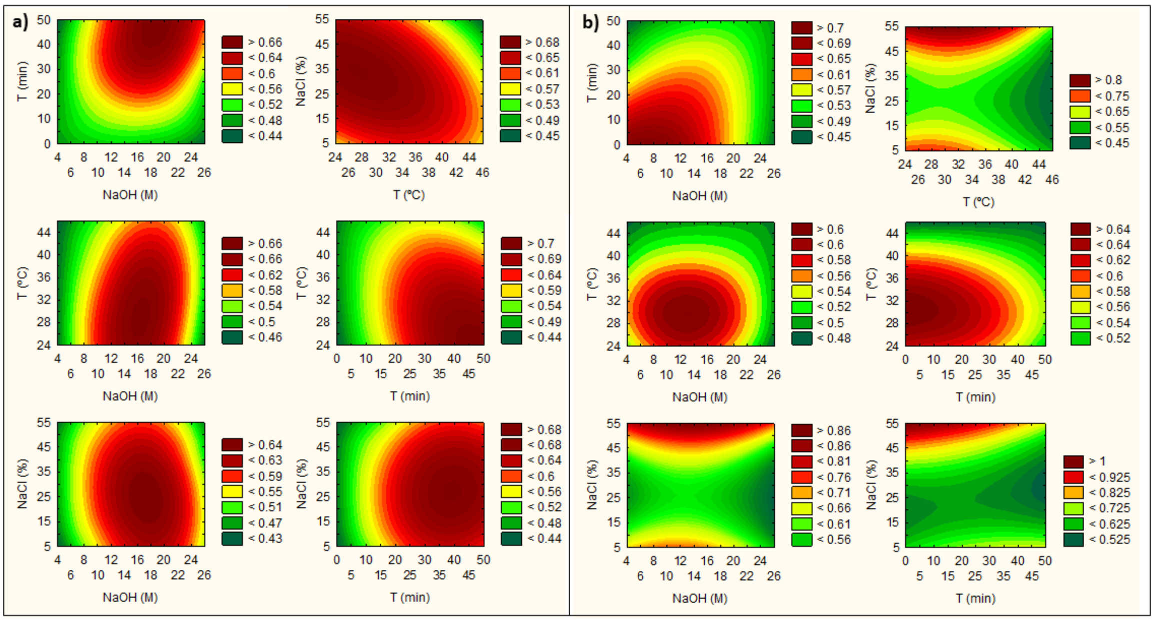

Fig. 3 SM – Desirability surface contour method analysis. **a)** shows the effects of all CMPs on TPA **b)** shows the effects of all CMPs on IP.

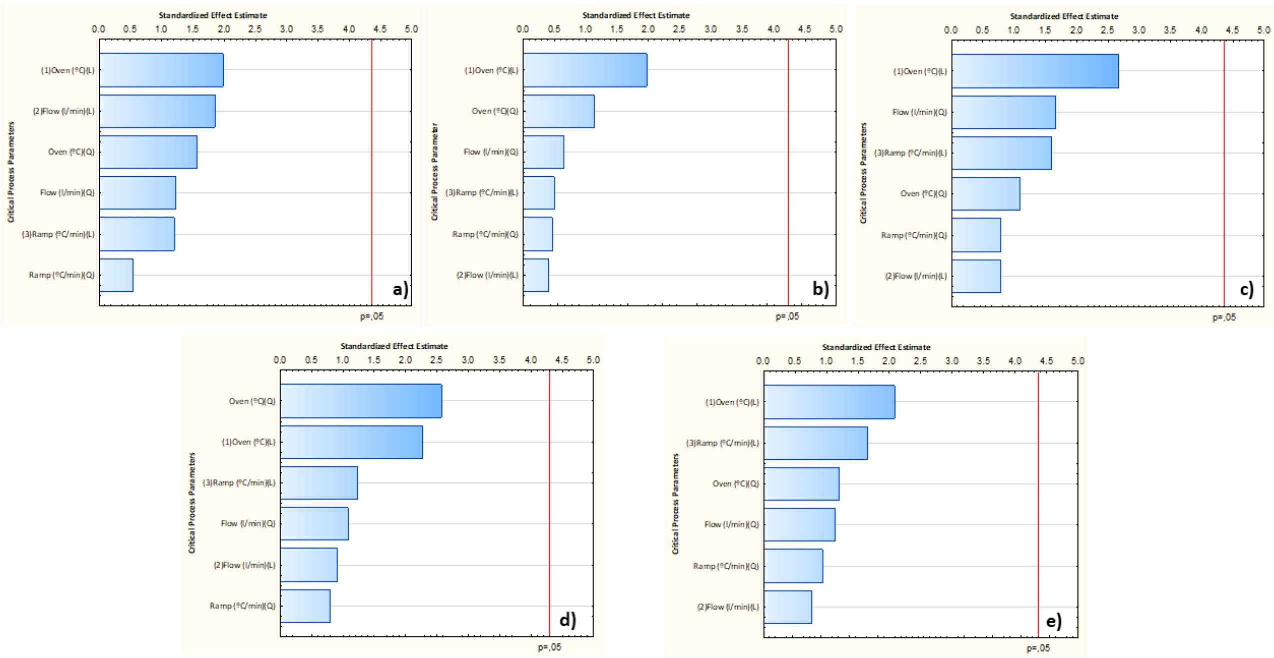

Fig. 4 SM - The Pareto ranking analysis for CG-MS$_{(BP20)}$. **a)** Total peak area; **b)** Intermediate precision; **c)** Peak resolution; **d)** Tailing factor; **e)** Symmetry factor.

Table 1 SM. Advantages and disadvantages of chemical methods used for fish freshness determination (Howgate 2010).

Method	Advantages	Disadvantages
Direct distillation (L&G)	-	Ammonia formation under unstandardized conditions. Total recovery of DMA is not achieved.
The Picrate Method	-	None of the alkali/formaldehyde combinations completely suppressed the interference of DMA.
Distillation with Formaldehyde	-	The procedure is not specific for TMA. Over estimation of the content (20%). Ammonia is not completely fixed.
Use of muscle extracts	-	Lack of uniformity in calculating the TVB concentration in the muscle tissue. Over estimation (7%) of TVB-N, is common.
Steam distillation (Khejdal)	Semi-automatic and fast.	-
Distillation under reduced pressure	Decomposition of nitrogen-containing substances during direct or steam distillation can be avoided.	Requires a complex apparatus. Time taken to dismantle and reassemble between analyses.
The Microdiffusion (Conway) Method	Distillation at room temperature. Simplicity of procedure and low cost.	Careful execution for accurate and precise results. Scrupulous cleaning of the cells between analyses. Care needed to avoid air bubbles.
Flow Injection Analysis (FIA)	Distillation at room temperature. Measure the intrinsic TVB content and specific amines.	Practical difficulties with the procedure. Moderately large discrepancies among results between laboratories.
Gas Liquid Chromatography	The analysis of headspace avoids the need for a distillation or extraction step.	Tailing of the amine's pikes. Column packing effect.

Table 2 SM. Reported works in literature regarding TMA and/or DMA analysis.

Gas-Chromatography						HS-SPME				
Detector	**GC-column**	**Temperature (ºC)**	**Ramp (ºC/min)**	**Flow (L/min)**	**Split mode**	**Fiber**	**Extr. Time (min)**	**Extr. Temperature (ºC)**	**Alkalizer agent**	**Reference**
MS	DB1 (60 m × 0.32 mm, 5 µm, Agilent)	80/230	15	-	Splitless	DVB/PDMS	10	22	Ammonia	(Wzorek et al., 2010)
MS	Stabilwax DB (30 m× 0.25 mm× 0.25 µm, Restek)	60/210	15	He 1.2	Split 5	CAR/PDMS	35	30	KOH	(Dehaut et al., 2016)
MS	PTA-5 column. (30 m × 0.25 mm i.d.)	35/200	40	He 1.3	Splitless	DVB/CAR/PDMS CW/DVB DVB/PDMS PDMS	5	25	NaOH 15%	(Chan et al., 2006)
FID	Rtx®-VolatileAmine (30 m x 0.32 mm I.D.)	40/120	10	He 1.3	Split 1:25	DVB/CAR/PDMS	15	21	NaOH 15%	(Barbosa-Pereira et al., 2012)
FID	SE-54 (25m × 0.32mm i.d.)	60/230	25	N 12-15	Split 1:100	PDMS-DVB PA Amide bridge-C4	-	-	-	(Li et al., 2004)
FID	-	-	-	He 2.0 H 25.0	-	DVB/PDMS	2	35	NaOH 40%	(Béné et al., 2001)

Table 3 SM. Scheme of the Quality Index Method (QIM) proposed for *Sparus aurata* (*GSB*). It is adapted from the work of Huidobro et al 2010 [34].

Attribute	Parameter	Description	
Appearance	Skin	Very bright, iridescent	0
		Loss of iridescence and bright	1
		Pale, dull	2
	Odor	Sea, seaweed, seafood	0
		Neutral	1
		Rancid, metallic	2
		Putrid	3
Texture	Firmness	Rigor-mortis	0
		Firm, recovers shape fast (≤ 2 sec.)	1
		Less firm, softness, recovers shape slow (> 2 sec.)	2
		Clearly marked; spongy	3
Eyes	Color	Black; shinny	0
		Black opaque/ slightly milky	1
		Greyish. Distinguishable eyeball	2
		White/ grey eyeball defined	3
	Shape	Convex	0
		Flat	1
		Concave	2
Gills	Color	Dark red	0
		Light red	1
		Brownish red	2
		Brown, grey, discolored, bacteria present	3
	Mucus	Absent	0
		Present, colorless	1
		Whitish/cream	2
		Abundant, brown, yellowish	3
	Odor	Sea, seaweed, seafood	0
		Neutral	1
		Rancid, metallic	2
		Putrid	3
Total Demerit Points			**0-22**

Table 4 SM Results from DoE 3^{4-1} level, for HS-SPME Knowledge of space, for all tested fibres.

Run	Time (min)	NaOH (M)	Temp. (ºC)	NaCl (%)	DVB/CAR/PDMS						CAR/PDMS						PDMS/DVB					
					TMA		DMA		Total		TMA		DMA		Total		TMA		DMA		Total	
					TPA ($x10^7$)	IP (%)	TPA ($x10^7$)	IP (%)	TPA ($x10^7$)	Mean (%)	TPA ($x10^7$)	IP (%)	TPA ($x10^7$)	IP (%)	TPA ($x10^7$)	Mean (%)	TPA ($x10^7$)	IP (%)	TPA ($x10^7$)	IP (%)	TPA ($x10^7$)	Mean (%)
RUN1	5	5	25	10	5.63	1.3	1.69	9.0	7.32	5.1	2.01	1.9	0.49	0.1	2.50	1.0	4.73	13.3	4.93	12.7	5.23	13.0
RUN2	5	5	35	50	8.49	4.2	1.86	0.3	10.3	2.3	2.35	8.7	0.62	9.1	2.97	8.9	4.77	12.2	1.29	9.8	6.07	11.0
RUN3	5	5	45	30	5.25	6.5	2.75	12.2	8.00	9.4	3.42	6.1	0.29	5.7	3.72	5.9	1.36	9.1	0.52	4.6	1.88	6.8
RUN4	5	15	25	50	9.74	3.9	2.45	2.5	12.2	3.2	4.87	10.0	1.31	2.1	6.18	6.0	2.94	14.4	1.56	10.6	4.51	12.5
RUN5	5	15	35	30	8.16	5.0	6.07	6.7	14.2	5.8	1.91	9.6	0.88	12.6	2.79	11.1	2.93	10.8	2.04	14.8	4.97	12.8
RUN6	5	15	45	10	8.80	11.0	8.18	5.7	17.0	8.3	5.10	2.9	1.78	11.8	6.88	7.4	1.56	11.5	1.66	13.8	3.23	12.7
RUN7	5	25	25	30	5.59	6.9	1.43	6.9	7.02	6.9	1.74	10.8	0.65	6.6	2.39	8.7	5.55	12.6	3.79	9.6	9.35	11.1
RUN8	5	25	35	10	6.46	12.5	3.41	5.0	9.86	8.7	3.60	8.0	1.38	10.4	4.98	9.2	1.52	9.5	1.90	12.6	3.42	11.0
RUN9	5	25	45	50	6.90	12.5	6.89	4.8	13.8	8.6	2.58	10.0	2.52	13.4	5.10	11.7	3.58	4.5	3.17	14.0	6.74	9.3
RUN10	25	5	25	50	7.54	2.4	3.12	8.1	10.7	5.2	3.60	3.8	1.54	11.0	5.14	7.4	2.71	13.8	2.59	7.9	5.29	10.8
RUN11	25	5	35	30	12.4	1.5	5.88	6.6	18.2	4.1	2.13	2.9	1.54	6.7	3.68	4.8	2.57	1.4	2.73	1.0	5.30	1.2
RUN12	25	5	45	10	1.95	7.7	2.28	12.2	4.23	9.9	1.16	11.7	0.74	3.1	1.90	7.4	2.07	0.7	2.59	11.2	4.67	5.9
RUN13	25	15	25	30	21.6	10.2	10.9	4.7	32.4	7.4	3.92	7.3	2.26	11.1	6.19	9.2	5.39	12.4	3.30	2.8	8.69	7.6
RUN14	25	15	35	10	15.0	5.4	8.87	6.8	23.9	6.1	2.83	0.8	1.15	3.6	3.97	2.2	0.82	6.5	0.67	0.0	1.49	3.3
RUN15	25	15	45	50	3.66	1.9	4.33	1.5	8.00	1.7	15.8	7.8	5.25	13.1	21.1	10.5	4.88	12.3	4.03	4.7	8.91	8.5
RUN16	25	25	25	10	11.3	8.2	5.20	7.2	16.5	7.7	9.84	8.0	6.83	4.6	16.7	6.3	3.69	15.4	3.12	9.8	6.80	12.6
RUN17	25	25	35	50	9.74	6.4	6.79	10.4	16.5	8.4	6.25	6.5	4.50	11.5	10.8	9.0	6.39	6.9	5.13	11.3	11.5	9.1
RUN18	25	25	45	30	8.15	3.6	6.88	15.0	15.0	9.3	15.3	5.9	7.58	9.5	22.9	7.7	2.47	11.0	2.76	5.6	5.22	8.3
RUN19	45	5	25	30	7.95	5.7	4.49	10.7	12.4	8.2	3.99	11.9	1.61	8.4	5.60	10.1	3.91	1.2	1.93	0.4	5.84	0.8
RUN20	45	5	35	10	4.20	4.9	3.63	3.2	7.83	4.1	0.98	11.2	0.79	1.8	1.78	6.5	2.10	0.9	2.46	11.0	4.56	6.0
RUN21	45	5	45	50	2.45	10.8	2.34	9.2	4.78	10.0	10.4	4.4	4.85	0.6	15.3	2.5	1.10	12.0	1.49	12.2	2.59	12.1
RUN22	45	15	25	10	9.85	6.3	10.2	4.3	20.0	5.3	7.59	5.9	3.21	2.2	10.8	4.1	6.60	5.9	5.15	1.7	11.7	3.8
RUN23	45	15	35	50	18.2	6.5	9.01	3.2	27.2	4.9	7.79	12.6	5.56	12.5	13.3	12.5	4.68	4.8	3.35	9.8	8.03	7.3
RUN24	45	15	45	30	11.9	14.3	8.94	10.1	20.9	12.2	18.9	8.1	7.02	5.3	26.0	6.7	2.28	10.2	2.20	6.8	4.48	8.5
RUN25	45	25	25	50	12.2	4.9	8.58	3.2	20.8	4.0	9.31	0.1	5.47	5.8	14.8	2.9	2.81	6.4	3.18	10.4	5.99	8.4
RUN26	45	25	35	30	4.60	6.7	6.66	9.2	11.3	7.9	8.84	5.6	4.67	12.8	13.5	9.2	2.36	3.3	2.78	7.2	5.13	5.2
RUN27	45	25	45	10	14.9	3.8	11.2	14.7	26.1	9.2	18.4	6.1	1.94	11.7	20.3	8.9	1.82	12.9	1.27	3.5	3.09	8.2

Table 5 SM The HS-SPME/MODR results for the observed vs predicted values agreement.

	Total Peak Area ($x10^8$)							Intermediate Precision (% RSD)			
	Observed 1	**Observed 2**	**Observed 3**	**Predicted**	**C.I. -95%**	**C.I. + 95%**		**Observed**	**Predicted**	**C.I. -95%**	**C.I. + 95%**
TMA	2.31	2.00	2.16	1.47	0.88	2.07	**TMA**	10.2	5.2	0.9	9.6
DMA	1.13	1.05	1.09	0.79	0.55	1.04	**DMA**	4.7	7.0	3.3	10.8
Sum	3.44	3.05	3.24	2.26	1.45	3.07	**Mean**	7.4	6.1	3.2	9.1

Table 6 SM. GC-MS MODR results, from DOE fractional factorial design 3^{3-1} level.

Run	Oven (ºC)	Flow (L/min)	Ramp (ºC/min)	TMA					DMA					Total				
				TPA ($x10^7$)	**IP (%)**	**PR**	**TF**	**SF.**	**TPA ($x10^7$)**	**IP (%)**	**PR**	**TF**	**SF.**	**TPA ($x10^7$)**	**IP (%)**	**PR**	**TF**	**SF**
RUN1	35	0.8	40	1.48	9.9	7.0	0.77	0.50	6.82	13.7	3.3	0.58	2.13	8.29	11.8	5.17	0.68	1.32
RUN2	35	1.0	60	1.71	13.8	9.2	0.81	0.60	1.48	15.0	26.1	0.07	0.21	3.20	14.4	17.6	0.44	0.41
RUN3	35	1.3	50	2.36	9.3	13.1	0.72	0.35	13.8	12.4	3.7	0.54	2.11	16.2	10.8	8.39	0.63	1.23
RUN4	60	0.8	60	1.62	1.6	4.8	0.93	0.85	5.73	14.5	4.0	0.73	0.33	7.35	8.1	4.41	0.83	0.59
RUN5	60	1.0	50	2.15	12.5	4.7	0.94	0.82	6.07	9.4	4.3	0.74	0.36	8.22	10.9	4.50	0.84	0.59
RUN6	60	1.3	40	2.02	8.2	4.8	0.99	0.86	5.64	6.6	4.9	0.76	0.38	7.66	7.4	4.83	0.87	0.62
RUN7	85	0.8	50	5.05	11.1	3.2	0.84	0.59	7.44	10.3	2.2	0.63	0.16	12.5	10.7	2.69	0.74	0.38
RUN8	85	1.0	40	6.44	7.7	3.0	0.92	0.75	7.57	8.0	2.2	0.60	2.15	14.0	7.8	2.64	0.76	1.45
RUN9	85	1.3	60	17.6	8.4	2.9	0.80	0.47	19.3	8.9	2.1	0.58	0.04	36.9	8.6	2.53	0.69	0.25

Table 7 SM. GC-MS /MODR observed vs predicted values analysis.

	Observed	Predicted	C.I. -95%	C.I. + 95%
TPA	1.40E+08	1.42E+08	-1.87E+08	4.71E+08
IP	7.83	8.99	2.28	15.69
PR	2.64	3.37	-11.46	18.19
TF	0.72	0.70	0.40	1.00
RS	0.57	0.40	-0.99	1.80

Table 8 SM. Fractional Factorial Design 3^{3-1} level, for HS-SPME$_{(DVB/CAR/PDMS)}$ robustness analysis.

Run	Temperature (°C)	NaOH (M)	Time (min)	TMA ($x10^8$)	IP (%)	DMA ($x10^8$)	IP (%)	Total ($x10^8$)	Mean (%)
Run1	33.0	14.0	28.0	1.73	11.9	3.86	10.0	5.59	11.0
Run2	33.0	15.0	32.0	2.42	9.2	4.35	5.6	6.77	7.4
Run3	33.0	16.0	30.0	2.19	7.3	4.83	4.0	7.02	5.7
Run4	35.0	14.0	32.0	2.34	7.6	3.51	10.6	5.85	9.1
Run5	35.0	15.0	30.0	2.34	2.3	4.71	1.1	7.04	1.7
Run6	35.0	16.0	28.0	2.43	4.6	4.52	9.6	6.95	7.1
Run7	37.0	14.0	30.0	2.26	8.0	4.61	9.4	6.87	8.7
Run8	37.0	15.0	28.0	1.80	5.8	3.56	7.0	5.35	6.4
Run9	37.0	16.0	32.0	1.69	11.4	3.51	8.6	5.20	10.0

Table 9 SM. GC-MS Robustness results, from DOE fractional factorial design 3^{3-1} level.

Run	Oven (ºC)	Flow (L/min)	Ramp (ºC/min)	TMA					DMA					Total				
				TPA ($x10^8$)	IP (%)	PR	TF	SF.	TPA ($x10^8$)	IP (%)	PR	TF	SF.	TPA ($x10^8$)	IP (%)	PR	TF	SF
RUN1	75	0.8	45	2.64	6.5	2.9	0.83	2.63	3.93	5.3	1.9	0.59	2.27	6.57	5.9	2.4	0.71	2.45
RUN2	75	1	55	2.32	2.1	2.2	0.74	2.61	4.88	3.0	2.2	0.56	2.23	7.19	2.5	2.2	0.65	2.42
RUN3	75	1.2	50	1.82	1.2	3.0	0.76	2.56	3.58	2.7	3.0	0.55	2.22	5.40	2.0	3.0	0.66	2.39
RUN4	80	0.8	55	2.64	6.4	2.0	0.76	2.48	4.83	5.0	2.0	0.58	2.26	7.47	5.7	2.0	0.67	2.37
RUN5	80	1	50	2.19	3.4	2.3	0.78	2.55	3.80	5.7	2.3	0.56	2.24	5.99	4.6	2.3	0.67	2.39
RUN6	80	1.2	45	2.12	8.7	2.5	0.76	2.57	3.97	4.7	2.5	0.55	2.23	6.09	6.7	2.5	0.66	2.40
RUN7	85	0.8	50	2.75	3.3	1.7	0.81	2.77	4.67	15.6	1.7	0.58	2.26	7.42	9.5	1.7	0.70	2.52
RUN8	85	1	45	2.58	7.6	2.0	0.76	2.53	3.79	9.3	2.0	0.55	2.23	6.36	8.4	2.0	0.66	2.38
RUN9	85	1.2	55	1.88	2.9	2.2	0.79	2.81	3.47	4.5	2.2	0.56	2.23	5.36	3.7	2.2	0.68	2.52

Table 10 SM. Analysis of agreement between observed and predicted values for HS-SPME robustness.

	Total Peak Area (x10^8)					Intermediate Precision (% RSD)			
	Observed	Predicted	*C.I. - 95%	*C.I. + 95%		Observed	Predicted	*C.I. - 95%	*C.I. + 95%
TMA	2.34	2.55	1.06	4.04	TMA	2.3	1.3	-4.3	6.9
DMA	4.71	4.84	2.95	6.74	DMA	1.1	1.9	-3.6	7.3
Total	7.04	7.39	4.12	10.7	Mean	1.7	1.6	-0.7	3.9

***C.I.** – Confidence Interval

Table 11 SM. GC-MS robustness evaluation by, observed vs predicted values analysis.

	Observed	Predicted	C.I. -95%	C.I. + 95%
TPA	5.99E+08	6.45E+08	3.39E+08	9.51E+08
IP	4.56	5.28	0.31	10.2
PR	2.27	2.21	1.48	2.94
TF	0.67	0.66	0.58	0.74
RS	2.39	2.36	2.10	2.63

Table 12 SM. Attributes evaluation for QIM analysis.

Days in ice	Skin	Odor	Anus	Firmness	Eye color	Eye shape	Gill color	Gill mucus	Gill odor	Q.I
0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.0	0.0	0.5
1	0.2	0.2	0.3	0.3	0.0	0.2	0.2	0.2	0.5	2.1
3	0.8	1.0	0.0	1.0	0.5	0.5	1.3	0.8	1.0	6.8
5	1.5	1.2	0.0	1.0	1.0	1.2	1.5	1.3	1.2	9.8
7	1.7	1.4	1.2	1.6	1.1	1.5	1.8	1.4	1.6	13.2
9	2.0	1.0	0.0	2.0	2.0	2.0	3.0	2.0	2.0	16.0
12	2.0	2.0	1.0	2.0	2.0	2.0	2.5	2.5	3.0	19.0
14	2.1	2.3	2.2	2.3	2.2	2.3	2.9	2.8	2.9	21.9

Improved approach based on MALDI-TOFMS for establishment of the fish mucus protein pattern for geographic discrimination of *Sparus aurata*

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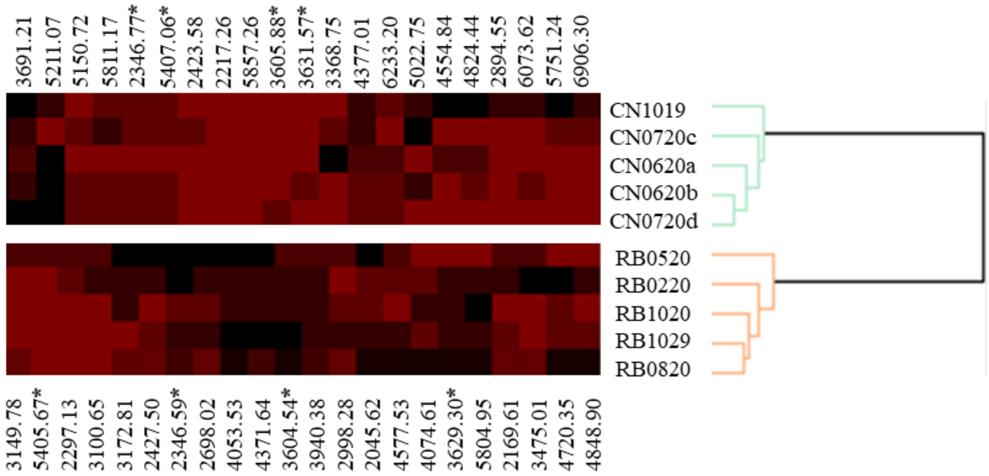
SUPPLEMENTARY MATERIAL

Supplementary figure 1



Supplementary Table 1 – Principal Component Analysis results, regarding Ribeira Brava samples (RB).

	PC0	PC1	PC2
Eigenvalue	190.09	68.11	62.70
Variance	0.31	0.11	0.10
Cumulative	0.31	0.42	0.53
ID			
RB_arrival (a)	13.22	-6.93	-7.08
RB_arrival (b)	8.76	-7.24	-1.96
RB_arrival (c)	15.35	-1.73	5.10
RB_3M (a)	-0.52	-3.87	-0.90
RB_3M (c)	0.79	-4.50	-1.59
RB_3M (b)	0.62	-3.21	-0.09
RB_3M (d)	-0.64	-3.32	-0.72
RB_6M (d)	-1.22	0.83	-1.60
RB_6M (b)	-1.30	0.35	-1.59
RB_6M (a)	-1.00	0.93	-1.89
RB_12M_1019	0.18	-9.07	1.56
RB_12M_0220	0.69	-8.87	1.22
RB_12M_0820	0.06	-7.62	1.15
RB_12M_1020	-0.43	-7.26	1.78
RB_12M_0520	1.28	-9.80	3.42

Supplementary Table 2 – Principal Component Analysis results, regarding Cainçal samples (CN).

	PC0	PC1	PC2
Eigenvalue	97.38	76.85	59.49
Variance	0.25	0.20	0.15
Cumulative	0.25	0.45	0.60
ID			
CN_arrival	11.54	-0.10	2.20
CN_3M (a)	2.91	-9.16	-0.94
CN_3M (b)	3.58	-10.94	-0.54
CN_12M_0720 (b)	0.73	-3.72	5.77
CN_12M_0620 (a)	0.84	-4.57	6.97
CN_12M_0620 (c)	0.90	-5.36	6.70
CN_12M_1019	2.63	-6.81	6.46
CN_12M_0720 (d)	-0.19	-2.30	5.03
CN_6M (a)	-2.39	-0.12	-1.13
CN_6M (b)	-3.01	0.16	-0.82

Supplementary Table 3 – Principal Component Analysis results, regarding locations analysis.

	PC0	PC1	PC2
Eigenvalue	395.38	63.01	60.06
Variance	0.51	0.08	0.08
Cumulative	0.51	0.59	0.67
ID			
CN_12M_0720 (b)	-8.49	-1.12	0.03
CN_12M_0620 (a)	-8.53	-0.22	0.05
CN_12M_0620 (c)	-8.80	-0.20	0.05
CN_12M_1019	-9.76	-8.64	-0.13
CN_12M_0720 (d)	-6.55	4.17	0.13
RB_12M_1019	10.70	-2.27	-2.02
RB_12M_0220	10.93	-2.89	-3.96
RB_12M_0820	9.84	-1.45	-2.05
RB_12M_1020	9.34	-1.40	-0.59
RB_12M_0520	10.51	-2.54	7.85

Supplementary Table 4 – Discriminant peaks list, for CN and Rb location.

Peak	p-value	q-value	CN_12M0720 (b)	CN_12M0620 (a)	CN_12M0620 (c)	CN_12M1019	CN_12M0720 (d)	RB_12M1019	RB_12M0220	RB_12M0820	RB_12M1020	RB_12M0520
2045.622	0.0079	0.0988	0.0	0.0	0.0	0.0	0.0	66.7	83.3	57.1	83.3	50.0
2169.612	0.0079	0.0988	0.0	0.0	0.0	0.0	0.0	83.3	66.7	85.7	100.0	75.0
2217.257	0.0079	0.0988	100.0	100.0	100.0	100.0	100.0	0.0	0.0	0.0	0.0	0.0
2297.135	0.0079	0.0988	0.0	0.0	0.0	0.0	0.0	100.0	83.3	100.0	100.0	75.0
2346.593	0.0079	0.0988	0.0	0.0	0.0	0.0	0.0	66.7	50.0	71.4	83.3	50.0
2346.768	0.0079	0.0988	83.3	100.0	83.3	83.3	83.3	0.0	0.0	0.0	0.0	0.0
2423.582	0.0079	0.0988	83.3	100.0	100.0	100.0	100.0	0.0	0.0	0.0	0.0	0.0
2427.502	0.0079	0.0988	0.0	0.0	0.0	0.0	0.0	83.3	66.7	85.7	100.0	50.0
2698.017	0.0079	0.0988	0.0	0.0	0.0	0.0	0.0	66.7	66.7	85.7	83.3	50.0
2894.546	0.0079	0.0988	100.0	100.0	100.0	66.7	100.0	0.0	0.0	0.0	0.0	0.0
2998.277	0.0079	0.0988	0.0	0.0	0.0	0.0	0.0	66.7	100.0	85.7	83.3	75.0
3100.648	0.0079	0.0988	0.0	0.0	0.0	0.0	0.0	100.0	66.7	100.0	100.0	75.0
3149.776	0.0079	0.0988	0.0	0.0	0.0	0.0	0.0	100.0	100.0	85.7	100.0	75.0
3172.809	0.0079	0.0988	0.0	0.0	0.0	0.0	0.0	100.0	66.7	85.7	66.7	50.0
3368.745	0.0079	0.0988	83.3	50.0	100.0	100.0	100.0	0.0	0.0	0.0	0.0	0.0
3475.013	0.0079	0.0988	0.0	0.0	0.0	0.0	0.0	100.0	50.0	85.7	100.0	75.0
3604.541	0.0079	0.0988	0.0	0.0	0.0	0.0	0.0	50.0	66.7	57.1	66.7	75.0
3605.88	0.0079	0.0988	100.0	100.0	100.0	100.0	83.3	0.0	0.0	0.0	0.0	0.0
3629.296	0.0079	0.0988	0.0	0.0	0.0	0.0	0.0	66.7	66.7	57.1	66.7	100.0
3631.568	0.0079	0.0988	100.0	100.0	83.3	100.0	100.0	0.0	0.0	0.0	0.0	0.0
3691.209	0.0079	0.0988	66.7	75.0	66.7	50.0	50.0	0.0	0.0	0.0	0.0	0.0
3940.376	0.0079	0.0988	0.0	0.0	0.0	0.0	0.0	66.7	66.7	71.4	66.7	75.0
4053.528	0.0079	0.0988	0.0	0.0	0.0	0.0	0.0	50.0	66.7	57.1	66.7	50.0
4074.609	0.0079	0.0988	0.0	0.0	0.0	0.0	0.0	83.3	83.3	57.1	66.7	100.0
4371.639	0.0079	0.0988	0.0	0.0	0.0	0.0	0.0	50.0	66.7	71.4	66.7	50.0
4377.014	0.0079	0.0988	66.7	75.0	83.3	66.7	83.3	0.0	0.0	0.0	0.0	0.0
4554.835	0.0079	0.0988	100.0	75.0	100.0	50.0	100.0	0.0	0.0	0.0	0.0	0.0
4577.531	0.0079	0.0988	0.0	0.0	0.0	0.0	0.0	66.7	83.3	57.1	100.0	75.0
4720.355	0.0079	0.0988	0.0	0.0	0.0	0.0	0.0	83.3	50.0	57.1	83.3	100.0
4824.44	0.0079	0.0988	100.0	75.0	83.3	50.0	100.0	0.0	0.0	0.0	0.0	0.0
4848.9	0.0079	0.0988	0.0	0.0	0.0	0.0	0.0	83.3	66.7	57.1	100.0	100.0

5022.752	0.0079	0.0988	50.0	100.0	66.7	66.7	100.0	0.0	0.0	0.0	0.0	0.0
5150.721	0.0079	0.0988	83.3	100.0	83.3	100.0	83.3	0.0	0.0	0.0	0.0	0.0
5211.073	0.0079	0.0988	100.0	50.0	50.0	66.7	50.0	0.0	0.0	0.0	0.0	0.0
5405.668	0.0079	0.0988	0.0	0.0	0.0	0.0	0.0	100.0	100.0	100.0	100.0	75.0
5407.056	0.0079	0.0988	83.3	100.0	83.3	83.3	83.3	0.0	0.0	0.0	0.0	0.0
5751.244	0.0079	0.0988	83.3	100.0	100.0	50.0	100.0	0.0	0.0	0.0	0.0	0.0
5804.951	0.0079	0.0988	0.0	0.0	0.0	0.0	0.0	66.7	66.7	57.1	50.0	100.0
5811.17	0.0079	0.0988	66.7	100.0	83.3	83.3	83.3	0.0	0.0	0.0	0.0	0.0
5857.26	0.0079	0.0988	100.0	100.0	100.0	100.0	100.0	0.0	0.0	0.0	0.0	0.0
6073.618	0.0079	0.0988	100.0	100.0	83.3	66.7	100.0	0.0	0.0	0.0	0.0	0.0
6233.197	0.0079	0.0988	100.0	75.0	83.3	83.3	83.3	0.0	0.0	0.0	0.0	0.0
6906.301	0.0079	0.0988	83.3	100.0	100.0	66.7	100.0	0.0	0.0	0.0	0.0	0.0
2116.548	0.0079	0.0988	0.0	0.0	0.0	0.0	0.0	100.0	33.3	100.0	83.3	50.0
2280.066	0.0079	0.0988	83.3	50.0	100.0	33.3	100.0	0.0	0.0	0.0	0.0	0.0
2626.618	0.0079	0.0988	0.0	0.0	0.0	0.0	0.0	83.3	83.3	42.9	100.0	75.0
2648.67	0.0079	0.0988	0.0	0.0	0.0	0.0	0.0	83.3	66.7	42.9	100.0	75.0
2669.625	0.0079	0.0988	100.0	100.0	100.0	33.3	100.0	0.0	0.0	0.0	0.0	0.0
2998.019	0.0079	0.0988	100.0	50.0	50.0	66.7	33.3	0.0	0.0	0.0	0.0	0.0
3228.632	0.0079	0.0988	100.0	100.0	100.0	33.3	83.3	0.0	0.0	0.0	0.0	0.0
3860.551	0.0079	0.0988	33.3	75.0	50.0	66.7	66.7	0.0	0.0	0.0	0.0	0.0
3979.782	0.0079	0.0988	0.0	0.0	0.0	0.0	0.0	66.7	66.7	42.9	66.7	75.0
4159.716	0.0079	0.0988	66.7	100.0	33.3	66.7	100.0	0.0	0.0	0.0	0.0	0.0
4292.12	0.0079	0.0988	0.0	0.0	0.0	0.0	0.0	66.7	83.3	57.1	33.3	75.0
4420.584	0.0079	0.0988	0.0	0.0	0.0	0.0	0.0	66.7	100.0	42.9	50.0	75.0
4554.46	0.0079	0.0988	0.0	0.0	0.0	0.0	0.0	66.7	50.0	71.4	33.3	75.0
4822.149	0.0079	0.0988	0.0	0.0	0.0	0.0	0.0	50.0	83.3	71.4	33.3	75.0
4890.661	0.0079	0.0988	50.0	75.0	66.7	33.3	83.3	0.0	0.0	0.0	0.0	0.0
5578.881	0.0079	0.0988	0.0	0.0	0.0	0.0	0.0	66.7	100.0	42.9	66.7	50.0
6009.675	0.0079	0.0988	100.0	75.0	33.3	50.0	66.7	0.0	0.0	0.0	0.0	0.0
6333.073	0.0079	0.0988	0.0	0.0	0.0	0.0	0.0	50.0	33.3	57.1	50.0	100.0
6536.392	0.0079	0.0988	83.3	50.0	50.0	33.3	50.0	0.0	0.0	0.0	0.0	0.0
2368.755	0.0179	0.1915	0.0	0.0	0.0	0.0	0.0	33.3	83.3	57.1	33.3	50.0
4058.668	0.0179	0.1915	33.3	75.0	66.7	33.3	50.0	0.0	0.0	0.0	0.0	0.0
4206.636	0.0179	0.1915	0.0	0.0	0.0	0.0	0.0	33.3	83.3	42.9	83.3	75.0

4459.864	0.0179	0.1915	0.0	0.0	0.0	0.0	0.0	50.0	33.3	42.9	83.3	75.0
4689.113	0.0179	0.1915	0.0	0.0	0.0	0.0	0.0	33.3	50.0	42.9	66.7	75.0
5223.873	0.0179	0.1915	0.0	0.0	0.0	0.0	0.0	33.3	33.3	57.1	83.3	100.0
5305.857	0.0179	0.1915	33.3	50.0	33.3	66.7	50.0	0.0	0.0	0.0	0.0	0.0
6286.329	0.0179	0.1915	50.0	75.0	33.3	83.3	33.3	0.0	0.0	0.0	0.0	0.0
6289.283	0.0179	0.1915	0.0	0.0	0.0	0.0	0.0	50.0	33.3	42.9	83.3	100.0
6395.496	0.0179	0.1915	0.0	0.0	0.0	0.0	0.0	50.0	33.3	42.9	50.0	75.0
2026.674	0.0476	0.2283	66.7	50.0	66.7	66.7	0.0	0.0	0.0	0.0	0.0	0.0
2068.244	0.0476	0.2283	0.0	0.0	0.0	0.0	0.0	83.3	50.0	100.0	16.7	50.0
2081.657	0.0476	0.2283	100.0	50.0	66.7	50.0	0.0	0.0	0.0	0.0	0.0	0.0
2095.58	0.0476	0.2283	0.0	100.0	83.3	66.7	83.3	0.0	0.0	0.0	0.0	0.0
2115.031	0.0476	0.2283	83.3	100.0	83.3	83.3	0.0	0.0	0.0	0.0	0.0	0.0
2136.622	0.0476	0.2283	0.0	50.0	100.0	66.7	100.0	0.0	0.0	0.0	0.0	0.0
2156.957	0.0476	0.2283	100.0	100.0	100.0	0.0	100.0	0.0	0.0	0.0	0.0	0.0
2217.25	0.0476	0.2283	0.0	0.0	0.0	0.0	0.0	83.3	83.3	0.0	83.3	50.0
2300.986	0.0476	0.2283	100.0	100.0	83.3	0.0	100.0	0.0	0.0	0.0	0.0	0.0
2313.054	0.0476	0.2283	0.0	0.0	0.0	0.0	0.0	83.3	16.7	100.0	100.0	50.0
2407.191	0.0476	0.2283	0.0	0.0	0.0	0.0	0.0	50.0	66.7	0.0	100.0	50.0
2563.198	0.0476	0.2283	83.3	100.0	83.3	16.7	83.3	0.0	0.0	0.0	0.0	0.0
2627.134	0.0476	0.2283	83.3	100.0	100.0	83.3	16.7	0.0	0.0	0.0	0.0	0.0
2756.045	0.0476	0.2283	50.0	25.0	50.0	50.0	100.0	0.0	0.0	0.0	0.0	0.0
2869.608	0.0476	0.2283	100.0	100.0	66.7	50.0	0.0	0.0	0.0	0.0	0.0	0.0
3205.638	0.0476	0.2283	100.0	100.0	100.0	0.0	100.0	0.0	0.0	0.0	0.0	0.0
3209.161	0.0476	0.2283	0.0	0.0	0.0	0.0	0.0	66.7	66.7	85.7	100.0	25.0
3332.42	0.0476	0.2283	0.0	0.0	0.0	0.0	0.0	50.0	50.0	57.1	0.0	75.0
3555.254	0.0476	0.2283	0.0	75.0	66.7	50.0	66.7	0.0	0.0	0.0	0.0	0.0
3576.089	0.0476	0.2283	16.7	75.0	50.0	83.3	50.0	0.0	0.0	0.0	0.0	0.0
3689.644	0.0476	0.2283	0.0	0.0	0.0	0.0	0.0	66.7	16.7	57.1	50.0	50.0
3788.156	0.0476	0.2283	0.0	0.0	0.0	0.0	0.0	50.0	83.3	14.3	83.3	50.0
3810.467	0.0476	0.2283	66.7	75.0	50.0	0.0	66.7	0.0	0.0	0.0	0.0	0.0
3836.133	0.0476	0.2283	66.7	75.0	50.0	50.0	16.7	0.0	0.0	0.0	0.0	0.0
4040.097	0.0476	0.2283	66.7	25.0	66.7	50.0	66.7	0.0	0.0	0.0	0.0	0.0
4209.407	0.0476	0.2283	100.0	75.0	50.0	16.7	100.0	0.0	0.0	0.0	0.0	0.0
4245.978	0.0476	0.2283	66.7	50.0	100.0	16.7	66.7	0.0	0.0	0.0	0.0	0.0

4255.873	0.0476	0.2283	0.0	0.0	0.0	0.0	0.0	100.0	100.0	14.3	100.0	50.0
4325.414	0.0476	0.2283	0.0	0.0	0.0	0.0	0.0	100.0	50.0	0.0	50.0	50.0
4342.026	0.0476	0.2283	66.7	25.0	83.3	66.7	50.0	0.0	0.0	0.0	0.0	0.0
4576.919	0.0476	0.2283	50.0	75.0	66.7	66.7	0.0	0.0	0.0	0.0	0.0	0.0
4714.317	0.0476	0.2283	0.0	50.0	66.7	50.0	50.0	0.0	0.0	0.0	0.0	0.0
4751.698	0.0476	0.2283	50.0	50.0	66.7	0.0	83.3	0.0	0.0	0.0	0.0	0.0
4759.419	0.0476	0.2283	0.0	0.0	0.0	0.0	0.0	16.7	66.7	71.4	100.0	75.0
5149.24	0.0476	0.2283	0.0	0.0	0.0	0.0	0.0	83.3	50.0	100.0	100.0	0.0
5197.067	0.0476	0.2283	0.0	0.0	0.0	0.0	0.0	66.7	16.7	71.4	66.7	100.0
5627.579	0.0476	0.2283	0.0	0.0	0.0	0.0	0.0	66.7	50.0	14.3	83.3	75.0
5856.111	0.0476	0.2283	0.0	0.0	0.0	0.0	0.0	100.0	50.0	85.7	100.0	0.0
6222.82	0.0476	0.2283	0.0	0.0	0.0	0.0	0.0	83.3	100.0	100.0	0.0	50.0
6325.322	0.0476	0.2283	50.0	75.0	50.0	50.0	0.0	0.0	0.0	0.0	0.0	0.0
6370.412	0.0476	0.2283	66.7	50.0	50.0	100.0	16.7	0.0	0.0	0.0	0.0	0.0
6536.137	0.0476	0.2283	0.0	0.0	0.0	0.0	0.0	66.7	50.0	71.4	83.3	0.0
6648.655	0.0476	0.2283	66.7	50.0	50.0	66.7	0.0	0.0	0.0	0.0	0.0	0.0
8563.903	0.0476	0.2283	0.0	0.0	0.0	0.0	0.0	66.7	50.0	85.7	66.7	25.0
8567.63	0.0476	0.2283	66.7	100.0	83.3	16.7	100.0	0.0	0.0	0.0	0.0	0.0
2043.05	0.0476	0.2283	33.3	75.0	50.0	66.7	16.7	0.0	0.0	0.0	0.0	0.0
2081.976	0.0476	0.2283	0.0	0.0	0.0	0.0	0.0	33.3	83.3	28.6	66.7	50.0
2138.792	0.0476	0.2283	0.0	0.0	0.0	0.0	0.0	66.7	0.0	42.9	66.7	75.0
2384.246	0.0476	0.2283	100.0	75.0	50.0	0.0	33.3	0.0	0.0	0.0	0.0	0.0
2386.009	0.0476	0.2283	0.0	0.0	0.0	0.0	0.0	33.3	83.3	0.0	66.7	75.0
2547.932	0.0476	0.2283	0.0	0.0	0.0	0.0	0.0	16.7	33.3	100.0	66.7	75.0
2570.415	0.0476	0.2283	0.0	0.0	0.0	0.0	0.0	50.0	16.7	42.9	50.0	75.0
2733.663	0.0476	0.2283	0.0	0.0	0.0	0.0	0.0	33.3	50.0	0.0	50.0	75.0
2820.809	0.0476	0.2283	83.3	75.0	33.3	66.7	0.0	0.0	0.0	0.0	0.0	0.0
2823.338	0.0476	0.2283	0.0	0.0	0.0	0.0	0.0	66.7	33.3	85.7	83.3	25.0
2868.632	0.0476	0.2283	0.0	0.0	0.0	0.0	0.0	83.3	50.0	85.7	33.3	0.0
3076.673	0.0476	0.2283	0.0	0.0	0.0	0.0	0.0	33.3	0.0	71.4	50.0	50.0
3449.885	0.0476	0.2283	0.0	0.0	0.0	0.0	0.0	83.3	33.3	14.3	66.7	50.0
3499.511	0.0476	0.2283	16.7	100.0	33.3	50.0	50.0	0.0	0.0	0.0	0.0	0.0
3705.745	0.0476	0.2283	0.0	0.0	0.0	0.0	0.0	33.3	66.7	57.1	66.7	25.0
3787.462	0.0476	0.2283	83.3	100.0	66.7	33.3	0.0	0.0	0.0	0.0	0.0	0.0

3904.458	0.0476	0.2283	100.0	50.0	50.0	33.3	16.7	0.0	0.0	0.0	0.0	0.0
3979.368	0.0476	0.2283	83.3	75.0	50.0	33.3	0.0	0.0	0.0	0.0	0.0	0.0
4015.286	0.0476	0.2283	33.3	50.0	66.7	50.0	0.0	0.0	0.0	0.0	0.0	0.0
4159.821	0.0476	0.2283	0.0	0.0	0.0	0.0	0.0	16.7	50.0	57.1	33.3	75.0
4288.752	0.0476	0.2283	100.0	75.0	33.3	50.0	0.0	0.0	0.0	0.0	0.0	0.0
4403.25	0.0476	0.2283	33.3	25.0	66.7	66.7	50.0	0.0	0.0	0.0	0.0	0.0
4420.242	0.0476	0.2283	33.3	75.0	66.7	16.7	66.7	0.0	0.0	0.0	0.0	0.0
4459.574	0.0476	0.2283	0.0	75.0	50.0	83.3	33.3	0.0	0.0	0.0	0.0	0.0
4478.799	0.0476	0.2283	0.0	0.0	0.0	0.0	0.0	66.7	50.0	71.4	33.3	25.0
4485.357	0.0476	0.2283	100.0	25.0	50.0	33.3	100.0	0.0	0.0	0.0	0.0	0.0
4507.176	0.0476	0.2283	0.0	0.0	0.0	0.0	0.0	66.7	0.0	57.1	33.3	50.0
4604.288	0.0476	0.2283	66.7	50.0	16.7	33.3	50.0	0.0	0.0	0.0	0.0	0.0
4632.832	0.0476	0.2283	33.3	25.0	66.7	50.0	50.0	0.0	0.0	0.0	0.0	0.0
4659.142	0.0476	0.2283	0.0	0.0	0.0	0.0	0.0	83.3	50.0	42.9	83.3	25.0
4924.319	0.0476	0.2283	50.0	75.0	33.3	100.0	0.0	0.0	0.0	0.0	0.0	0.0
4967.627	0.0476	0.2283	0.0	0.0	0.0	0.0	0.0	83.3	33.3	0.0	100.0	50.0
5008.627	0.0476	0.2283	0.0	0.0	0.0	0.0	0.0	50.0	33.3	28.6	50.0	50.0
5025.812	0.0476	0.2283	0.0	0.0	0.0	0.0	0.0	33.3	66.7	57.1	83.3	0.0
5057.506	0.0476	0.2283	50.0	100.0	33.3	83.3	0.0	0.0	0.0	0.0	0.0	0.0
5083.68	0.0476	0.2283	33.3	25.0	50.0	66.7	50.0	0.0	0.0	0.0	0.0	0.0
5781.714	0.0476	0.2283	66.7	100.0	16.7	66.7	33.3	0.0	0.0	0.0	0.0	0.0
5935.523	0.0476	0.2283	0.0	0.0	0.0	0.0	0.0	83.3	50.0	71.4	33.3	0.0
6073.215	0.0476	0.2283	0.0	0.0	0.0	0.0	0.0	33.3	66.7	0.0	50.0	75.0
6175.637	0.0476	0.2283	0.0	0.0	0.0	0.0	0.0	33.3	83.3	71.4	66.7	25.0
6848.713	0.0476	0.2283	0.0	0.0	0.0	0.0	0.0	66.7	50.0	42.9	66.7	0.0
6906.245	0.0476	0.2283	0.0	0.0	0.0	0.0	0.0	50.0	33.3	71.4	100.0	0.0
6987.705	0.0476	0.2283	0.0	0.0	0.0	0.0	0.0	33.3	66.7	14.3	50.0	75.0
8516.076	0.0476	0.2283	33.3	75.0	50.0	50.0	0.0	0.0	0.0	0.0	0.0	0.0
2239.758	0.1071	0.47	0.0	0.0	0.0	0.0	0.0	33.3	83.3	0.0	33.3	50.0

Bacterial diversity analysis of coastal superficial seawaters near aquaculture facilities, using MALDI-TOF approach and Ribopeaks database.

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SUPPLEMENTARY MATERIAL

Supplementary Table 1

Season	Spring			Winter			Summer		
Local	CNL	RB	STCZ	CNL	RB	STCZ	CNL	RB	STCZ
Total Hits	700	550	320	410	940	430	530	220	240
Number Genus	107	112	58	125	126	60	132	76	92
Acinetobacter	2.4	3.6	3.8	0.8	6.6	2.8	0.8	5.1	0.5
Aeromonas	1.0	0.7	0.9	0.8	1.0	0.7	0.8	0.0	0.0
Alcaligenes	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0
Aliivibrio	0.1	0.2	0.3	0.0	0.1	0.2	0.0	0.0	0.0
Alteromonas	0.1	0.0	0.0	35.8	0.0	0.0	2.1	6.1	8.0
Anaerosporomusa	0.0	0.0	0.0	1.6	0.0	0.0	0.0	0.0	0.0
Alcanivorax	0.0	0.0	0.0	0.8	0.1	0.0	0.2	1.0	0.0
Agrococcus	0.0	0.0	0.0	1.6	0.0	0.0	0.0	0.0	0.0
Actinotignum	0.0	0.0	0.0	0.8	0.0	0.0	0.0	0.0	0.0
Actinobaculum	0.0	0.0	0.0	0.8	0.0	0.0	0.2	0.0	0.0
Acholeplasma	0.1	0.4	0.3	0.8	0.1	0.0	0.2	0.0	0.9
Agrobacterium	1.1	0.0	0.0	0.8	0.1	0.0	0.4	0.0	2.3
Adlercreutzia	0.0	0.0	0.0	0.8	0.0	0.0	0.0	0.0	0.0
Acidaminococcus	0.0	0.0	0.0	0.8	0.0	0.0	0.0	0.0	0.0
Alishewanella	0.0	0.0	0.0	1.6	0.0	0.0	0.2	0.0	0.9
Actinobacillus	0.3	0.0	0.0	0.0	0.2	0.0	0.4	0.5	0.0
Acidithiobacillus	0.1	0.0	0.0	0.0	0.1	0.0	0.0	0.5	0.0
Aggregatibacter	0.1	0.0	0.0	0.0	0.1	0.0	0.4	0.0	0.0
Azospirillum	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0
Arsenophonus	0.1	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0
Arcobacter	0.1	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0
Azorhizobium	0.1	0.2	0.0	0.0	0.1	0.0	0.0	0.0	0.0
Achromobacter	0.3	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0
Acetobacterium	0.1	0.0	0.0	0.0	0.2	0.0	0.2	0.0	0.0
Aureimonas	0.1	0.2	0.3	0.0	0.0	0.0	0.0	0.0	0.0
Alloprevotella	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Actinomadura	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Acetivibrio	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Amycolatopsis	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Actinomycetospora	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Aerococcus	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Andreprevotia	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Altererythrobacter	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0
Actinomyces	0.0	0.0	0.3	0.0	0.0	0.0	0.4	0.0	0.5
Aliiglaciecola	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.5
Aromatoleum	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5
Aphanocapsa	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5
Alloiococcus	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5

Actinosynnema	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5
Ardenticatena	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.0
Azospira	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.0
Arenibacter	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0
Actibacterium	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0
Aquitalea	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0
Alistipes	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0
Actinoplanes	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.0
Amantichitinum	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0
Bradyrhizobium	0.0	0.4	0.0	0.8	0.1	0.0	0.0	0.0	0.0
Bacillus	1.7	1.6	1.9	0.0	2.9	0.9	4.4	1.5	1.4
Bartonella	0.0	0.5	0.3	2.4	0.2	0.0	0.6	0.0	0.0
Bdellovibrio	0.0	0.0	0.0	0.8	0.0	0.0	0.0	0.0	0.0
Bacteroides	0.0	0.0	0.0	0.8	0.0	0.5	0.0	0.0	0.5
Brucella	0.9	0.0	0.6	1.6	0.9	0.2	0.0	0.0	0.0
Bordetella	0.1	0.0	0.0	2.4	0.2	0.2	0.8	0.0	0.0
Buchnera	0.1	3.3	0.0	3.3	0.4	0.9	1.5	3.5	1.4
Brevibacillus	0.0	0.0	0.3	0.0	0.0	0.7	0.0	0.0	0.0
Burkholderia	1.9	0.9	1.6	0.0	0.3	0.2	1.7	0.0	0.5
Brevibacterium	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.9
Bibersteinia	0.0	0.0	0.0	0.8	0.0	0.0	0.0	0.0	0.0
Bartonella	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.4
Brachybacterium	0.1	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Bergeyella	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0
Brochothrix	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0
Beijerinckia	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Bifidobacterium	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.5
Blautia	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.5	0.0
Bradyrhizobium	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0
Campylobacter	0.1	0.2	0.6	6.5	0.3	0.2	1.1	0.5	3.3
Catenovulum	0.0	0.0	0.0	2.4	0.3	0.2	0.2	0.0	0.0
Clostridium	0.1	1.1	0.6	8.1	0.2	0.5	2.9	3.5	2.3
Cellulophaga	0.0	0.0	0.0	0.8	0.0	0.0	0.0	0.0	0.0
Corynebacterium	0.1	0.2	0.0	0.8	0.0	0.0	0.8	0.0	0.0
Cycloclasticus	0.0	0.0	0.0	0.8	0.0	0.0	0.2	0.0	0.0
Caloramator	0.0	0.0	0.0	0.8	0.0	0.0	0.0	0.0	0.0
Curtobacterium	0.0	0.0	0.0	0.8	0.0	0.0	0.0	0.0	0.0
Caldithrix	0.0	0.0	0.0	3.3	0.0	0.0	0.4	0.0	0.0
Carnobacterium	0.0	0.0	0.0	0.8	0.0	0.0	0.0	0.0	0.9
Christensenella	0.0	0.0	0.0	0.8	0.0	0.0	0.0	0.0	0.0
Cupriavidus	0.0	0.0	0.0	0.8	0.0	0.0	0.0	0.0	0.0
Chlamydia	0.1	0.2	0.0	0.8	0.4	0.0	0.0	0.5	0.5
Celeribacter	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0
Citrobacter	0.0	0.0	0.0	0.0	0.0	0.5	0.0	0.0	0.0

Cryptosporangium	0.1	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0
Comamonas	0.1	0.0	0.0	0.0	0.1	0.0	0.2	0.0	0.5
Caldisalinibacter	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0
Cronobacter	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0
Cellvibrio	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Chelatococcus	0.1	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Crocosphaera	0.1	0.0	0.6	0.0	0.0	0.0	0.0	0.0	0.0
Cedecea	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Chitinophaga	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Caldisericum	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Colwellia	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Cellulomonas	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5
Cecembia	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5
Clavibacter	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.5
Clostridiales	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5
Cobetia	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0
Crenobacter	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.0
Calditerrivibrio	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.0
Coriobacterium	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0
Coprococcus	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0
Coprobacillus	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0	0.0
Collimonas arenae	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0
Cardinium	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0
Cellulophaga	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0
Derxia	0.0	0.0	0.0	1.6	0.0	0.0	0.0	0.0	0.0
Desulfovibrio	0.0	0.4	0.3	1.6	0.1	0.2	0.0	0.5	0.0
Desulfosporosinus	0.0	0.0	0.0	0.8	0.0	0.0	0.2	0.0	0.0
Dyella thiooxydans	0.1	0.0	0.0	0.8	0.1	0.0	0.0	0.0	0.5
Dickeya	0.1	0.4	0.0	0.0	0.1	0.0	0.0	0.0	0.0
Demetria	0.1	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0
Deinococcus	0.1	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.0
Dehalococcoides	0.3	0.5	0.0	0.0	0.1	0.0	0.0	0.0	0.0
Defluviimonas	0.0	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Dysgonomonas	0.0	0.2	0.0	0.0	0.0	0.0	0.2	0.0	0.0
Denitrovibrio	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Dermatophilus	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Diplosphaera	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Desulfonatronospira	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Desulfococcus	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5
Deefgea	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5
Dorea	0.0	0.0	0.0	0.0	0.0	0.0	0.2	1.5	0.5
Dielma	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5
Dolichospermum	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0
Desulfurobacterium	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0

Desulfitobacterium	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0
Devosia	0.0	0.0	0.0	0.0	0.0	0.0	0.6	0.0	0.0
Exiguobacterium	0.0	0.0	0.0	0.8	0.0	0.0	0.0	1.5	0.0
Eggerthella	0.0	0.0	0.0	0.8	0.0	0.0	0.0	0.0	0.0
Escherichia	4.1	4.9	5.6	12.2	4.6	2.1	1.3	2.5	0.9
Enterococcus	0.1	0.5	0.0	0.8	0.2	0.2	0.0	0.0	0.0
Enterobacter	1.1	0.5	3.1	4.1	1.4	0.0	0.6	0.0	0.0
Eubacterium	0.0	0.2	0.0	1.6	0.0	0.0	0.0	0.5	0.5
Enterovibrio	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0
Endozoicomonas	0.3	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0
Erwinia	0.1	0.2	0.0	0.0	0.0	0.0	0.0	2.0	0.0
Emticicia	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Eggerthia	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0
Enhydrobacter	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5
Erysipelothrix	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5
Eikenella	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0
Flavobacterium	0.0	0.0	0.0	0.8	0.0	0.0	0.0	0.0	0.0
Ferrimonas	0.1	0.2	0.0	0.0	0.1	0.2	0.0	0.5	0.5
Fusobacterium	0.7	0.4	0.0	2.4	0.9	0.2	1.0	0.0	0.0
Fervidicella	0.1	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0
Fictibacillus	0.1	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0
Flavihumibacter	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Faecalitalea	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Francisella	0.0	0.2	0.9	0.0	0.0	0.0	0.2	0.0	0.0
Fulvivirga	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0
Fructobacillus	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.0
Faecalicoccus	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0
Grimontia	0.1	0.0	0.0	0.0	0.0	0.5	0.0	0.0	0.0
Gallaecimonas	0.3	0.0	0.0	0.0	0.2	0.2	0.0	0.0	0.0
Geothrix	0.0	0.0	0.0	1.6	0.0	0.0	0.0	0.0	0.0
Glaciecola	0.0	0.0	0.0	0.8	0.0	0.0	0.2	0.0	0.0
Gemmatimonas	0.0	0.0	0.0	0.8	0.1	0.0	0.0	0.0	0.0
Gluconobacter	0.0	0.0	0.0	0.8	0.0	0.0	0.2	0.0	0.0
Gardnerella	0.0	0.0	0.0	1.6	0.0	0.0	0.0	0.0	0.5
Geobacillus	0.0	0.0	0.0	2.4	0.0	0.0	0.0	0.0	0.0
Granulicoccus	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5
Geodermatophilus	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Gallibacterium	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.9
Geobacter	0.0	0.0	0.0	0.0	0.0	0.0	0.6	0.0	0.0
Helicobacter	0.1	2.2	1.6	6.5	0.7	0.9	1.9	3.5	2.3
Halomonas	0.0	0.2	0.3	0.0	0.1	0.2	1.1	0.5	2.3
Hahella	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0
Herbidospora	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0
Haemophilus	0.4	0.2	0.3	0.0	0.3	0.0	0.4	1.0	0.5

Haliscomenobacter	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0
Halothiobacillus	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0
Holospora	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0
Hylemonella	0.0	0.0	0.3	0.0	0.0	0.0	0.2	0.0	0.0
Hyphomicrobium	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0
Herminiimonas	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0
Hymenobacter	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0
Hydrogenobacter	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0
Idiomarina	0.0	0.0	0.0	0.8	0.3	0.2	0.0	0.0	0.5
Ilyobacter	0.0	0.0	0.0	0.8	0.0	0.0	0.0	0.0	0.0
Janibacter	0.1	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0
Jeotgalibaca	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.0
Kangiella	0.0	0.0	0.0	3.3	0.0	0.0	0.0	0.0	0.0
Kingella	0.0	0.2	0.0	0.0	0.0	0.2	0.4	0.0	0.0
Klebsiella	1.6	1.5	1.3	1.6	1.6	0.5	0.0	0.5	0.5
Kineococcus	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0
Kluyvera	0.1	0.0	0.0	0.0	0.1	0.0	0.2	0.0	0.0
Kandleria	0.0	1.1	0.0	0.0	0.0	0.0	0.0	0.0	0.5
Kocuria	0.0	0.2	0.0	0.0	0.0	0.0	0.2	0.0	0.0
Kibdelosporangium	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.9
Kytococcus	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0
Ketogulonicigenium	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.0
Komagataeibacter	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0
Lelliottia	0.3	0.0	0.0	1.6	0.3	0.0	0.0	0.0	0.0
Lactobacillus	0.9	1.8	0.6	2.4	0.4	0.9	0.4	3.5	0.9
Lampropedia	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0
lactococcus	0.0	0.0	0.0	0.8	0.1	0.0	0.0	0.0	0.0
Legionella	0.1	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.9
Listeria	0.0	0.9	0.3	2.4	0.0	0.0	0.2	0.0	0.0
Litoreibacter	0.0	0.0	0.0	0.8	0.0	0.0	0.0	0.0	0.0
Luteibacter	0.1	0.2	0.0	0.0	0.0	0.2	0.0	0.0	0.0
Labrenzia alba	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0
Lawsonia	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0
Lysinibacillus	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0
Leisingera	0.0	0.0	0.0	0.0	0.1	0.0	0.0	2.5	0.0
Lysobacter	0.0	0.2	0.3	0.0	0.0	0.0	0.0	0.0	0.0
Leptospira	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.5	0.5
Leclercia	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0
Leminorella	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0
Luminiphilus	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5
Leptotrichia	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.0
Lacinutrix	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.0
Longispora	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0
Marinagarivorans	0.0	0.0	0.0	0.8	0.0	0.0	0.0	0.0	0.0

Myroides	0.0	0.0	0.0	0.8	0.0	0.0	0.4	0.0	0.0
Methylobacter	0.0	0.0	0.0	0.8	0.0	0.0	0.2	0.0	0.0
Mycoplasma	0.0	0.5	0.3	2.4	0.0	0.0	0.0	2.0	0.9
Mizugakiibacter	0.0	0.0	0.0	0.8	0.0	0.0	0.0	0.0	0.0
Methylophaga	0.4	0.5	0.3	0.0	0.2	0.5	0.4	0.0	0.0
Mageeibacillus	0.0	0.4	0.0	0.0	0.0	0.2	0.2	0.0	0.0
Moritella	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0
Moraxella	0.0	0.2	0.0	0.8	0.0	0.2	0.0	0.0	0.0
Mycobacterium	1.6	2.7	0.3	6.5	1.4	0.7	3.0	1.0	2.3
Methylosinus	0.0	0.0	0.0	0.8	0.0	0.0	0.0	0.0	0.0
Mesoplasma	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.5	0.9
Meganema	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0
Maritalea	0.1	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0
Microbulbifer	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.9
Microcoleus	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0
Muricauda	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Marinobacterium	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Mannheimia	0.1	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0
Marinomonas	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.5
Marinospirillum	0.0	0.2	0.0	0.0	0.0	0.0	0.2	0.0	0.0
Microvirga	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Microcystis	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0
Magnetospirillum	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5
Methylobacterium	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.5	2.8
Methylobacillus	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5
Marinobacter	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.5	0.0
Mesoaciditoga	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.0
Microbacterium	0.0	0.0	0.0	0.0	0.0	0.0	0.6	0.0	0.0
Micrococcus	0.0	0.0	0.0	0.0	0.0	0.0	1.3	0.0	0.0
Megasphaera	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0
Mesorhizobium	0.0	0.0	0.0	0.0	0.0	0.0	0.6	0.0	0.0
Nitratireductor	0.1	0.0	0.3	0.0	0.2	0.5	0.2	0.0	0.0
Niastella koreensis	0.0	0.0	0.0	0.8	0.0	0.0	0.0	0.0	0.0
Neisseria	0.0	0.2	0.3	4.9	0.2	0.2	0.0	0.0	0.0
Novosphingobium	0.1	0.2	0.0	1.6	0.2	0.0	0.8	0.0	1.4
Nitriliruptor	0.0	0.0	0.0	0.8	0.0	0.0	0.0	0.0	0.0
Nannochloropsis	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0
Nocardia	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.5
Nonlabens	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0
Nocardiopsis	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.5	0.0
Nautella	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0
Nitritalea	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0
Oblitimonas	0.0	0.0	0.0	4.1	0.0	0.0	0.0	0.0	0.9
Oceanobacter	0.0	0.0	0.0	0.8	0.0	0.0	0.0	0.0	0.0

Oenococcus	0.1	0.4	0.0	0.0	0.1	0.0	0.0	0.5	0.0
Ochrobactrum	0.1	0.0	0.0	0.0	0.1	0.0	0.2	0.0	0.0
Oligella	0.1	0.2	0.0	0.0	0.1	0.0	0.0	0.0	0.0
Oceanobacillus	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0
Orientia	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Oscillibacter	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Octadecabacter	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Oceanibulbus	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.4
Odoribacter	0.0	0.0	0.0	0.0	0.0	0.0	1.5	0.0	0.5
Paraburkholderia	0.1	0.4	0.0	0.8	0.2	0.0	0.8	0.0	0.0
Planococcus	0.0	0.0	0.0	0.8	0.2	0.0	0.0	0.0	0.0
Prochlorococcus	0.0	1.3	0.0	2.4	0.5	0.0	0.4	0.0	0.9
Photobacterium	3.7	3.1	6.9	7.3	3.0	2.8	0.0	0.0	0.9
Pseudoalteromonas	1.3	11.8	1.9	41.5	4.9	2.6	21.5	2.0	1.4
Pseudomonas	2.0	4.7	2.5	9.8	6.2	0.9	1.7	2.5	6.1
Paenibacillus	0.7	1.1	0.3	2.4	0.5	0.7	0.0	0.5	0.9
Polaribacter	0.0	0.0	0.0	3.3	0.0	0.0	0.0	0.0	0.0
Prevotella	0.0	0.4	0.0	0.8	0.1	0.0	0.0	0.0	0.0
Planomonospora	0.0	0.0	0.0	0.8	0.0	0.0	0.0	0.0	0.0
Propionibacterium	0.1	0.0	0.0	0.8	0.3	0.0	0.2	0.0	0.0
Paulinella	0.0	0.0	0.0	0.8	0.0	0.0	0.0	0.0	0.0
Pseudopedobacter	0.0	0.0	0.0	0.8	0.0	0.0	0.0	0.0	0.0
Plautia	0.2	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0
Peptoclostridium	0.0	0.2	0.0	0.2	0.0	0.0	0.0	0.0	0.0
Pandoraea	0.0	0.5	0.0	0.0	0.1	0.0	0.0	0.0	0.5
Paramesorhizobium	0.1	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0
Parasutterella	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0
Paenarthrobacter	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0
Persephonella	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0
Paracoccus	0.1	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.0
Pantoea	0.0	0.0	0.6	0.0	0.0	0.0	0.0	0.0	0.5
Parachlamydia	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0
Piscirickettsia	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0
Pasteurella	0.0	0.7	0.0	0.0	0.0	0.0	0.8	0.0	0.0
Phaeobacter	0.0	0.4	0.0	0.0	0.0	0.0	0.0	2.5	0.0
Pectobacterium	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Pontibacillus	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Paraoerskovia	0.0	0.2	0.6	0.0	0.0	0.0	0.0	0.0	0.0
Pediococcus	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Porphyromonas	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Pseudoclavibacter	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5
Plantibacter	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.4
Parabacteroides	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5
Pelobacter	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5

Pectobacterium	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.5	0.0
Pseudorhodobacter	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.0
Prochlorococcus	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.5	0.0
Pedobacter	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0
Parvularcula	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0
Paenibacillus	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0
Porphyrobacter	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0
Phenylobacterium	0.0	0.0	0.0	0.0	0.0	0.0	0.6	0.0	0.0
Paenirhodobacter	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0
Rothia	0.0	0.0	0.3	1.6	0.0	0.0	0.0	0.5	0.0
Roseivivax	0.0	0.0	0.0	0.8	0.0	0.0	0.0	0.0	0.0
Rhodobacter	0.0	0.0	0.0	3.3	0.1	0.0	0.4	0.0	0.0
Ruania	0.0	0.0	0.0	0.8	0.0	0.0	0.0	0.0	0.0
Rhizobium	0.0	0.4	0.9	1.6	0.0	0.0	0.4	0.5	1.4
Roseovarius	0.0	0.2		1.6	0.0	0.0	0.2	1.0	0.5
Rhodothermus	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0
Rubrivivax	0.1	0.0	0.0	0.0	0.1	0.2	0.0	0.0	0.0
Roseobacter	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0
Ruegeria	0.3	0.0	0.0	0.0	0.3	0.0	0.0	1.5	0.0
Rubrobacter	0.1	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0
Roseburia	0.1	0.0	0.0	0.0	0.2	0.0	0.2	1.0	1.9
Rahnella	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.9
Rothia	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5
Ruminococcus	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.0	0.5
Rhodococcus	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.0
Runella zeae	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.0
Roseivirga	0.0	0.0	0.0	0.0	0.0	0.0	0.2	1.0	0.0
Rhodospirillum	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0
Rummeliibacillus	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.0
Rhizobacter	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0
Rhodonellum	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0
Salinimonas	0.0	0.0	0.0	0.8	0.0	0.0	0.0	0.0	0.0
Serratia	0.0	0.0	0.0	1.6	0.0	0.0	0.4	0.0	0.0
Streptomyces	0.7	0.4	0.6	0.8	0.3	0.0	0.8	0.5	0.0
Streptococcus	0.3	0.5	0.3	2.4	0.6	0.5	0.4	1.5	1.4
Staphylococcus	3.9	3.3	3.8	8.9	4.0	3.1	3.2	5.6	4.2
Sphingomonas	0.1	0.0	0.0	1.6	0.1	0.2	0.2	4.0	0.0
Spiroplasma	0.1	0.4	0.0	3.3	0.1	0.2	0.0	0.0	0.0
Salmonella	0.4	0.5	1.6	3.3	0.9	0.0	1.1	1.5	0.0
Slackia	0.0	0.0	0.0	0.8	0.0	0.0	0.0	0.0	0.0
Sinorhizobium	0.0	0.0	0.0	2.4	0.0	0.0	0.0	1.0	0.0
Sporichthya	0.0	0.0	0.0	0.8	0.0	0.0	0.0	0.0	0.0
Stenotrophomonas	0.1	0.4	0.6	0.8	0.1	0.0	0.0	0.0	0.0
Sphingobium	0.0	0.2	0.0	0.8	0.1	0.0	0.0	0.0	0.0

Selenomonas	0.0	0.2	0.0	0.8	0.0	0.0	0.0	0.0	0.9
Scytonema	0.0	0.0	0.0	0.8	0.0	0.0	0.0	0.0	0.0
Syntrophomonas	0.0	0.0	0.0	0.8	0.0	0.0	0.0	0.0	0.0
Spiribacter	0.0	0.0	0.0	0.8	0.0	0.0	0.0	0.0	0.0
Sodalis	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0
Shewanella	0.1	0.0	0.0	0.0	0.2	0.5	0.2	0.0	0.0
Shigella	0.9	0.0	0.0	0.0	0.6	0.0	0.0	0.0	0.0
Salinicoccus	0.1	0.0	0.0	0.0	0.1	0.0	0.2	0.0	0.0
Sphingopyxis	0.1	0.0	0.0	0.0	0.2	0.0	0.4	0.0	0.0
Stappia	0.1	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0
Sulfitobacter	0.0	0.0	0.0	0.0	0.4	0.0	0.0	0.0	0.0
Sporolactobacillus	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0
Simiduia	0.1	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0
Salmonella	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5
Shimazuella	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5
Sharpea	0.0	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Sulfurospirillum	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Sporocytophaga	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Serpens	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Synechococcus	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5
Saccharospirillum	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5
Spiroplasma	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.0
Salinispora	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.0
Sutterella	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.0
Synergistes	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0
Stanieria	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0
Thiothrix	0.0	0.0	0.0	0.8	0.0	0.0	0.8	0.0	0.0
Thioclava	0.1	0.0	0.0	1.6	0.2	0.0	0.0	0.0	0.0
Tetragenococcus	0.0	0.0	0.0	0.8	0.0	0.0	0.0	0.0	0.0
Tetrasphaera	0.0	0.0	0.0	1.6	0.0	0.0	0.0	0.0	0.0
Thauera	0.0	0.0	0.0	0.8	0.0	0.0	0.0	0.0	0.0
Thiomicrospira	0.0	0.0	0.0	0.8	0.0	0.0	0.4	0.0	0.0
Tolypothrix	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0
Tenacibaculum	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0
Thalassobacter	0.1	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0
Treponema	0.0	0.4	0.0	0.0	0.0	0.0	0.2	0.0	0.5
Terrisporobacter	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Taylorella	0.0	0.2	0.3	0.0	0.0	0.0	0.0	0.0	0.0
Tolumonas	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Tatumella	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Thermocrinis	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.0	0.0
Terrabacter	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.5	0.0
Tropicibacter	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0
Thermobacillus	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0

Tanticharoenia	0.0	0.0	0.0	0.0	0.0	0.0	0.8	0.0	0.0
Uliginosibacterium	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0
Ureaplasma	0.0	0.4	0.0	0.0	0.0	0.0	0.2	0.0	0.0
Vibrio	44.1	13.1	30.3	28.5	30.4	51.1	12.4	1.0	6.1
Variibacter	0.0	0.0	0.0	0.8	0.0	0.0	0.0	0.0	0.0
Virgibacillus	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.0
Veillonella	0.0	0.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Ventosimonas	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Xylella	0.0	0.0	0.0	0.0	0.1	0.2	0.0	0.0	0.0
Xanthomonas	11.1	8.4	15.9	8.1	9.5	15.1	0.2	0.0	1.9
Yersinia	0.0	0.0	0.0	0.0	0.1	0.2	0.0	0.0	0.5
Weissella	0.0	0.0	0.0	1.6	0.0	0.2	0.0	0.0	0.0
Weeksella	0.0	0.0	0.0	0.8	0.0	0.0	0.0	0.0	0.0
Wolbachia	0.0	0.2	0.0	0.0	0.2	0.0	0.0	1.0	0.0
Wigglesworthia	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0