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EMERGED AND EMERGING THREATS IN MARINE FOOD SYSTEMS AND FOOD SAFETY

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ABSTRACT

Marine food systems are increasingly shaped by anthropogenic pressures—warming, biodiversity loss, pollution, and intensified exploitation—that alter hazard landscapes and challenge conventional food-safety paradigms. The One Health framework, which links human, animal, and environmental health, offers an integrated lens for surveillance and risk management spanning ecosystems, primary production, processing, and retail exposure. This thesis operationalizes that perspective across four complementary lines of evidence: (i) retail-level antimicrobial resistance (AMR) in psychrotrophic *Pseudomonas* from fresh finfish fillets; (ii) a critical review of microplastics (MPs) in edible aquatic products and their implications for human exposure; (iii) the first metataxonomic baseline of the swordfish (*Xiphias gladius*) gut microbiota; and (iv) governance and hygiene guidance for a neglected/under-utilized species (NUS), the Patagonian red octopus (*Enteroctopus megalocyathus*), in artisanal value chains. Together, these studies generate empirical measurements, methodological synthesis, and context-appropriate controls intended to protect consumers while supporting sustainable seafood systems.

Chapter 1 quantified process-relevant spoilage flora and resistance phenotypes at the point of pack-out. Seventy-five commercial fillets (salmon, plaice, cod) were sampled across five lots (March–May), transported chilled, and analyzed for Total Viable Count (TVC), *Pseudomonas* loads, biochemical identification, and disc-diffusion susceptibility under psychrotrophic conditions. Presumptive *Pseudomonas* isolates were identified and tested against 15 agents following EUCAST execution guidance (with an ≤ 10 mm zone interpreted as highly resistant, given the absence of species-level breakpoints for these taxa). *Pseudomonas* counts were generally on the order of 10^4 cfu/g at day-0, with frequent excursions toward 10^5 – 10^6 cfu/g, especially in plaice and defrosted cod. Salmon displayed significantly lower loads, and TVC averaged $\sim 10^5$ cfu/g across species. Psychrotrophic *Pseudomonas* constituted a major share of the initial microbiota and a determinant of shelf-life trajectories. Resistance profiles revealed high non-susceptibility prevalence to multiple classes (e.g., macrolides, glycopeptides, lincosamides, diaminopyrimidines), comparatively lower resistance to meropenem (16.22%), and multidrug resistance in 76.47% of *Pseudomonas fluorescens* strains tested—underscoring these opportunists as potential reservoirs and vectors of resistance in chilled distribution. Methodologically, the study highlights the need for retail-level process-hygiene indicators that include psychrotrophic spoilers, validation of anti-biofilm sanitation, and defensible shelf-life targets calibrated to starting loads and time–temperature discipline.

Chapter 2 synthesizes the evidence on microplastics in edible aquatic products (finfish, bivalves, crustaceans), mapping sources, occurrence, exposure routes, and toxicological mechanisms (particle effects, leaching of additives and sorbed pollutants, and biofilm-mediated transport of microbes/ARGs). Cross-study comparability is presently limited by heterogeneity in sampling, digestion, identification, and reporting units, precluding robust dose–response appraisal. While MPs can carry potentially toxic elements and organic pollutants, current estimates indicate that, for several contaminant classes (e.g., PAHs, PCBs), the incremental dietary contribution via MPs in seafood is small relative to total dietary intake; for certain additives, effects may be negligible under current scenarios. However, the overall risk characterization remains uncertain due to methodological variability and incomplete exposure metrics. The review calls for harmonized methods, standardized units, and integrated surveillance designs that connect occurrence to co-exposures (metals, POPs, antibiotic residues) and to microbiological endpoints, while risk management proceeds via upstream source reduction and proportionate supply-chain controls (e.g., water/ice hygiene, depuration where applicable, prevention of cross-contamination).

Chapter 3 reports the first metataxonomic baseline of the swordfish gut microbiota using a sizeable dataset spanning across FAO areas and weight classes. Proteobacteria (Pseudomonadota) dominated, with *Photobacterium* consistently leading. Alpha-diversity differences were more pronounced by weight category than by FAO fishing area, indicating within-species ecological structuring that likely reflects ontogeny, diet, or physiology. *Photobacterium*'s known spoilage potential (trimethylamine and biogenic amine formation) and presence in swordfish flesh metagenomes elevate its relevance for spoilage and histamine-related risks, despite swordfish not being among high-histidine families under EU criteria; historical incidents in swordfish underline this vigilance. This baseline supports targeted verification sampling at critical control points in pelagic fish chains, and motivates compartment-resolved (mucosa vs digesta) and functional (metagenome/resistome) follow-ups to translate composition into risk-relevant traits.

Chapter 4 turns to a neglected and under-utilized (fish) species (NUS): the Patagonian red octopus (*Enteroctopus megalocyathus*) in Chilean artisanal fisheries. Biology, fishing management, and concrete hygiene guidance adapted to infrastructure-limited contexts were integrated. The analysis emphasizes immediate icing on vessels, potable water/ice verification, hygienic evisceration/cleaning, and landing-site sanitation as practical levers to reduce post-harvest microbial proliferation and cross-contamination. Where ice is scarce, a simple 0.1% acetic-acid dip can extend shelf-life up to five days with quality comparable to ice, offering a low-cost adjunct in remote areas. Regulatory anchors (e.g., Chilean cold-chain temperature requirements) exist but require training,

subsidies, and technical assistance for effective uptake. A Quality Index Method tailored to *E. megalocyathus* is proposed to standardize organoleptic checks and spur continuous improvement at the community level.

Across chapters, the thesis advances a prevention-first agenda. At retail, managing initial *Pseudomonas* loads and realistic shelf-life windows is essential for sensory and safety endpoints; species effects (lower loads in salmon vs plaice; cod intermediate) argue for product-specific expectations and controls. For MPs, science-policy progress hinges on method harmonization and exposure standardization to move from presence toward dose–response. For swordfish, the microbiome baseline provides a scaffold to interrogate spoilage trajectories, ARG carriage, and handling effects across the chain; extending to functional ‘omics will increase decision relevance. For NUS, embedding simple, verifiable hygiene practices in co-management, coupled with training and modest infrastructure (potable ice, clean tanks, hand hygiene), can de-risk market expansion while supporting equitable livelihoods.

Limitations map a clear research program: broaden spatio-temporal AMR surveillance beyond *Pseudomonas* and link phenotypes to resistomes; standardize MP analytics and exposure metrics that capture co-exposures; resolve gut compartments and functions in swordfish to connect taxa with spoilage and resistance traits; and validate Good Hygiene Practices adaptations for small-scale octopus chains under real constraints. Nonetheless, the thesis contributes a One Health-rooted coherent set of measurements, baselines, and practices that tie laboratory, vessel, landing site, plant, and retail into a single surveillance-and-control continuum. The overarching conclusion is pragmatic: invest in integrated AMR/MP monitoring with harmonized metadata, validated anti-biofilm sanitation and time–temperature discipline, species-specific microbiome baselines, and context-appropriate hygiene for NUS. Such integration strengthens consumer protection while aligning seafood safety with ecosystem stewardship and community resilience in an era of environmental change.

INTRODUCTION

The modern world is marked by constant, profound, and often rapid transformations across diverse human activities. Advances in science, technology, and industry—applied to fields such as medicine, agriculture and animal husbandry, and other productive sectors—have profoundly reshaped human life, frequently generating collateral effects that remain poorly understood. Over the past century, this technological acceleration has contributed, in one hand, to an improved quality of human life as well as an exploitation of human activities conducted on a large scale, but, in the other, it has also drawn criticism for its major drawback that threaten global stability, sustainability and safety, namely the environmental alterations. These anthropogenic environmental changes, occurring at local, regional, continental, and planetary scales, pose significant threats to the stability of the Earth system, affecting both biotic and abiotic components. Many types of environmental alterations have been observed and described over past decades in marine food systems, like biodiversity loss [1,2], ocean and freshwater resources degradation [3-6], climate change [7,8], and pollution from an extremely wide group of natural and synthetic substances [9,10], like microplastics (MPs) and antibiotic compounds.

All these profound global changes and threats have led researchers to develop a conceptual framework that views human, animal, and environmental health as components of a single, interconnected and dynamic system, namely the so called “One Health” approach. This approach emphasizes the numerous and complex interactions between biotic and abiotic factors that alter natural equilibria, ultimately affecting both ecosystem integrity and individuals health. It is an integrated, unifying approach that aims to sustainably balance and optimize the health of people, animals, and ecosystems, recognizing that health of humans, domestic and wild animals, plants, and the wider environment (including ecosystems) are closely linked and interdependent [11].

Although originally developed to address zoonotic threats, the One Health framework has become increasingly relevant in the Anthropocene era, where large-scale environmental and societal changes have introduced new global health challenges including food insecurity, water scarcity, social inequalities, and the widespread dissemination of emerging pollutants such as plastic, antibiotics, and antimicrobial resistance across multiple ecosystems. This is in line with the European need to enhance resilience of food systems to various issues and stresses by ensuring sufficient, safe, healthy, nutritious, and affordable food for all as well

as with the advance of the 2030 Agenda for Sustainable Development framework adopted by the United Nations for an improvement in the living conditions of the current generation and a better perspective for the future. The exponential expansion of the world population and its demand for food, combined with climate changes and the transformation of the geopolitical situation, poses under great pressure the food industry, its stakeholders, and policymakers. Intrinsic in this context is the issue of providing not only enough food to fulfill rising demand but also safe and nutritious food that meets regulations in force as well as consumers' health standards.

In this context, the One Health approach is fundamental for preventing and combating both emerged and emerging global hazards, including those affecting food safety in seafood products. With the global consumption of wild-caught and farmed seafood steadily increasing, it is essential to adopt and promote a One Health perspective to prevent the spread of health risks [12]. Indeed, access to sufficient amounts of safe and nutritious food is key to sustaining life and promoting good health. Food safety is essential to the marketability of products. It is well known that rapid and profound anthropogenic changes to both society and the environment have made aquatic pollution one of the most critical threats to ecosystem functionality and human health [13]. Multiple human activities contribute to the contamination of freshwater and marine systems. Some pollution sources have been recognized and extensively studied for decades, such as industrial and urban wastewater discharges [14], heavy metal contamination [15], and eutrophication linked to synthetic fertilizers [16]. Others are more recent concerns, gaining increasing scientific attention as evidence accumulates regarding their potential hazards. These include pesticides [17], pharmaceutical residues [18], persistent organic pollutants [19], plastics and MPs, and the phenomenon of antimicrobial resistance (AMR) spread [20,21]. Also, human have also contributed to overfishing in freshwater and marine systems. The increasing demand for fish products—driven by population growth, greater awareness of health benefits, and changing dietary preferences—is progressively intensifying anthropogenic pressure on marine ecosystems. Without proper management, this rising demand could result in the overexploitation of aquatic resources, endangering biodiversity and hindering the regeneration of natural biomes [22-24]. For example, swordfish (*Xiphias gladius*) is a valuable fish species but in the recent decades its consumption has been growing in an unsustainable way and the consequent intensive fishing worldwide has compromised swordfish marine stocks. Therefore, both local and international policies have been starting to protect this species [25]. This overfishing

coupled with climate change, alien species invasions, and other anthropogenic activities may disrupt in a short time the fragile balance established by millions of years of underwater evolutionary process. Approximately 60% of fish stocks in the Mediterranean and Black Sea are classified as being in critical condition [12], and, at the same time, it is estimated that between 7 and 10 million tons of commercially caught fish are discarded each year worldwide [26]. The reason behind discards depends on several factors, which are not the core of this PhD thesis, but, among the discards generated there are the unwanted catches, known as neglected and underutilized species, also referred to as underutilized fish like the Patagonian red octopus (*Enteroctopus megalocyathus*).

In this complex scenario, while long-recognized threats have been widely investigated and are now the target of established mitigation strategies, more recent issues require further research to fully understand their mechanisms, impacts, and effective prevention measures. Among these emerging threats, plastic waste and its associated MPs dissemination, along with the emerged global AMR challenge, are of particular concern due to their ubiquity and widespread distribution across ecosystems, particularly in marine environment. Whereas, in relation to the adaptation of marine vertebrates to the environment in which they were caught, literature on fish gut microbiota (even of major commercialized fish species) as well as on neglected and underutilized fish species are still limited.

It is widely described how these issues are posing emerging serious threats to marine ecology and seafood production, representing a global health risk for people. Therefore, the overarching aims of this thesis are to analyze and evaluate emerged and emerging seafood-related threats to public health (principally AMR and MPs), to characterize the gut microbiota of commercially important seafood species (swordfish, *Xiphias gladius*), and to assess food-safety risks in neglected seafood taxa (Patagonian red octopus, *Enteroctopus megalocyathus*). Adopting a One Health perspective, the present PhD thesis treats these issues not as isolated events but as components of an interconnected, dynamic system with consequences for human, animal, and environmental health.

Antimicrobial Resistance

Antimicrobial resistance (AMR) represents a critical and urgent global public health crisis that jeopardizes the efficacy of treatments for infections. It is defined as the phenomenon where microorganisms, including bacteria, viruses, fungi, and parasites, develop the ability to withstand the effects of antimicrobial treatments that were previously effective against them. Antimicrobial agents encompass various categories such as antibiotics, antifungals, antivirals, disinfectants, and food preservatives, all of which function by either inhibiting microbial growth and reproduction or by eliminating the microorganisms. Antibiotic resistance (AR) is a specific subset of AMR, referring to the resistance developed by bacteria against antibacterial drugs [27-29]. AMR is listed by Regulation (EU) 2022/2371 as a serious cross-border threat to health, across threats of biological origin.

The emergence of AMR is fundamentally a natural evolutionary phenomenon. Microorganisms have always developed robust mechanisms to evade destruction from toxic substances, including naturally produced antimicrobial molecules. However, this natural process has been significantly accelerated and intensified by human activities, particularly the imprudent overuse of antimicrobial compounds in medical, veterinary, and agricultural sectors. This widespread use creates selective pressure, allowing resistant strains to survive and proliferate [30-32]. AMR arises via three broad categories: intrinsic (chromosome-encoded structural/physiological traits), acquired (mutations or horizontal gene transfer (HGT): transformation, conjugation, transduction), and adaptive (reversible, stress-induced phenotypes) [33-35]. Mechanistically, four strategies dominate: (i) reduced permeability via porin/membrane changes, (ii) higher active efflux, (iii) target alteration/protection, and (iv) drug inactivation (β -lactamases; enzymatic modification) [36-39]. Biofilm formation could amplify these mechanisms by creating a protective extracellular polymeric matrix that hinders antibiotic penetration, alters local physicochemical conditions, induces a slow-growth state, and supports persister cells. Biofilm-associated bacteria often upregulate efflux pumps, evade immune responses, and sustain chronic or recurrent infections. Collectively, these strategies contribute to the persistence and dissemination of AMR in clinical, environmental, and food-related contexts [40,41].

AMR, though rooted in ancient evolutionary processes, has been dramatically accelerated by anthropogenic activities, particularly the excessive and indiscriminate use of antimicrobials in human medicine, livestock, aquaculture, and agriculture since the 1940s. Inappropriate prescribing practices, prophylactic use,

and the application of antibiotics for growth promotion have intensified selective pressures, driving the persistence and dissemination of resistant strains across ecosystems. Once released into the environment via wastewater, animal manure, and industrial effluents, antibiotics, antibiotic-resistant bacteria (ARB) and genes (ARGs) contaminate soils, surface and groundwater, and marine systems, entering the food chain through contaminated meat, fish, and produce. Environmental compartments, including sediments and air, act as reservoirs and vectors, while heavy metals and biocides from industrial and consumer sources promote co-selection by favoring bacteria carrying multiple resistance determinants [42,43]. HGT is the primary engine of AMR spread, with plasmid-mediated conjugation enabling bundled transfer of multiple resistance genes, and transformation and bacteriophage-mediated transduction further mobilizing DNA across bacteria. Dissemination is amplified by co-selection—when several resistance determinants reside on the same mobile element—and by cross-resistance, both of which can be driven by non-antibiotic stressors such as biocides and metals. Clinically, resistance typically arises from synergistic mechanisms rather than a single change, exemplified by methicillin-resistant *Staphylococcus aureus* (MRSA), which couples target modification with efflux, enzymatic inactivation, cell-wall remodeling, and biofilm formation [44-47]. These complexities demand integrated surveillance and targeted mitigation within a One Health framework, alongside development of alternative interventions. Globally, the WHO GLASS platform harmonizes AMR and antimicrobial-use (AMU) data across many countries, while in Europe EARS-Net reports sustained high AMR burdens with recent declines in MRSA but increases in carbapenem-resistant *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*, and vancomycin-resistant *Enterococcus faecium*.

The dissemination of AMR exemplifies the complex interdependence of human, animal, and environmental health, underscoring the necessity of a One Health approach. Resistance determinants and bacteria circulate freely across ecological compartments via direct and indirect pathways, making coordinated cross-sectoral interventions essential. A key driver is the extensive and often indiscriminate use of antibiotics in human medicine and food-producing animal sectors which accounts for approximately 73% of global antimicrobial sales. This selective pressure fosters the emergence and amplification of resistant strains [43]. Active antibiotic residues, ARB and ARGs are excreted in human and animal waste and enter sewage networks, where Wastewater Treatment Plants reduce but often do not eliminate them, creating hotspots for HGT and microbial adaptation. From these systems, ARB/ARGs disseminate into soils, surface waters, and sediments and return to

humans and animals via multiple pathways, including the food chain, direct animal contact, drinking or recreational waters, and irrigated crops, with mobile genetic elements (e.g., plasmids, bacteriophages) accelerating interspecies exchange [48,49]. Environmental compartments thus act as dynamic reservoirs seeded by cross-sector effluents—from hospitals, intensive livestock, aquaculture, and pharmaceutical production—that maintain residual ARG loads even after treatment [50-53]. Also MPs/NPs could further concentrate resistant communities within biofilm-rich “plastisphere” niches, enhancing HGT; their surfaces sorb antibiotics, metals, pesticides, and biocides, creating co-selection hotspots intensified by particle aging and the high reactivity of small particles. These processes contaminate biota and harvesting areas, enabling ARG carriage in seafood—particularly bivalves eaten raw—and elevate consumer exposure risks [54-58]. Collectively, this ecology underscores the need to integrate plastic pollution control and advanced effluent management into One Health AMR surveillance and mitigation.

Contamination of aquatic environment with pathogenic and resistant bacteria is an emerging problem worldwide. Rapid expansion of aquaculture and intensive harvesting of wild stocks have positioned the aquatic food system at the center of the AMR challenge. Antimicrobials are still widely used in parts of global aquaculture for therapy, metaphylaxis, and (in some jurisdictions) routine prophylaxis; point estimates suggest consumption on the order of 10,000+ tonnes in 2017 with a projected rise of ~33% by 2030. Such use fosters selection for resistant pathogens and enriches mobile resistance determinants in farmed waters, sediments, and associated biota, with direct implications for seafood safety and public health [59,60]. Within farming units, AMR selection emerges from the combined effects of antimicrobial exposure, high host density, fluctuating water quality, and extensive biofilm formation on nets, tanks, pipes, and equipment. Fish and shellfish harbor complex mucosal microbiota (gill, gut, skin) that can act as reservoirs and vectors of ARB and ARGs. Common aquatic and seafood-associated genera (including *Vibrio*, *Aeromonas*, *Pseudomonas* and Enterobacterales) carry integrons, plasmids, transposons and prophages encoding resistance to tetracyclines, sulfonamides, quinolones, β -lactams, and phenicols, among others. These mobile genetic elements enable HGT across environmental, commensal, and pathogenic taxa and can persist in farm sediments long after antimicrobial inputs cease [61,62].

Food safety risks arise pre-harvest and post-harvest. Pre-harvest, animals raised in cages, ponds, or bivalves filtering coastal waters can accumulate ARB/ARGs from

impacted waters and sediments; wild-caught seafood harvested near river plumes or effluent discharges may exhibit similar patterns. Evidence from retail monitoring demonstrates the presence of clinically relevant ARGs in both aquaculture and wild fisheries products. Post-harvest, processing plants, ice, and water used for rinsing and glazing can introduce or stabilize ARB in biofilms that resist routine sanitation [63-65].

Synthesis across multiple surveys indicates that resistance to medically important drugs is common in aquatic bacteria and seafood. A meta-analysis of 103 studies in marine bivalves reported resistant isolates across indicator and zoonotic taxa, with detection of Extended-Spectrum β -Lactamases (ESBLs) and fluoroquinolone resistance in *Escherichia coli* and notable carbapenem resistance in *Aeromonas* spp., underscoring exposure to highest-priority critically important antimicrobials [66]. Culture-independent data are consistent with this: targeted metagenomics of retail oysters and shrimp identified ESBL families more sensitively than shotgun sequencing or culture, revealing a broader resistome in these products [67]. Region-specific syntheses also show substantial burdens of antibiotic-resistant seafood-borne pathogens at retail (overall $\approx 56\%$ prevalence in Malaysia), highlighting consumer-relevant exposure pathways [65]. In seafood, the principal human bacterial hazards comprise both autochthonous marine taxa and faecally derived enterics. Across key aquatic genera implicated in seafood safety (including *Vibrio* spp. and *Aeromonas* spp.) multidrug-resistant profiles (commonly to ampicillin/tetracyclines, and in some settings to broader-spectrum agents) are repeatedly documented, reinforcing the public-health rationale for integrated AMR surveillance along seafood supply chains [68,69]. A U.S. retail study detected ARGs to carbapenems and ESBLs in seafood, while meta-analyses and regional surveys report AMR in *Vibrio*, *Enterococcus*, and coliforms across shrimp and finfish supply chains. *Vibrio* spp. (especially *V. parahaemolyticus* and *V. vulnificus*, with occasional non-O1/non-O139 *V. cholerae*) are intrinsically linked to warm, brackish waters and raw bivalves; recent EU appraisals anticipate higher occurrence with coastal warming, and collated data indicate detection in $\sim 20\%$ (*V. parahaemolyticus*), $\sim 6\%$ (*V. vulnificus*), and $\sim 4\%$ (non-cholerae *V. cholerae*) of tested seafood, underscoring climate-sensitive risk in Baltic/North Sea transitional waters [70]. *Listeria monocytogenes* remains a key microbiological hazard in ready-to-eat fish (e.g., cold-smoked salmon) owing to its environmental persistence and psychrotrophic growth. Multiple studies have documented AMR in seafood-derived isolates. In Poland, Wieczorek and Osek [71] reported resistance to oxacillin, ceftriaxone and clindamycin, including multidrug-resistant strains, among isolates from fresh and

smoked fish. In Iran, Jamali et al. [72] and Abdollahzadeh et al. [73] described resistant isolates from seafood, including resistance to ampicillin, penicillin and cefotaxime.

Contamination of farmed and wild-caught seafood with *Salmonella* spp. generally reflects fecal inputs during harvest or processing rather than a true marine reservoir. Zhang et al. [74] documented ceftriaxone–ciprofloxacin co-resistance in *S. enterica* serovar Thompson isolated from seafood in China, and other authors likewise reported resistant *Salmonella* strains in seafood products [75,76]. *Clostridium* spp. also pose public-health risks due to toxigenicity and emerging AMR. Li et al. [77] isolated resistant *Clostridium perfringens* from aquatic mollusks (clams, oysters and mud snails) in China. Marine bivalves have additionally been identified as a potential source of resistant *Clostridioides difficile* in the North Adriatic Sea [78], and a systematic review reported the highest prevalence of *Clostridioides difficile* among food categories in seafood (~10.3%) [79]. Taken together, these findings suggest the potential for AMR exposure via seafood (most notably with raw or minimally processed items) while underscoring that consumer risk is context-specific and should be managed through risk-based controls along the supply chain [65,80,81].

Along fish and seafood value chains, several factors facilitate the dissemination of AMR. These include substandard sanitation and hygiene on fishing vessels, at landing sites, within processing plants, and throughout distribution; inappropriate antimicrobial use in both aquatic and terrestrial animal production; the inclusion of antibiotics or strong biocides in cleaning formulations used in fish-processing environments; and the prophylactic or therapeutic overuse of antimicrobials in food-producing animals, which selects for AMR microorganisms and resistance determinants that can enter the food chain. Additional contributors are the contamination of foods of animal origin with antimicrobial residues, the use of antimicrobials as feed additives in livestock, improper disposal of expired or unused medicines with consequent environmental release, and the application of banned chemicals in aquaculture. Collectively, these practices sustain selective pressures and environmental reservoirs that promote the persistence and transmission of AMR from production to consumers.

Finally, current evidence shows that seafood can carry AMR to consumers, making routine retail monitoring a public-health priority. Clinically relevant resistance genes are found even when phenotypic resistance appears low, and while culture alone underestimates risk, targeted metagenomics consistently detect broader

resistome. Risk is heterogeneous and depends on species, origin, processing, and cold-chain integrity, and may be higher for raw or minimally processed products. Within a One Health approach, surveillance should combine standardized sampling, phenotypic testing, and molecular detection of key ARGs, alongside detailed product metadata.

Within seafood critical control point (CCP) programs, preventing the dissemination of AMR requires a coordinated suite of pre- and post-harvest measures. Regarding fish farms management, priorities include systematic management of antimicrobial residues; rigorous sanitation and hygiene across production and distribution; substitution of antibiotics with non-antimicrobial alternatives and stewardship to reduce and rationalize therapeutic use in aquaculture; and policy instruments that regulate dispensing and utilization of antimicrobials. Reducing selective pressure at the farm is central to AMR control. Evidence-based vaccination, biosecurity, water-quality management, and the targeted use of probiotics/postbiotics and immunostimulants can lower disease incidence and therapeutic antibiotic demand. Additional tools (phage therapy, anti-virulence strategies, selective breeding for robustness) may further reduce antimicrobial inputs when embedded in farm-level stewardship plans and verified by AMR monitoring. These actions should be complemented by monitoring of antibiotic consumption in human and animal populations, integrated risk-based surveillance for resistance in priority foodborne pathogens and hotspot areas, sound waste management to limit environmental dissemination, and sustained training and capacity building. Related to wild-caught seafood and capture fisheries, AMR carriage typically reflects environmental contamination rather than selection pressure on board; consequently, control measures focus on preventing fecal contamination and minimizing cross-contamination along the harvest–landing–processing continuum. Priorities can include: harvesting only from approved/classified waters and enforcing temporary closures after sanitary incidents; strict onboard hygiene (cleanable surfaces, potable water and ice, verified ice-plant hygiene, hygienic evisceration when performed at sea, and proper offal management to avoid re-contamination); rapid time–temperature control (prompt chilling and continuous cold chain) and physical separation of raw and ready-to-eat streams at landing sites and processing plants; validated sanitation programs for vessels, bins, and landing infrastructure, with appropriate detergent/disinfectant selection and rotation to avoid sub-lethal exposures that foster biocide tolerance; traceability of harvest area/date and verification of water/ice quality certificates. For high-risk matrices such as bivalve mollusks, risk reduction further relies on sanitary surveys of

growing areas, depuration/relaying where applicable, and targeted microbiological verification at receiving plants. As part of CCP programs, competent authorities and industry should implement risk-based surveillance for AMR in indicator organisms and priority pathogens at landing and early processing nodes, integrate AMR findings with environmental data (e.g., proximity to wastewater outfalls), and invest in training and capacity building for crews, auction staff, and processors to maintain hygiene and cold-chain discipline.

In a One Health perspective, systematically assessing AMR along seafood supply chains is essential to quantify consumer exposure, prioritize risk-based controls, and guide stewardship beyond clinical settings. Even when classical pathogens are infrequent, harvested fish and processing environments can harbor ARB/ARGs that persist in biofilms, spread via cross-contamination, and affect both safety and shelf-life. Within this context, focusing on *Pseudomonas* spp. in fish fillets is extremely important: these psychrotrophic, biofilm-forming taxa dominate spoilage at refrigeration temperatures, tolerate sub-inhibitory biocides, and act as reservoirs and vectors of resistance determinants. Documenting AMR in *Pseudomonas* spp. therefore fills a surveillance gap with direct operational value (informing validated sanitation (anti-biofilm), water/ice management, and strict time–temperature controls) and provides actionable markers for routine retail monitoring of AMR in seafood.

Microplastics

Plastics are a broad class of synthetic organic polymers, made of long carbon chain, that have revolutionized human society due to their exceptional versatility, durability, moldability, light weight, insulation and low production costs, and are now among the most widely produced human-made materials, alongside steel and concrete. Nowadays, they are widely used across various sectors, including packaging, construction, automotive, electronics, clothing, and agriculture [82]. According to Napper and Thompson [83], global plastic (and single-use throw-away products) production increased exponentially (from approximately 2 million metric tons in the 1950s to over 350 million metric tons in 2019, with a subsequent acceleration during SARS-CoV-2 pandemic) and is projected to continue rising in the coming decades [84-87]. As highlighted by Chamas et al. [88] and Stabnikova et al. [89] the key characteristics that make plastic so useful, contribute to transform these materials into one of the most pressing environmental challenges of our time. Their presence has now been documented across virtually all ecosystems, from terrestrial to marine environments, raising significant concerns regarding their ecological impact [90-92]. Little is known about pollutant effects of plastics debris and is reported that they have been found in all seas and marine environments [93,94], air and food [95]. Marine ecosystems alterations caused by plastic litters can cause harmful effects on global scale [96].

Given the widespread detection of MPs across aquatic ecosystems and seafood value chains, a rigorous understanding of their behavior in food webs and their One Health implications is essential to guide proportionate policy and risk management. However, current evidence on MPs contamination in fish and fishery products (finfish, bivalves, crustaceans) is limited with also several not negligible uncertainties.

A particularly concerning consequence of plastic overuse and mismanagement is the generation of MPs and nanoplastics (NPs), tiny plastic particles formed either intentionally (primary MPs) or through the degradation of larger plastic debris (secondary MPs). These particles have been identified as emerging pollutants, capable of bioaccumulating in organisms, entering food webs, and potentially affecting human and animal health [95,97]. The term “microplastics” has been firstly introduced by Thompson et al. [98] and has later been defined as all plastic fragments smaller than 5 mm in diameter [99].

In case of primary MPs, they are intentionally manufactured in small dimensions for specific industrial or domestic uses (toothpaste, skin cleaners) [100-103].

Secondary MPs are defined as small plastic fragment resulting from the breakdown, fragmentation or deterioration of larger plastic debris which are already present in the environment [104-108]. This occurs once plastics are released in the environment, where they are affected by different external factors like ultraviolet radiation, biological metabolism and physical abrasion from water mechanical stress, resulting in systematic fragmentation in smaller pieces [109-111].

Terrestrial activities represent the primary source of plastic pollution [112] and have been identified different dispersal pathways from terrestrial sources. Agriculture and Wastewater Treatment Plants have been reported as human activities with high environmental impact, and an important source of plastic pollution [113-117]. Synthetic microfibers, shed from textiles during laundering processes, represent an additional and significant source of environmental MPs pollution. Moreover, small plastic fragments can originate from the degradation of larger plastic items (secondary MPs), including packaging materials, bottles, electronic components, vehicle parts, and tires [118-120]. A significant quantity of plastic debris originates from continental sources that primarily enter the marine environment via rivers [121-123]. Any solid waste of anthropic origin entering the sea forms the marine litter, of which is estimated that up to 80% is made of plastic debris [124]. In addition to terrestrial sources, another important source of marine MPs pollution is human activities at sea. Human offshore industrial activities, such as aquaculture, and oil and gas extraction release huge amount of plastic debris in the environment. A notable and direct contributor is aquaculture, as evidenced by the presence of MPs in the water, feed, and cultured fish, such as European seabass, across various production systems including cage, pond, and recirculation aquaculture systems. For farmed fish, water and feed are identified as principal pathways of MP exposure [125-128]. Also, navigation and fishing activities are a substantial source of plastic pollution, mainly for the loss of gears and fishing nets, and more general waste release during various maritime activities (like tourism and shopping) [83].

MPs and NPs are globally mobilized via atmospheric, oceanic, and fluvial pathways, leading to deposition across ecosystems (including remote regions) and creating inhalation and ingestion exposure for humans and biota [129-134]. In the marine realm, currents disperse plastics widely, while organismal uptake and biofouling-driven vertical flux promote trophic transfer and deep-sea accumulation [93,128,135-139]. Widespread ingestion has been documented in finfish and shellfish across multiple basins, underscoring seafood as a relevant human exposure route [140-146].

MPs and NPs readily enter aquatic food webs. Bioaccumulation, the net uptake from all exposure routes, is well established [147-151]. Ingestion is widespread across freshwater and marine taxa (fish, mollusks, crustaceans, zooplankton), with the gastrointestinal tract as the primary reservoir [152-159]. Smaller MPs—especially NPs—can translocate beyond entry epithelia to secondary organs, heightening concern for human health via seafood exposure [151,160-163]. Accumulation depends on environmental concentration, polymer type, particle size/shape, and color [95]. By contrast, evidence for biomagnification is still inconclusive: trophic transfer is documented but does not necessarily imply stepwise increases across trophic levels [159,164-168], and some datasets suggest higher burdens in lower trophic organisms [169]. Distinguishing the potential magnification of plastic particles themselves from that of sorbed additives or co-transported contaminants is therefore critical when evaluating seafood-related public-health risks.

MPs reach organisms via ingestion, inhalation, and dermal contact, with ingestion the dominant pathway across marine and terrestrial taxa [97] (Prata et al., 2020). Numerous marine groups—including zooplankton, bivalves, crustaceans, fish, and whales—ingest MPs; in fish (>150 species reported), particles accumulate primarily in the gastrointestinal tract and can cause mechanical injury, with occasional extra-digestive localization [95,122,141,170-173]. Commercial bivalves consumed whole can contain notable MP burdens (e.g., 4.3–57.2 items/individual in Chinese markets), highlighting a direct human dietary exposure route [174-177]. Human exposure also arises through a wide range of foods and beverages (including seafood) and via inhalation, with animal models showing airway accumulation and inflammation [178-182]. Post-exposure, size-dependent translocation allows MPs, especially smaller particles, to enter circulation and secondary tissues; consistent with this, MPs have been detected in human stool, placenta, lungs, liver, sputum, breast milk, blood, and saphenous vein tissue [175,183-199].

The biological and toxicological impacts of MPs occur through a complex array of mechanisms at the cellular, tissutal, and systemic levels, and can induce cellular toxicity, oxidative stress, inflammatory lesions, and can possibly trigger dysbiosis [149,200-206].

Beyond their physical presence, MPs can serve as carriers for a range of hazardous substances, posing additional risks and concerns to public health following seafood consumption. Their high surface area allows them to adsorb environmental pollutants like toxic metals (e.g., mercury, cadmium, lead, arsenic, etc.), and toxic

organic and inorganic compounds, like persistent organic pollutants (POPs), dichlorodiphenyltrichloroethane (DDT), phthalates and antibiotics [207-213]. MPs are also reported to act as potential vectors for pathogenic microorganisms, antibiotic-resistant bacteria (ARB) and ARGs, which pose a risk for disease transmission and microbial dysbiosis. The surface of MPs in both aquatic and terrestrial environments is rapidly colonized by a wide array of organisms that form a complex microbial community known as the “plastisphere.” This plastisphere is of particular concern for public health due to its capacity to host and facilitate the dissemination of pathogenic and invasive species [214-216]. Interactions with ARB can lead to implications for food safety. MPs may enhance the bioaccumulation of antibiotics in edible organisms such as bivalves by disrupting detoxification processes. Co-exposure to MPs and antibiotics has been shown to result in significantly high antibiotic levels in bivalve tissues, increasing risks for consumers [217,218]. Moreover, ingestion of MPs can lead to intestinal dysbiosis, altering the composition and function of the gut microbiota in aquatic animals and mammals. Such alterations can impair host immunity and promote infections and have been associated with inflammatory bowel diseases and increased abundance of ARGs [206,219-221].

The widespread presence of MPs in marine ecosystems has direct implications for the seafood sector, as numerous commercially important species are exposed to these particles through their diet and surrounding environment. Marine fish and invertebrates, including bivalves, crustaceans, and cephalopods, are particularly vulnerable to MP ingestion due to their feeding strategies, habitat preferences, and trophic interactions. Demersal fish can ingest MPs both directly, by consuming contaminated sediment or water, and indirectly, through trophic transfer from lower-level organisms. Filter-feeding bivalves and suspension-feeding crustaceans are especially prone to accumulate MPs, as they process large volumes of water and retain particulate matter, while cephalopods may be exposed through the ingestion of prey organisms already contaminated with MPs [171,222,223].

From a food safety perspective, the ingestion of MPs by edible marine species represents a potential pathway for human exposure. Studies have documented MP presence in seafood products destined for human consumption, including fish fillets, mollusks, and crustaceans, with particle abundance and characteristics varying according to species, habitat, and processing methods. Although the gastrointestinal tract is often removed prior to consumption, smaller MPs and nanoplastics have the potential to translocate into edible tissues, raising concerns

about their persistence in seafood products. Furthermore, MPs can act as carriers of chemical pollutants and pathogenic microorganisms, including antibiotic-resistant bacteria, potentially amplifying health risks along the seafood value chain [224,225]. In parallel, fish represent one of the most widely consumed sources of animal protein worldwide, and their role as both indicators and vectors of MP pollution is increasingly recognized.

A comprehensive synthesis of available data on MP contamination in fish is essential to identify knowledge gaps, standardize analytical approaches, and support the development of effective monitoring and mitigation strategies [226,227]. In conclusion, there is a clear need for a focused synthesis to guide seafood-safety work on MPs.

Fish Gut Microbiota

The gut microbiota of fish has become a frontier research field. Due to the complexity of the gut microbiota, the structure and function of the gut microbiota in fish have not been studied in depth. Teleost fish harbor complex intestinal microbial communities that are integral to host nutrition, immunity, and overall fitness, while also mediating interfaces with the aquatic environment and food systems. Across species and habitats, fish gut consortia typically comprise Proteobacteria, Firmicutes, and Bacteroidota in varying proportions, with pronounced differences driven by host ecology (diet/trophic level), salinity, temperature, geography, and captive state (wild vs. farmed) [228-230]. Within a One Health perspective, these communities matter beyond fish health: harvesting, evisceration, and processing can mobilize gut-associated bacteria and resistance genes into seafood, facilities, and effluents, influencing both product quality (spoilage) and consumer risk (AMR and specific hazards).

Although a single “core microbiome” for all fishes is unlikely, broad patterns recur. Intestinal microbiota are either indigenous (colonizing the epithelial surface or microvilli) or transient (in the lumen, passing through the gut), and the latter are largely dependent on the diet. Fish with different dietary habits have distinct transient gut microbial communities. Marine carnivores often show Proteobacteria-dominated profiles (e.g., Vibrionaceae/Photobacterium), whereas many freshwater fishes feature high Fusobacteriota/Cetobacterium, a putative vitamin B₁₂ producer linked to protein digestion [230-232]. In salmonids, multiple studies report a Mycoplasma-dominated gut, sometimes >50–70% relative abundance, suggesting an intimate host association [233,234]. Overall composition shifts with trophic level: herbivores often enrich fermenters (e.g., clostridia), while carnivores enrich proteolytic taxa, a pattern seen across controlled and field surveys [228,235]. For apex, highly migratory taxa (e.g., tunas), available data indicate Proteobacteria-rich assemblages with site, temperature, and diet effects. Multi-site work in southern bluefin tuna mapped microbiomes across mucosa and digesta; other studies link thermal stress to shifts in juvenile yellowfin tuna gut communities [236,237]. Systematic gut profiling of large pelagic and economically important fishes remains sparse, underscoring the value of dedicated studies on these species.

Microbiome assembly begins at first feeding and is shaped by water, diet, and habitat; captive/farming conditions can present diversity relative to wild fish. The composition and activity of microorganisms colonizing the gut are closely related to the conditions of the host itself, including its genetic information and

physiological stage, and environmental factors, including feed nutrition, environmental bacteria, stress, etc. Likewise, microbiota colonizing the gut can participate in and influence a variety of physiological activities of the host, including its nutrition, energy absorption, and immunization, especially in adapting to extreme environments. The gut microbiota contributes to nutrient harvest, bile acid and amino-acid metabolism, and gut–brain/endocrine axes affecting feeding and energy homeostasis [229,238]. Dysbiosis has been linked to stress, low-grade inflammation, and heightened pathogen susceptibility in aquaculture settings [239].

Salinity, temperature, diet, and season are major determinants of community structure; habitat and host phylogeny also play an important role. Captivity, feeds (including novel ingredients like insect meal), and husbandry alter diversity and function, with digesta typically showing stronger diet signals than mucosa [228,230,240]. Climate-linked stressors (warming, hypoxia) and pollutants (metals, pesticides, MPs/NPs) can shift gut consortia and barrier function. MPs, in particular, can carry biofilms that enrich opportunists and alter immunity [241,242].

Psychrotrophic Gram-negative bacteria such as *Pseudomonas* spp. and *Shewanella* spp. are key specific spoilage organisms (SSOs) of chilled fish; *Photobacterium phosphoreum* contributes under modified-atmosphere conditions, with species- and packaging-dependent differences. Growth of these taxa shortens shelf-life via proteolysis, lipolysis, and volatile amine production (e.g., TMA), and can be accelerated by lapses in the cold chain [243-245]. Although many SSOs originate from skin, gills, and water/ice, evisceration and handling can spread gut-derived flora to fillets and work surfaces—relevant where *Pseudomonas* and *Shewanella* thrive in processing biofilms. Several fish-associated bacteria (*Morganella morganii*, *Klebsiella*, *Enterobacter*, *Photobacterium damsela*/*phosphoreum*, *Vibrio* spp.) can decarboxylate histidine in muscle to histamine, causing scombroid poisoning when time/temperature control fails. Psychrotrophic *Photobacterium* on gills/viscera and processing surfaces can drive histamine formation even at refrigeration temperatures; strict cold chain, sanitation, and rapid chilling remain the primary controls [246,247]. *Vibrio parahaemolyticus* and *V. vulnificus* risks are well documented in raw mollusks and warm-water seafood; warming seas and lower salinity regimes are expanding environmental suitability and seasonal windows in Europe, with some *Vibrio* showing concerning resistance trends. Risk management emphasizes harvest-area classification, temperature controls, and consumer guidance for raw products [70]. In sum, these microorganisms can contaminate and alter the flesh of fish, shortening shelf-life and, in some cases (e.g., histamine

formers), creating a food-safety hazard. Beyond skin, gills, water and ice, a critical route is gut-derived contamination during evisceration, which can seed fillets and processing surfaces with SSOs and decarboxylase-positive taxa. Once established, they may proliferate even at refrigeration temperatures, driving proteolysis/lipolysis, volatile amine and biogenic amine formation.

Large pelagic predators are ecologically pivotal, widely consumed, and traverse broad thermal, trophic, and geographic gradients, yet—compared with aquaculture mainstays—their gut microbiota remain under-characterized. Emerging tuna studies already demonstrate temperature- and site-linked shifts in intestinal communities [236,237], but compartment-resolved datasets for other large pelagics, notably swordfish (*Xiphias gladius*), are still scarce. This gap matters for food hygiene: the fish gastrointestinal tract harbors dense, diverse microbial consortia that, during capture, evisceration, filleting, and distribution, can cross-contaminate edible tissues and processing surfaces. Such transfer can seed spoilage organisms, introduce opportunistic or zoonotic bacteria, and mobilize ARGs, with direct consequences for shelf-life, product quality, and public health. Robust risk assessment therefore requires species-specific baselines of gut microbiota for commercially important taxa. Against this backdrop, a dedicated swordfish gut-microbiota analysis fills a clear knowledge gap by (i) providing baseline data for an apex pelagic teleost, (ii) contextualizing microbial ecology in relation to diet and migratory behavior, and (iii) informing seafood safety and quality by identifying spoilage-relevant taxa. Establishing a baseline for a widely traded, high-value pelagic species provides the evidence base for targeted interventions at critical control points within a One Health framework, ultimately strengthening seafood safety and consumer protection.

Neglected and Underutilized (Fish) Species

Aquatic foods provide highly bioavailable nutrients that are difficult to replace in many diets, most notably long-chain n-3 PUFAs, vitamin B12, iodine, and selenium [248,249]. Yet global production, research, and policy still focus on a relatively narrow set of major species, while numerous edible fishes and invertebrates remain undervalued or locally underexploited. These are commonly referred to as neglected and underutilized (fish) species (NUS). Although there is no generally accepted definition of NUS [250], they are usually characterized as (i) species included in bycatches [251,252]; (ii) species rarely or never used for human consumption, but instead utilized for other purposes such as fertilizer or bait [253]; (iii) species for which quotas and licenses are established but not fully exploited (i.e., quota limits are not reached) [253]; (iv) species not currently included in licensing frameworks but that could be harvested sustainably [253]; and most importantly, (v) species underutilized due to low market demand [254]. Diversifying toward NUS has been identified as a lever to improve nutrition, livelihoods, and supply resilience within the FAO's "Blue Transformation" agenda and recent global fisheries outlooks [12]. In fisheries science, "underutilized" typically denotes biologically available species with weak markets or high by-catch fractions; from a food-systems perspective, "neglected and underutilized species" emphasizes nutrient density, climate resilience, and historically limited market development [250,255,256]. These framings converge in NUS: taxa with nutritional and livelihood potential but limited evidence and fragile value chains. Crucially, underutilized does not mean safe by default; responsible expansion demands explicit attention to public health and hygiene constraints.

From a systems perspective, bringing NUS to market can (i) reduce pressure on over-targeted stocks, (ii) valorize by-catch and locally abundant species, and (iii) broaden access to nutrient-dense aquatic foods [255]. Risk–benefit analyses further suggest that shifting consumption toward overlooked, often lower-trophic or regional species can yield net health gains when chemical and microbial hazards are effectively managed [257]. However, many NUS lack basic hazard profiles (microbiological, parasitological, toxin, and chemical), validated processing guidelines, and systematic monitoring—gaps that complicate risk assessment and regulatory alignment. Codex's *General Principles of Food Hygiene* and the HACCP annex provide the preventive framework for all foods, but novel taxa and product forms require fresh validation and verification studies [258].

NUS frequently originate in small-scale fisheries (SSF) and artisanal supply chains where infrastructure can be variable. Classic hygiene and temperature-control challenges—rapid post-mortem quality loss, limited access to potable water and ice, handling on unprotected surfaces, and long distances to cold storage—select for psychrotrophic spoilage flora and raise cross-contamination risks if evisceration and chilling are delayed [259,260]. In these contexts, validated standard operating procedures for evisceration, sanitation, and icing can markedly improve shelf life and safety, but they require context-appropriate training, equipment, and verification. Strengthening governance (e.g., traceability, territorial use rights for fisheries) and embedding hygiene metrics in co-management can align sustainability and food-safety objectives [12,260].

Global cephalopod landings and prices have drawn attention to non-traditional cephalopod sources and species, expanding markets beyond well-studied octopuses and squids [261-263]. Cephalopods pose distinctive public-health and hygiene considerations: rapid autolysis and high water activity drive fast quality decline unless rapidly chilled; skins, gills, and viscera carry abundant microbiota; product diversity (whole, eviscerated, raw, marinated) demands product-form-specific controls; and the digestive gland can accumulate metals [264,265].

Along the coasts of South America, cephalopod fishing is a well-developed traditional and industrial activity. Large, regionally traded cephalopods such as the Patagonian red octopus (*Enteroctopus megalocyathus*) illustrate the dual opportunity and challenge of NUS. This benthic cephalopod is harvested in nearshore habitats often influenced by human activities, increasing the likelihood of exposure to microbial contamination (e.g., fecal indicators, *Vibrio* spp., *Aeromonas* spp.), chemical pollutants (notably metals accumulated in cephalopod digestive glands), and marine biotoxins associated with harmful algal blooms. Post-harvest issues typical of artisanal supply chains—variable hygiene at landing sites, delays in cleaning/evisceration, inadequate chilling, and cross-contamination during transport—can further reduce shelf life and increase consumer risk, especially when the product is eaten raw or minimally processed. On the one hand, such species contribute to nutrition and livelihoods in coastal communities and offer diversification potential; on the other, they highlight evidence gaps typical of NUS: limited species-specific hazard profiles (e.g., tissue-specific metals), variable hygiene infrastructure in small-scale fisheries, and product-form heterogeneity that complicates the development of comprehensive guidance. Situating *E. megalocyathus* within this framework clarifies why there is the need of studies that

focus on different NUS following the whole supply chain: to document baseline hazards, identify critical control points (rapid evisceration, cold chain, sanitation), and align artisanal practices with Good Hygiene Practices/HACCP, thereby derisking NUS integration into markets while preserving their socioeconomic value.

OBJECTIVES

The main aims of this PhD work are 1) describe emerged (AMR) and emergent (MPs) safety hazards connected to different seafood products; 2) enhance the understanding of the fish gut microbiota of major commercial species and establishes a baseline for swordfish (*Xiphias gladius*); 3) evaluates food-safety concerns in neglected and underutilized species (*Enteroctopus megalocyathus*) providing an update review on fisheries, including artisanal fisheries, in the Los Lagos region, with the aim of detailing general food safety aspects in octopus fisheries and products.

This PhD thesis will be divided in chapters as follows:

- i) the first two chapters will deal with the first objective showing, in one hand, resistance phenotypes in *Pseudomonas* spp. isolated from fresh fillets of major commercial species sold in Italy—*Salmo salar*, *Gadus morhua*, and *Pleuronectes platessa*, and, in the other, synthesizing current evidence on MPs contamination in fish and fishery products (finfish, bivalves, crustaceans) and the associated risks linked to human dietary exposure.

Chapter 1: The documented environmental dissemination of AMR has heightened concerns for consumers of both farmed and wild seafood. Accordingly, robust surveillance of antimicrobial-resistant bacteria and ARGs in fish products is essential to protect human health. In this context, we quantified resistance phenotypes in *Pseudomonas* spp. isolated from fresh fillets of major commercial species sold in Italy—*Salmo salar*, *Gadus morhua*, and *Pleuronectes platessa*. This provides tangible evidence that AMR concerns are not confined to clinical settings or aquaculture facilities; rather, they extend to consumer-level products where exposure pathways are immediate. These data underscore the need to monitor not only clinically dominant pathogens but also environmental and opportunistic bacteria that can act as reservoirs, vectors, or exchange partners for ARGs.

Chapter 2: To address additional emerging hazards with potential public-health implications, we conducted a critical review of MPs in seafood, synthesizing evidence on their occurrence in edible products and the associated risks linked to dietary exposure. This review synthesizes current evidence on MPs contamination in fish and fishery products (finfish, bivalves, crustaceans), including sources, occurrence across species and processing steps. It also delineates consumer exposure pathways and explains plausible health mechanisms—particle-mediated

effects, leaching of additives and sorbed pollutants, and biofilm-enabled transport of microbes and antimicrobial resistance genes—and highlights key gaps and major uncertainties in human toxicokinetic and dose–response that limit risk assessment. It also highlights methodological constraints that hinder cross-study comparability and robust risk quantification, and sets priorities for harmonized surveillance and standardized exposure metrics. The implications for public health are clear: risk assessments must move beyond single-agent paradigms to consider co-occurrence, co-selection, and the cumulative burden of exposures arising from complex seafood matrices. Framed within a One Health perspective, these insights provide a policy-relevant basis for upstream source reduction and targeted supply-chain controls (e.g., depuration and processing hygiene) aimed at minimizing consumer exposure and safeguarding public health.

- ii) The third chapter will focus on the second objective, describing the first metataxonomic characterization of gut microbiota of Swordfish using next-generation sequencing.

Chapter 3: Microbiological hazards remain central to the epidemiology of foodborne illness; therefore, understanding the microbial composition of seafood products and identifying potential contaminants are fundamental to risk assessment. To provide an ecological baseline for a high-value species, we produced the first characterization of the swordfish (*Xiphias gladius*) gut microbiota, offering insights into the core community structure of this important commercial taxon. Establishing the structure and putative functions of the *X. gladius* gut community is not merely descriptive: it provides a scaffold against which perturbations due to capture methods, handling, environmental stressors, or dietary shifts can be detected, and it informs hypotheses about how commensal microbiota mediate spoilage trajectories, ARG carriage, and potential pathogen colonization in large pelagic fish.

- iii) The last chapter will finally address the last objective, describing a neglected and underutilized fish species in terms of its fishery and food safety aspects.

Chapter 4: applying the same integrative approach to neglected aquatic species, we developed a concise review of the Patagonian red octopus (*E. megalocyathus*) covering core biology and fisheries management, with a specific emphasis on public-health considerations and actionable measures to reduce risk in harvesting

areas. This integrated body of work advances a forward-looking agenda for seafood safety at the interface of human, animal, and environmental health. This highlights how public health and food safety considerations must be integrated into local fisheries management for species that are rising in global markets but often lack comprehensive risk profiles. For *E. megalocyathus*, targeted guidance on harvest practices, cold-chain integrity, and hygiene at landing sites—as well as surveillance for microbial and chemical hazards—is essential to preempt risk transfer to consumers and to support equitable value chains for artisanal fishers communities.

Collectively, the evidence assembled here advances three strategic priorities. First, strengthen integrated surveillance for AMR and MPs across the aquatic food chain. Second, invest in preventive health and biosecurity in aquaculture to reduce therapeutic pressure and consequent resistance selection and spread while protecting product hygiene. Third, extend food-safety frameworks to emerging and neglected species, coupling biological and fishery knowledge with tailored hygiene protocols and community-appropriate interventions. By weaving together the presence of AMR phenotypes in retail products, the role of MPs, the foundational microbiome baselines in key commercial species, and management guidance for under-studied invertebrates, this thesis argues for a forward-looking seafood safety agenda. Such an agenda is premised on One Health integration, sensitive to the realities of global environmental change, and oriented toward actionable risk mitigation that protects consumers while supporting sustainable aquatic production systems.

CHAPTER 1 - PREVALENCE AND ANTIBIOTIC RESISTANCE PHENOTYPES OF *PSEUDOMONAS* SPP. IN FRESH FISH FILLETS

Mhenni, N. B., Alberghini, G., Giaccone, V., Truant, A., & Catellani, P. (2023). Prevalence and antibiotic resistance phenotypes of *Pseudomonas* spp. in fresh fish filets. *Foods*, 12(5), 950. <https://doi.org/10.3390/foods12050950>

1.1 INTRODUCTION

The microbiota of fishery products is made up of a set of microbial populations (bacteria, yeasts and/or molds), and the bacteria belonging to the Pseudomonadaceae family are one microbial population, alongside enterobacteria, coliforms or lactic acid bacteria. The genus *Pseudomonas* counts over 200 species divided into 11 subspecies. These bacteria are widely spread in the terrestrial and aquatic environment, both in fresh water and salt water. Based on currently available references, the species of *Pseudomonas* (hereafter sometimes only “*P.*”) most commonly isolated in fish are *P. fluorescens*, *P. lundensis*, *P. fragi*, *P. anguilliseptica* and *P. putida* [1–3]. The species of *Pseudomonas* that is mainly considered a human pathogen is *P. aeruginosa*, but it is not so common in food products [4].

Pseudomonads are one of the most relevant specific spoiling microorganisms (SSOs) because with their excessive proliferation, they enhance the splitting of nitrogenous compounds leading to product deterioration [5]. It is widely demonstrated that Gram-negative bacteria belonging to the Pseudomonadaceae family are one of the most active

microbial populations in the production of proteolytic, lipolytic and saccharolytic enzymes (also extracellular ones). Furthermore, many species of *Pseudomonas* spp. are able to synthesize yellow, green fluorescent, blue and red pigments that can lead to the development of anomalous colors [5–8]. The discoloration and the development of unpleasant off-odors and slime coats characterize the spoilage of fresh fish, whole or portioned if they are stored in the air and even if stored at refrigeration temperatures [5,9]. In fact, Pseudomonadaceae are aerobic microorganisms and grow only in the presence of molecular oxygen. In vacuum- and CO₂-packed stored fish, the number of *Pseudomonas* spp. is reduced [5].

Grooming and filleting operations bring additional loads of microbial community to the product being processed, with the risk of shortening the commercial life of the product itself [10].

Pseudomonas spp. can produce tough microbial biofilms on all surfaces they come into contact with, and these biofilms can offer shelter and protection to foodborne disease bacteria. The presence of high loads of *Pseudomonas* spp. in food or in any other substrate

can favor the survival of *Listeria monocytogenes*, *Staphylococcus aureus* and enteropathogenic strains of *E. coli* (STEC) [11–14]. Due to the biofilm produced by

Pseudomonas spp., it is not easy to eliminate the bacteria that are hosted there and, on the contrary, they are able to resist various environmental stresses, including chlorine disinfectants. Nikel and co-authors reported that *P. putida* possesses metabolic mechanisms that allow it to resist oxidative stress [15]. It is also known that Pseudomonadaceae are able to withstand a certain osmotic pressure (salinity of the medium) thanks to specific molecules present in their cytoplasm that can hook osmoprotective compounds such as choline, betaine and carnitine from the environment in which the bacteria are found, favoring the survival of species such as *P. syringae* and *P. aeruginosa* [16].

In general, it is accepted that a low number of *Pseudomonas* spp. does not pose a health problem for the consumer. Instead, Pseudomonadaceae can produce histamine and become a risk to human health if they proliferate excessively in fish that are inherently high in free histidine [17]. For this and for the reasons considered above, it is therefore wise that FBOs take into due consideration the presence of *Pseudomonas* spp. in whole fresh fish as well as in fresh fillets, formally giving to it the qualification of “process hygiene criterion” pursuant to EC Regulation n.2073/2005 [18] that establishes some “microbiological criteria for foodstuffs”.

The aforementioned Regulation is addressed to the FBOs who use it as a reference to check and validate their self-monitoring plan as well as the safety level of their products as required by European laws. The process hygiene criteria currently provided by the Regulation n.2073/2005 for fishery products are the loads of *E. coli* and coagulase positive staphylococci; in addition, only shelled and shucked products of cooked crustaceans and shellfish are the fishery products considered.

The consumption of fishery products is regularly increasing, especially in low- and middle-income countries. Between 1961 and 2016, the demand for fish products increased by about 3.2% per year, while the need for meat from terrestrial animals increased by only 2.8% [19,20]. According to the FAO Report 2022 on “The State of World Fisheries and Aquaculture”, in recent years, there has been a sharp increase in the consumption of fresh, chilled and frozen fish products, while the consumption of salted smoked or canned products remains stable. At the same time, aquatic food production is increasing; China, Norway, Vietnam, Chile and India are the major producers and exporters in the world [19]. In 2020, the global production of fishery products reached 178 million tons (with aquaculture representing 49.2% of the total), and it is forecasted to grow another 15% by 2030 [19].

Aquatic food systems are under growing pressure, and many reports document that the use of antimicrobials in fish farming is consequently growing. Researchers estimate that in 2030, about 13,495 tons of antimicrobials will be used in

aquaculture, which, therefore, represents 5.7% of the total amount used in the world (by adding those used in humans and in all other animals) [21]. This also draws much attention to the fact that most classes of antimicrobials are used for the treatment of bacterial infections in both veterinary and human medicine [22]. This increases the speed of selection of drug-resistant bacteria [23], and antimicrobial-resistant bacteria can reduce the effectiveness of treatments, especially in Gram-negative bacterial infections [24]. Thus, the careless use of antibiotics in aquaculture is a contributing factor to the rise in antimicrobial resistance (AMR), leading to potential animal, human and ecosystem consequences [21].

The relationship between the detection of antibiotic-resistant bacteria in humans and antibiotic use in food animals continues to be a topic of discussion among researchers.

Various studies have suggested that the use of antibiotics in animals can directly affect human health through direct contact with antibiotic-resistant bacteria from food animals [23]. Other authors argue indirect effects that result from contact with resistant organisms that have spread to various components of the ecosystem (e.g., water, soil etc.), and so they can indirectly pass from animals to humans and vice versa. This indirect effect can be greatly increased by the horizontal transfer of mobile genetic elements such as conjugative plasmids, phages and transposons [25]. From a holistic view of AMR in different sectors, there are few studies that directly consider foods and not clinical isolates. Moreover, current AMR research, surveillance programs and dietary risk assessment studies (as the potential transmission of AMR), focus mainly on food-producing terrestrial animals and a few indicator bacteria. So, considering that world seafood production is increasing every year, our study aims to provide additional data to implement those already known regarding *Pseudomonas* spp. which is considered a dominant bacterial genus in food processing facilities and in the microbial community of many fish products [4].

Many strains of *Pseudomonas* have been shown to possess a high level of intrinsic resistance to most antibiotics [26]. This intrinsic resistance is mainly conferred by concurrent mechanisms, such as low outer membrane permeability, efflux systems that pump antibiotics out of the cell and the production of antibiotic-inactivating enzymes such as β -lactamases [17,27]. For now, various resistance genes have been described in the literature, and as it is a possible source of new resistance genes, *Pseudomonas* spp. should be better monitored [28] in their evolution.

Considering only the strains of *Pseudomonas* spp. isolated from food, previous studies reported in the literature confirm that various strains are able to resist some

antimicrobial agents of different classes, especially β -lactams such as penicillins, cephalosporins, carbapenems, and monobactams [29,30]. For example, the study reported by Kačániová and co-authors [31] reveals a high proportion of resistant strains among the *Pseudomonas* spp. isolated originated from fish. Moreover, all *Pseudomonas* were resistant to meropenem. According to Fazeli and Momtaz [32], bacterial strains exhibited the highest level of resistance to penicillin (100%) followed by tetracycline (90.19%), streptomycin (64.70%) and erythromycin (43.13%). Shabana and co-authors [33] showed that *P. fluorescens* strains were resistant to more than two classes of antibiotics and, therefore, they are considered multidrug-resistant (MDR) strains and accounted for 29.7%.

The aim of our study was to determine and characterize a *Pseudomonas* spp. population in fresh fish fillets at the beginning of their shelf life and evaluate the degree of antimicrobial resistance of the isolated strains in order to provide useful data for monitoring its development from the point of view of food safety.

1.2 MATERIALS AND METHODS

1.2.1 Samples collection

A total of 75 fish fillets intended for large retailers were collected from a fish industry located in the Venetian area (Italy) in 5 different sampling times from March to May 2022. Each time, 15 samples were analyzed, namely:

- Five salmon (*Salmo salar*) fillets reared in Norway;
- Five plaice (*Pleuronectes platessa*) fillets caught using trawling in the Northeast Atlantic Ocean;
- Five northern cod (*Gadus morhua*) fillets caught using longlining in the Northeast Atlantic Ocean.

The fish arrived in Italy by ship and under melting ice or frozen, as established by the EC Regulation n.853/2004. The samples of salmon and plaice were fresh, while the cod fillets were defrosted at the processing site. The fillets were collected from the fish industry early in the morning after packaging and immediately transferred to the laboratory. During transport (less than one hour), they were preserved at refrigeration temperature in expanded polystyrene containers with eutectic plates. In the laboratory, the fillets were immediately subjected to microbiological analysis for Total Viable Count and *Pseudomonas* spp. count using the following methods: UNI EN ISO 6887-1: 2017, UNI EN ISO 7218: 2013, UNI EN ISO 4833-1: 2013, UNI EN ISO 11133: 2014, UNI EN ISO 13720:2010.

1.2.2 Samples processing

According to the guidelines provided by UNI EN ISO 6887-1:2017, 10 g of each sample was moved into a sterilized container with 90 mL of Buffered Peptone Water (BPW) under strict hygienic measures, and homogenization was performed using a Stomacher 400 Lab blender.

From this mixture, 1 mL was transferred to a sterilized test tube containing 9 mL of BPW, from which ten-fold serial dilutions were processed up to 10^{-5} . The prepared samples were subjected to the determination of *Pseudomonas* counts using *Pseudomonas* Agar Base (PAB) supplemented with CFC Supplement (Liofilchem®) and Total Viable Count (TVC) using Plate Count Agar (Merck®). The *Pseudomonas* Agar Base inoculated plates were incubated in a fridge-thermostat at 25 °C for 48 h, while the Plate Count Agar inoculated plates were incubated at 31 °C for 48–72 h.

1.2.3 Plate counting and Statistical Analysis

The colonies were counted using the UNI EN ISO 7218:2013 standard as a reference. The results were expressed in the colony-forming unit (cfu)/g and then subjected to the determination of arithmetic mean and statistical analysis.

Log-transformed data (Total Viable Count and *Pseudomonas* spp.) were analyzed using a linear ANOVA model that included the fixed effects of fish type and sample ID (nested within the fish type). Least squares means (ls-means) were calculated and post hoc pairwise comparisons were performed using the Bonferroni correction. Data expressed as an index [formula: number of figures-1; $10^{\text{(order of magnitude with powers of 10)}}$] were submitted to non-parametric analysis (Kruskall–Wallis test) to assess the effect of the type of fish.

Post hoc pairwise comparisons were estimated using the Steel–Dwass–Critchlow–Flinger correction. $p < 0.05$ was considered significant. All the analyses were carried out with SAS (SAS Institute Inc, Cary, NC, USA 2017) and XLStat (XLSTAT statistical and data analysis solution. New York, NY, USA).

1.2.4 Isolation of *Pseudomonas* spp. and Species Identification

At least 5 colonies grown on PAB were selected from each plate and their oxidase and catalase activities were tested. The oxidase test was performed by smearing a fresh colony of each isolate onto a sterile oxidase filter paper disc soaked in distilled water. The appearance of purple color indicates a positive result of oxidase activity. The catalase test was carried out using hydrogen peroxide and the positive reaction was indicated by immediate bubbling.

At this stage, if the colonies gave positive results to the two tests mentioned, the nonfermentation activity was evaluated using the Kligler Iron test. In the Kligler Iron Agar inflexion test, bacterial growth was evaluated after 24 h of incubation at 37 °C (time and temperature are those written in the technical data sheet of the media). Presumptive *Pseudomonas* spp. developed only on the surface because they are strictly aerobic bacteria. Furthermore, this bacterium does not ferment glucose or lactose but can split peptones, so the indicator turned purple (alkaline pH) on the surface. Finally, the counts were corrected proportionally to the results of the tests provided by the ISO mentioned above. Some of the colonies which proved positive for the 3 tests were identified by a unique alphanumeric code, purified on Brain Heart Infusion agar (Thermo Scientific™ Oxoid™), inoculated into Plate Count Agar tubes and stored in the refrigerator at 4 °C. Then, the isolated colonies ($n = 55$) were subjected to biochemical identification with the BIOLOG® system. Biolog's carbon source utilization technology identifies microorganisms depending on their characteristic metabolic pattern produced as a consequence of some pre-selected test. The reading is performed by comparing the results to an extensive database.

1.2.5 Determination of *Pseudomonas* spp. Phenotypic Antibiotic Resistance

The analyses were performed following the recommendation of EUCAST 2022 for the method of execution [34]. Antimicrobial resistance was tested using the standard disc diffusion method.

Suspensions of *Pseudomonas* spp. were cultivated on Mueller Hinton agar (Merck®) with modifications regarding incubation temperature for the psychrotrophy of the bacterium itself. Then, antimicrobial discs (Liofilchem®) were placed on the agar surface taking care to preserve as much sterility as possible. All the strains of *Pseudomonas* spp. were tested against 15 different commercial antimicrobial agents chosen among the most used and important in human medicine and aquaculture worldwide [21,35,36]; the concentrations were instead chosen on the basis of preliminary studies. The antimicrobials were ciprofloxacin (5 µg/disk), enrofloxacin (5 µg/disk), tetracycline (30 µg/disk), rifampicin (30 µg/disk), erythromycin (15 µg/disk), vancomycin (30 µg/disk), clindamycin (10 µg/disk), meropenem (10 µg/disk), trimethoprim (5µg/disk), penicillin G (10 IU/disk), ampicillin (10 µg/disk), amoxicillin (30 µg/disk), flumequine (30 µg/disk), florfenicol (30 µg/disk) and sulfadiazine (300 µg/disk). Inoculated plates were incubated at 31 °C for 24 h. Then, the diameters were measured in millimeters with a caliper.

For the interpretation of the results, neither EUCAST nor CLSI (Clinical and Laboratory Standards Institute) provides criteria and reference values for

Pseudomonas spp., so, it was decided to consider as “highly resistant” those cases in which the diameter of the inhibition zone was ≤ 10 mm.

1.3 RESULTS AND DISCUSSION

1.3.1 *Pseudomonas* spp. Count, Total Viable Count, Data Elaboration

The load of *Pseudomonas* spp. in the samples analyzed has been reported in Figure A1. Indeed, in the salmon fillets, in 19 samples out of 25 (76%), the initial loads were between 10^4 and 10^5 cfu/g, a rather significant load considering that these are fresh fish products. In the remaining samples, the charges of *Pseudomonas* spp. were between 10^3 and 10^4 cfu/g. In only one sample, 10^6 cfu/g has been detected. In the plaice fillets, 13 out of 25 (52%) of the samples analyzed showed a load of *Pseudomonas* spp. between 10^4 and 10^5 cfu/g, and the remaining 12 samples showed loads between 10^5 and 10^6 cfu/g since the beginning of their shelf life. According to Silbande and co-authors [37], *Pseudomonas* loads equal to or greater than 10^6 cfu/g may already be sufficient to compromise the organoleptic qualities of fresh fish products. Therefore, in the salmon and plaice fillets, the loads of *Pseudomonas* spp. were, on average, between 10^4 and 10^5 cfu/g; nevertheless, some of the samples presented an initial load between 10^5 and 10^6 cfu/g of the fillet. Higher loads in some fillets could be due to transport or to the processing environment and also to the moment of processing (beginning vs. end) of that specific product compared to the other products belonging to the same batch.

In the defrosted northern cod fillets, the number of samples that contained 10^4 – 10^5 cfu/g was 14 out of 25 (56%), while the number of samples that showed amounts of *Pseudomonas* spp. between 10^5 and 10^6 cfu/g was 10. In only 1 sample of northern cod fillet, an exceptionally low load of *Pseudomonas* spp. (i.e., 10^2 cfu/g) was detected. In addition to the reasons described above for salmon and plaice, the remarkable loads of cod fillets could be also explained by the fact that cod fillets were a defrosted product, so the load of *Pseudomonas* spp. could have been affected by the specific pre-existing microbial population in the fish prior to freezing, although it is admitted that freezing may have inactivated part of this specific microbial population [38].

It is also interesting to note that loads of *Pseudomonas* spp. in plaice and cod fillets have progressively increased with the progress of the season and, therefore, going towards higher environmental temperatures. Indeed, the loads of *Pseudomonas* spp. were relatively low in the samples analyzed in March and April, while they increased in the April/May period [39]. However, this was not observed in salmon

fillets that presented a rather “homogeneous” microbial distribution and, in any case, further and larger studies should be performed to confirm this hypothesis. Generally, the average for the counts of pseudomonads detected in all three species analyzed is around 10^4 cfu/g (see Table 1) with some occasional increases towards higher loads (10^5 – 10^6 cfu/g) rather than lower levels.

Table 1. TVC and *Pseudomonas* spp. back-transformed ls-means with 95% CI in the three species of fish fillets.

Fish Fillets	TVC Mean Value (cfu/g)	<i>Pseudomonas</i> spp. Mean Value (cfu/g)
<i>Gadus morhua</i>	2.57×10^5 (1.83 – 3.61×10^5) ^a	4.67×10^4 (2.68 – 8.16×10^4) ^{ab}
<i>Pleuronectes platessa</i>	2.56×10^5 (1.82 – 3.60×10^5) ^a	6.71×10^4 (3.85 – 1.17×10^5) ^a
<i>Salmo salar</i>	1.07×10^5 (7.65 – 1.51×10^5) ^b	2.33×10^4 (1.34 – 4.06×10^4) ^b

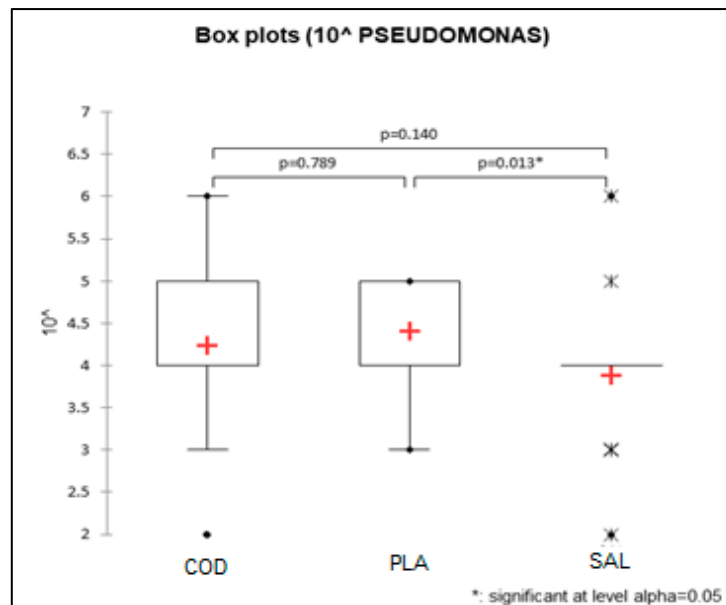
^{a,b} Different letters along columns mean significantly different values ($p < 0.05$)

Regarding the Total Viable Count (TVC) in the three species of fillets analyzed (see Figure A2), the values were in most cases comprised between 10^4 and 10^5 cfu/g or between 10^5 and 10^6 cfu/g. In 10 samples out of 75, the Total Viable Count was higher, between 10^6 and 10^7 cfu/g. The arithmetic mean values were around 10^5 cfu/g (see Table 1). These values lead to the conclusion that a large part of the microbiota found in the analyses was made up of Pseudomonadaceae with other minor microbial populations. This confirms that the loads of *Pseudomonas* spp. have an important influence on the shelf life of the fish fillets considered.

The ANOVA model using log-transformed Total Viable Count data showed a significant effect of the fish type ($p < 0.001$). Back transformed ls-means pointed out that the loads of *Pseudomonas* spp. in salmon fillets were significantly lower than those in cod and, respectively, in plaice fillets (1.07×10^5 vs. 2.57×10^5 vs. 2.56×10^5 cfu/g, $p < 0.01$, see Table 1).

The effect of fish type was also significant for *Pseudomonas* spp. loads, with salmon being significantly lower than plaice (2.33×10^4 vs. 6.71×10^4 cfu/g, $p = 0.028$, Table 1) and cod being between the two above (4.67×10^4 cfu/g). The Kruskal–Wallis test showed a significant effect of fish type only for *Pseudomonas* spp. ($p = 0.021$) with the same results as the previous linear ANOVA model (Figure 1).

Figure 1. Box plot of the $10^{\wedge}$ index (order of magnitude with powers of 10) for *Pseudomonas* spp. The boxes contain 50% of the data distribution, the red crosses represent the average values, the whiskers are proportional to the interquartile range and the points and stars are the outliers as a function of the different interquartile ranges. "COD" refers to northern cod, "PLA" to plaice and "SAL" to salmon.



1.3.2 Phenotypic Identification of *Pseudomonas* Species

During our study, from the analyzed fish fillets, we selected 55 strains of presumptive *Pseudomonas* spp. that have been biochemically identified with the BIOLOG® system. Overall, 67,27% (37 up to 55) of the strains were definitively confirmed as *Pseudomonas* spp., while the other genera were all Gram-negative bacteria, such as *Kluyvera*, *Proteus*, *Citrobacter*, *Klebsiella*, *Serratia*, *Roseomonas* and *Providencia*. This finding is very similar to the one of another recent study carried out on the salmon processing environment where, out of 222 presumptive *Pseudomonas* isolates, 68% were confirmed as *Pseudomonas* [40]. This means that the identification of species on a certain number of bacterial colonies isolated is necessary to establish a higher degree of precision in the loads recorded. The bias of this methodological approach is represented by the economic costs and waiting times to obtain the final results.

Pseudomonas isolates originating from salmon, cod and plaice fillets are diverse with many species represented. Our isolated *Pseudomonas* species were *P. fluorescens*, *P. fragi*, *P. lundensis*, *P. marginalis*, *P. syringae*, *P. taetrolens*, *P. chlororaphis*, *P. tolaasii* and *P. viridilivida*. Indeed, 45.95% of the strains are represented by *P. fluorescens*, which largely dominates the Pseudomonadaceae population in the analyzed fillets, followed by *P. fragi* (21.62%), *P. marginalis* (10.81%), *P. taetrolens* (8.11%), *P. lundensis*, *P. syringae*, *P. tolaasii*, *P. viridilivida* and *P. chlororaphis* (2.70% each one).

It should be noted that this study has some limitations, mainly the lack of molecular identification using 16S rRNA and some housekeeping genes. It is, however, true that the genus *Pseudomonas* is very large and includes several hundred unclassified strains. So, for this complex, genus sequencing of the 16S rRNA gene can often only

identify the three main lineages (*P. aeruginosa*, *P. pertucinogena* and *P. fluorescens*) but cannot give information about the species [40].

These data confirm what has already been described in the literature, which indicates that among the major components of Pseudomonadaceae in fishery products, there are *P. fluorescens* and *P. fragi* [1–3]. It should be noted that *P. aeruginosa* was never found among the strains isolated, and this is in line with what is reported by other studies [4] which highlight a low prevalence of the *P. aeruginosa* species in foods, including in drinking water. As it is known, *P. fluorescens* and *P. marginalis* were able to produce fluorescent pigments on culture medium when illuminated with a Wood's lamp light (Figure A3). Additionally, some strains of *P. chlororaphis* also produced fluorescent pigments.

1.3.3 Antimicrobial Resistance

In order to ensure the best growing conditions, in our study we changed the temperature of incubation of the Mueller–Hinton plates as the tested isolates were psychrophiles and could not grow well at high temperatures. The assay for these was conducted at 31 °C for 24 h. It was decided to consider as “highly resistant” those cases in which the diameter of the inhibition zone was ≤ 10 mm as it was impossible to find guidelines that provided identification criteria. The reasons were that, on the one hand, the incubation temperature was different and, on the other hand, neither EUCAST nor CLSI give enough information about the *Pseudomonas* species. Resistance was found with a prevalence greater than 50% (see Figure A4) in the following cases: penicillin G (penicillins), ampicillin (penicillins), amoxicillin (penicillins), tetracycline (tetracyclines), erythromycin (macrolides), vancomycin (glycopeptides), clindamycin (lincosamides) and trimethoprim (diaminopyrimidines). It should be noted that, according to the WHO classification (2019) [36], macrolides and glycopeptides are CIAs (Critically Important Antimicrobials), while lincosamides and diaminopyrimidines are HIAs (Highly Important Antimicrobials).

Unlike Kačániová and co-authors [31], who found resistance of all *Pseudomonas* strains to meropenem, in our study, only 16.22% of the isolates were resistant to this antibiotic. Instead, according to Fazeli and Momtaz [32], the bacterial strains showed a high level of resistance to penicillin (86.49%), tetracycline and erythromycin but with greater resistance to erythromycin (64.86%) rather than to tetracycline (54.05%). In our case, these two were surpassed (or equaled) by clindamycin (70.27%), vancomycin and trimethoprim (both 64.86%).

In the literature, various resistance mechanisms are described that could explain some of the cases detected in our study, such as β -lactamases against penicillins, efflux pump against erythromycin and dihydrofolate reductase against trimethoprim [24,27,28,41].

Countless acquired resistance genes have also been discovered [28]; however, few studies on the possible mechanisms of the resistance owned by *Pseudomonas* spp. against clindamycin and vancomycin are reported in the scientific literature.

In our research, we noticed that 76.47% of the strains of *P. fluorescens* were resistant to more than two antibiotic classes and so we considered them as multi-drug resistant (MDR) according to Shabana and co-authors' definition of "MDR strains" [33].

1.4 CONCLUSIONS

Based on the data obtained and the statistical analyses mentioned above, we can conclude that, under the basic hygienic conditions of production established by the EC Regulations n.852/2004 and 853/2004, fresh fish fillets (salmon, plaice, cod) intended for largescale retailers have a load of *Pseudomonas* spp. of about, on average, 10^4 cfu/g. In salmon fillets, the counts of *Pseudomonas* spp. recorded have sporadically reached even 10^5 and 10^6 cfu/g. In contrast, in plaice and cod fillets, the upward swing of *Pseudomonas* loads to between 10^5 and 10^6 cfu/g were more frequent.

Moreover, in our study, we noted a significant effect of the fish type on the *Pseudomonas* population of the fillets. The loads of *Pseudomonas* spp. in salmon fillets were significantly lower than in plaice fillets ($p = 0.028$), while in cod fillets, they ranged between the two above-mentioned species.

Considering the Total Viable Count of around 10^5 cfu/g (mean value), we can also conclude that a large part of the microbial community found on the analyzed fish fillets was made up of Pseudomonadaceae with other minor microbial populations. The most frequently isolated *Pseudomonas* species was *P. fluorescens*.

As *Pseudomonas* is the main SSO of fresh fish fillets, it is certainly worth keeping an eye on the colony-forming units per gram of fish muscle. In our opinion, fresh fish fillets should contain low loads of *Pseudomonas* spp. during production in order to avoid rapid degradation of the product during its shelf life. It would be advisable that in the fresh fish fillets, the initial *Pseudomonas* load should be lower than 10^4 or 10^5 cfu/g to not exceed the concretely spoiling loads of these bacteria. In general, in the literature [37,42], it is admitted that up to 10^6 cfu/g of SSO, the organoleptic quality is fine. Furthermore, considering the normal growth potential of

Pseudomonas spp. in refrigerated fresh fish products, it is possible to act both on conservation techniques (for example vacuum packing) and on greater hygiene in the fish processing environment to limit their proliferation. It would be advisable that, at the refrigeration temperature, fresh fish fillets have a shelf life of no more than six days; five days would be even better. Moreover, our antimicrobial resistance study on the 37 isolated strains of *Pseudomonas* spp. showed that these bacteria have a high level of phenotypic resistance to various antimicrobial classes. More specifically, 8 out of 15 antimicrobials tested proved ineffective in more than 50% of the strains. These eight antimicrobials are penicillin G 10 IU, ampicillin 10 µg, amoxicillin 30 µg, tetracycline 30 µg, erythromycin 15 µg, vancomycin 30 µg, clindamycin 10 µg and trimethoprim 5 µg. This highlights that 76.47% of *P. fluorescens* strains were multi-drug resistant.

In the literature, various resistance mechanisms are described (both intrinsic and acquired) such as low outer membrane permeability, β -lactamases synthesis and efflux pump systems. However, it is worth noting that few studies on the possible mechanisms of the resistance of *Pseudomonas* spp. against clindamycin and vancomycin are available. In order to deepen our research and obtain more relevant results, our recommendation is to complete this study with a genomic analysis by performing a whole genome sequencing of the isolated strains of *Pseudomonas* (WGS). Therefore, our results suggest that *Pseudomonas* spp. should be monitored as possible source of other resistance genes. It would be preferable to complete this not only on strains isolated from clinical specimens but also on the strains isolated from food samples.

1.5 APPENDIX A

Figure A1. Representation of *Pseudomonas* spp. loads detected in the analyzed samples of the three studied fish species.

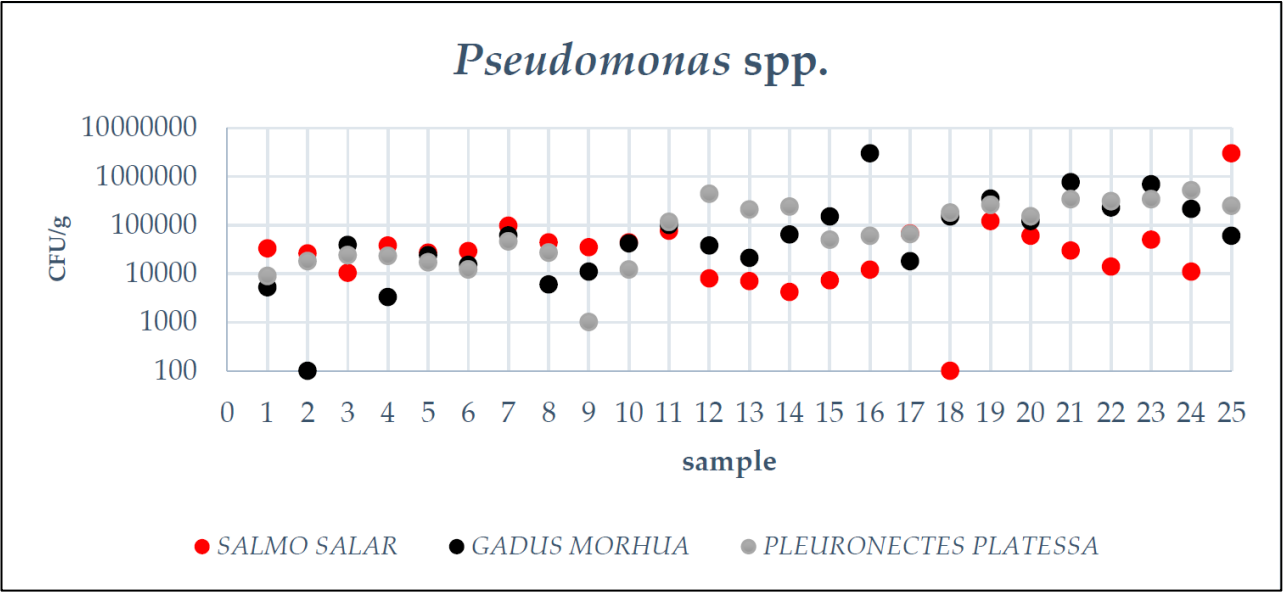


Figure A2. Representation of TVC in the analyzed samples of the three studied species.

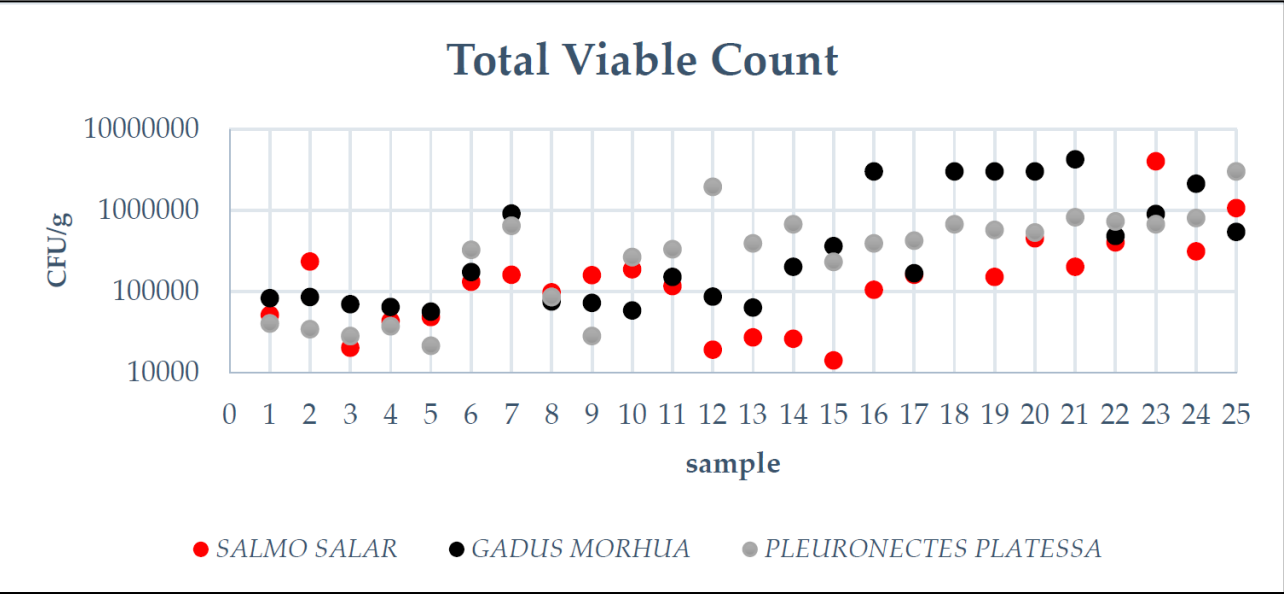


Figure A3. Fluorescence of two selected strains under Wood's lamp light.

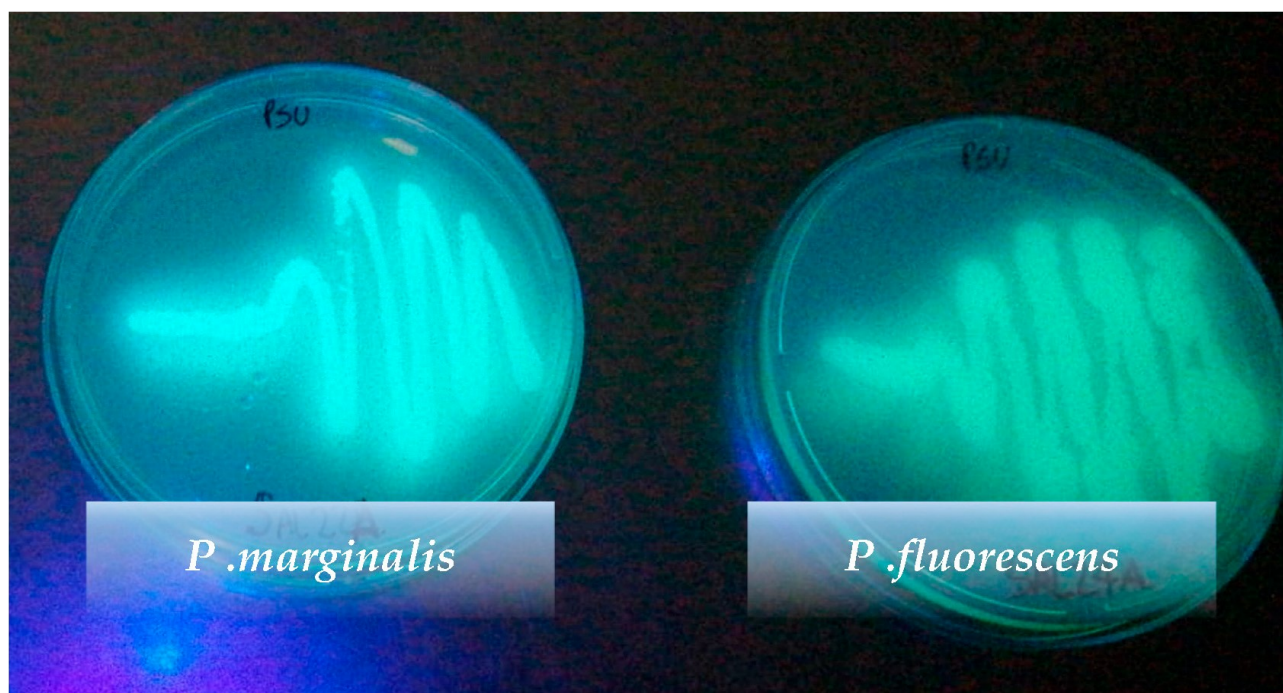
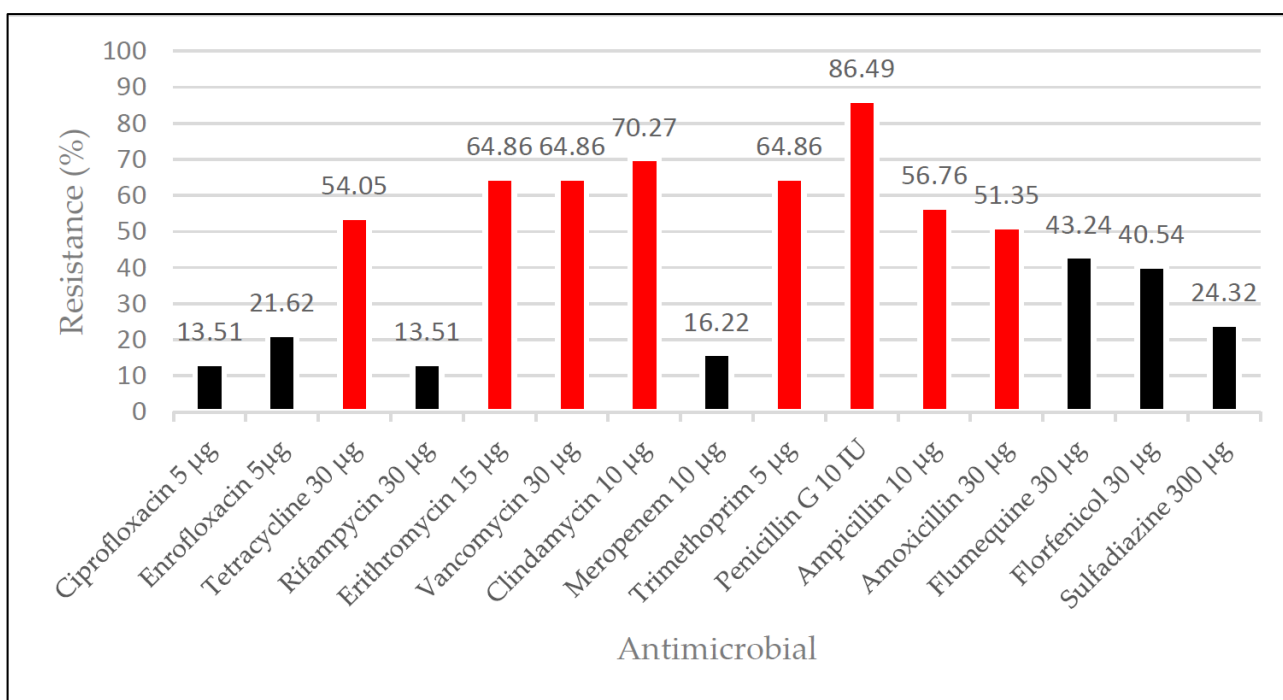


Figure A4. Percentage of *Pseudomonas* spp. resistance against different tested antimicrobials (the antimicrobial concentration is expressed as dose/disk). Having no criteria in the literature, cases where the diameter of the inhibition zone was ≤ 10 mm were considered "highly resistant". The red color highlights resistance in more than 50% of the strains. The black color, on the other hand, indicates resistance in less than 50% of the strains.



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CHAPTER 2 - MICROPLASTICS IN FISH AND FISHERY PRODUCTS AND RISKS FOR HUMAN HEALTH: A REVIEW

Alberghini, L., Truant, A., Santonicola, S., Colavita, G., & Giaccone, V. (2022).
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2.1 INTRODUCTION

Over the past seventy years there has been a sustained increase in plastic production, rising from 1.5 million tons (Mt) in the 1950s to 367 million tons in 2020 [1]. The production of plastic and the consequent production of waste are related to the growth of the human population, which has increased from about 3.1 billion in 1961 to about 7.3 billion in 2015 and is projected to exceed 9 billion by 2050 [2]. The demands of this growth will drive the plastics commodity market, as will the demand for fishery and aquaculture products. Estimates based on current growth rates indicate that plastic production is expected to double by 2025 and more than triple by 2050 [3]. The mass production and consumption of plastics have led to the accumulation of these materials in natural habitats resulting in negative impacts on biota and the economy [4].

Among the approximately 2.5 billion tons of solid waste produced globally in 2010, approximately 275 million tons were plastic waste generated and improperly managed by coastal countries and it is estimated that between 4.8 Mt and 12.7 Mt of this plastic waste has entered the oceans [5]. Once in the environment, plastic objects degrade and give rise to smaller fragments, which can directly enter the food chain or indirectly contaminate it due to the leaching of their potentially harmful chemicals [6]. Most humans ingest a significant amount of microplastic and even nanoplastic particles through food, particularly through the consumption of fish and other seafood [4]. To date, microplastics have been found in various foods such as beer, drinking water, honey, seafood, bivalve mollusks, sugar, and cooking salt [7,8]. To try to stem the problem of plastic pollution, many countries have set goals for the elimination or reduction of certain products such as plastic bags and disposable items [9]. In particular, in May 2018, a new proposal was approved by the European Commission, which bans several single-use plastic items and imposes stricter regulations on others [10]. Currently, 60 countries have banned or taxed the use of single-use plastics [11].

2.1.1 Management of Plastic Waste

Worldwide, the production of plastic waste is estimated to be 381 Mt per year and is expected to double in the next 10 years [12]. Only 9% and 12% of plastic waste was recycled and burned, respectively, while the remaining 79% ends up in landfills [13]. Waste treatment differs from country to country: developing countries are heavily dependent on landfills, while others focus on recycling and producing energy (in the form of heat, steam, and electricity).

To date, in the European Union, for example, 31% of plastic waste is destined for landfills [10]. However, not all plastics are recyclable or recycled and, if plastic waste is handled inappropriately, it can escape from waste management streams, enter the environment, and eventually reach the sea [14].

In general, plastic waste accounts for over 70% of all solid waste present in the natural environment [5]. The most recent data indicate that in 2020, mismanaged plastic globally was around 100 Mt and forecasts for the future suggest that it will reach around 220 Mt in 2060 if preventive actions are not taken to minimize the dispersion of plastic [15].

When dispersed into the environment, plastic waste is exposed to the intermittent combination of solar radiation, cooling, heating, drying, and rain, and begins to degrade and fragment, creating the so-called microplastics [16]. Over the years, the slow rate of plastic degradation has led to a significant accumulation of plastic debris on the surface of the sea, on the seabed, and on coasts around the world [17,18].

Current studies estimate that 88% of the sea surface is contaminated with plastic waste [19], of which 80% derives from terrestrial sources [20]. It has recently been estimated that 15 Mt of plastic enter the oceans each year and it has been hypothesized that these emissions of plastic waste will increase to around 20–53 Mt by 2030, potentially even reaching 90 Mt [21].

2.1.2 The Problem of Marine Waste

Marine litter is defined as any solid waste of human origin discarded at sea or which reaches the sea by dispersion from other environments [22,23]. They can be divided into nano-, micro-, meso-, and macro-wastes based on their size, and further divided into terrestrial and oceanic wastes based on their sources [24]. It is estimated that 40–80% of marine litter is made up of plastic [25]. Due to the high durability of plastic, such waste, reported all over the world, is destined to remain in the marine environment for a long period of time [26]. Household, river, marine, and fishing waste are generally considered chronic sources, as they represent an almost continuous input. These chronic sources combined with extreme weather events contribute to a large amount of plastic waste entering the marine environment [27]. Inevitably, in the marine environment, plastics undergo processes of mechanical abrasion, photodegradation, and biodegradation with the consequent formation of micro and nanoplastics [28,29]. Plastic debris in the oceans varies widely in size, shape, and chemical composition [30].

2.2 THE MICROPLASTICS

Although the presence of small plastic particles in the environment was already known since the early seventies [31], it is only recently that microplastics have attracted increasing interest from the scientific community. In particular, in the last decade, the attention to the problem of microplastics as a new pollutant has seen a great increase in investments in this new field of research on a global level [32]. These plastic fragments cause many concerns as they are small enough to be ingested by living organisms and therefore reach humans through the consumption of contaminated food [33,34] in which the presence of microplastics was only reported at the beginning of 2010 [35].

Definitions

To date, there is still no clear consensus on a definition broad enough to encompass all the criteria needed to describe microplastics [32]. An initial classification takes into consideration the dimensions of the fragments themselves, which can vary from several meters to such small dimensions as not to be visible to the naked eye (see Table 1) [36].

Table 1. Size categories of plastic marine litter, assuming a near-spherical form, showing common definitions and alternative options that may be appropriate for operational reasons from [36].

Fiel Descriptor	Common Size Division	Measurement Units	Alternative Options	Remarks
Mega	> 1 m	Meters	-	-
Macro	25 – 1000 mm	Meters	25 – 50 mm	-
		Centimeters	1 – 25 mm	
		Millimeters		
Meso	5 – 25 mm	Centimeters	< 25 mm	MARPOL Annex V (pre-revision)
		Millimeters		
Micro	< 5 mm	Millimeters	1 – 5 mm	[37]
		Micron	< 1 mm	
			> 330 μ m	
Nano	< 1 μ m	Nanometers	< 100 nm	Not considered for monitoring

So far, the most widely used definition is that microplastics are particles smaller than 5 mm in their longest dimension. This definition has been adopted in practical terms as it considers the size below which ingestion by many species of marine biota occurs [38] and has been accepted by the NOAA (National Oceanographic and

Atmospheric Administration) of the USA and by the MSFD (Marine Strategy Framework Directive) of the EU [14].

In the 2016 EFSA (the European Food Safety Authority) report, microplastics are defined as a heterogeneous mixture of materials of different shapes (fragments, fibers, spheroids, granules, pellets, splinters, or beads) whose dimensions are between 0.1 µm and 5000 µm, while nanoplastics are defined as plastic particles ranging from 0.001 µm to 0.1 µm [33]. Furthermore, microplastics are classified into primary and secondary. Primary microplastics are plastic particles intentionally produced in a size range of less than 5 mm and intended for special domestic or industrial uses [38]. Secondary microplastics, on the other hand, are the product of the fragmentation and degradation, caused by atmospheric agents, of larger plastics disseminated in the environment [38]. They appear to be the prevalent form in the marine environment, where it is estimated that approximately 68,500–275,000 tons of secondary microplastics are emitted annually [33]. Furthermore, it has been estimated that they constitute more than 90% of the 5 trillion microplastics floating on the sea [37].

2.3 PREVALENCE AND DISTRIBUTION OF MICROPLASTICS IN FISHERY PRODUCTS

According to FAO (the Food and Agricultural Organization of the United Nations) data, world production of fishery products reached about 179 million tons in 2018, of which 82 million tons came from aquaculture. Of the combined total, 156 million tons were used for human consumption, equivalent to an estimated annual supply of 20.5 kg per capita. The remaining 22 million tons were destined for non-food uses, mainly for the production of fishmeal and oil [39]. In the last decade, numerous articles have been published concerning the presence of microplastics in food, including fishery products (see Table 2) and sea salt [40–62].

Table 2. Microplastics in fishery products.

Species	Number of MPs Detected	% of Samples Positive to MPs (*)	Sampling Site	Reference
Fish				
<i>Merluccius</i>	0.40 ± 0.89 ^a	29%	Atlantic Ocean, Portugal	[41]
<i>Merluccius merluccius</i>	1.38 ^a	26.8%	Mediterranean Sea	[42]
<i>Merluccius merluccius</i>	1.6 ± 0.6 ^a	40%	Central Adriatic Sea, Italy	[43]
<i>Mullus barbatus</i>	1.08 ^a	19.7%	Mediterranean Sea	[42]

<i>Mullus barbatus</i>	1.8 ± 0.24 ^a	22.47%	Western Mediterranean Sea, Italy	[44]
<i>Mullus barbatus</i>	2 ^a	20%	Central Adriatic Sea, Italy	[43]
<i>Engraulis encrasicolus</i>	1.25 ^a	91%	Adriatic Sea, Italy	[45]
<i>Engraulis encrasicolus</i>	2.5 ± 0.3 ^a	83.4%	Eastern Mediterranean Sea, Lebanon	[46]
<i>Engraulis encrasicolus</i>	0.12 ± 0.12 ^a	34%	Eastern Ligurian Sea, Italy	[47]
<i>Sardinia pilchardus</i>	4.63 ^a	96%	Adriatic Sea, Italy	[45]
<i>Scomber scombrus</i>	0.29 – 2.5 ^a	100%	Atlantic Ocean, Portugal	[48]
<i>Mugil cephalus</i>	2.5 ^a	13.8%	Hong Kong coast	[49]
<i>Mugil cephalus</i>	10 ± 9 ^a	97%	Minho estuary, Iberian Peninsula	[50]
Crustaceans				
<i>Penaeus semisulcatus</i>	0.36 ^b	64.1%	Persian Gulf, Iran	[51]
<i>Parapenaeopsis hardwickii</i>	0.25 ^b	45%	East China Sea, China	[52]
<i>Fenneropenaeus indicus</i>	0.04 ^b	30.9%	Kerala coast, India	[53]
<i>Portunus armatus</i>	0.26 ^b	64.1%	Persian Gulf, Iran	[51]
<i>Crangon crangon</i>	0.55 ^b	63%	Channel area and Southern North Sea between France, Belgium, the Netherlands, and the UK	[54]
Mixed crab species	0.53 ^b	91%	Hong Kong coast	[55]
Mollusks				
<i>Mytilus</i> spp.	3.2 ± 0.52 ^c	-	Edinburgh	[56]
<i>Mytilus galloprovincialis</i>	0.8 - 0.9 ^c 2.5 – 5.3 ^d	46.25%	Ionian Sea, Greece	[57]
<i>Mytilus galloprovincialis</i>	0.69 ^c 0.23 ^d	48%	Turkish coasts	[58]
<i>Mytilus edulis</i>	0.61 ± 0.56 ^c 0.23 ± 0.20 ^d	65-90%	Nantes, France	[59]
<i>Mytilus edulis</i>	0.76 ± 0.40 ^c 0.15 ± 0.06 ^d	34-58%	Channel coastlines, France	[60]
<i>Crassostrea</i> spp.	2.93 ^c 0.62 ^d	84%	Coastline of China	[61]
<i>Crassostrea gigas</i>	2.10 ± 1.71 ^c 0.18 ± 0.16 ^d	80-93%	Nantes, France	[59]
<i>Crassostrea gigas</i>	0.369 – 3 ^c 0.02 – 0.14 ^d	63%	Salish Sea, USA	[62]

<i>Cerastoderma edule</i>	2.46 ± 1.16 ^c 0.74 ± 0.35 ^d	34-58%	Channel coastlines, France	[60]
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* In this context, the term “positive” expresses the number of fish samples, which, during the analysis, were found to be contaminated by MPs. ^a MP detected in the gastrointestinal tract. ^b MP content in the edible section. ^c MPs/individual. ^d MPs/g ww.

The ingestion of microplastics has been observed in many species of fish intended for human consumption from the Pacific, Atlantic, and Indian Oceans, and the Mediterranean Sea, although only one or two microplastic particles have been detected per fish [23,63]. For example, microplastics have been observed in chub mackerel (*Scomber japonicus*), erring (*Clupea harengus*), mackerel (*Scomber scombrus*), Japanese anchovy (*Engraulis japonicus*), northern cod (*Gadus morhua*), blue whiting (*Micromesistius poutassou*), sprat (*Sprattus sprattus*), king mackerel (*Scomberomorus cavalla*), shortfin scad (*Decapterus macrosoma*), horse mackerel (*Trachurus trachurus*), hake (*Merluccius merluccius*), bream (*Pagellus acarne*), and common sole (*Solea solea*) [64–73]. Microplastics have also been found in migratory commercial fish species (e.g., *Thunnus thynnus*), seasonal migratory fish (e.g., sea bass and *Dicentrarchus labrax*) and sedentary fish (e.g., plaice and *Pleuronectes platessa*) [34] and in Mediterranean fish of great commercial importance [74] such as sardines (*Sardina pilchardus*) and anchovies (*Engraulis encrasicolus*) which are often consumed entirely [34]. In these studies, microplastics were identified predominantly in the digestive tract and rarely in edible tissues, such as muscles [40].

Given that most fish species are eviscerated before human consumption, direct human exposure to microplastics would be negligible [14]. However, evisceration does not necessarily eliminate the risk of human intake of microplastics. In fact, in a study conducted on two species of dried fish among the most commonly consumed (the green back mullet *Chelon subviridis* and sin croaker *Johnius belangerii*), it was found that the presence of microplastics was significantly greater in gutted fish (whole fish excluding viscera and gills) compared to excised organs (viscera and gills) [75]. Despite this, small pelagic fish such as sardines, herring, and other small freshwater fish are commonly eaten whole, posing a greater threat than gutted fish [76]. However, little is known about microplastic levels in small fish.

2.3.1 Bivalve Mollusks

Bivalve mollusks can filter and retain microplastics of various sizes in quantities that depend on their concentration and distribution in the marine environment. Studies have revealed the presence of microplastics in two species of mussel commonly consumed as food by humans (*Mytilus edulis* and *M. galloprovincialis*) from five

European countries (France, Italy, Denmark, Spain, and the Netherlands) [77]. In a study conducted on wild and commercial Belgian mussels (*M. edulis*, *M. galloprovincialis*, and *M. edulis/galloprovincialis* hybrid form), the number of total microplastics ranged from 0.26 to 0.51 per gram of mussels [78].

In samples of mussels (*M. edulis*) reared in the North Sea and of Pacific oysters (*Crassostrea gigas*) reared in the Atlantic Ocean, an average content of 0.36 and 0.47 particles/g, respectively, was detected; the quantities of particles were reduced to 0.24 and 0.35 particles/g, respectively, after a purification period of 3 days [79]. Another study counted, on average, 0.2 ± 0.3 particles/g with up to 1.1 particles/g of mussels collected in six locations along the coast of France, Belgium, and Holland and left for purification for 24 h. However, this study, having excluded fibers from the count, may have underestimated the concentration of microplastics [59]. In a study carried out on mussels (*M. edulis*) sampled in coastal waters and in UK supermarkets, Li and co-authors [80] found that in wild mussels the total microplastics, ranging from 0.7 to 2.9 particles/g of tissue and from 1.1 to 6.4 particles/specimen, exceeded those contained in farmed mussels purchased at the supermarket. Furthermore, in supermarket-bought mussels, the abundance of microplastics was significantly greater in pre-cooked mussels with 1.4 particles/g compared to fresh mussels with 0.9 particles/g [80]. These results suggested a possible contamination during processing rather than a real environmental contamination [81]. In contrast, higher quantities of microplastics were found in nine species of bivalves purchased in Chinese markets than in wild bivalves. In this study, in fact, the authors counted from 2.1 to 10.5 particles/g and from 4 to 57 elements per single specimen coming from the markets [82]. To date, the studies that have investigated the prevalence of microplastics in bivalve mollusks sampled in nature or on farms are numerous, while those on mollusks sampled in markets and supermarkets are still very few [72,81–83].

2.3.2 Crustaceans

The ingestion of microplastics in crustaceans can be both accidental and related to their dietary strategies [84]. Swimming crustaceans can ingest more microplastics than the sessile species. Crustaceans can be filter feeders, such as copepods and shrimps, opportunistic feeders such as the brown shrimp (*Crangon crangon*), or active hunters of small fish and other organisms, such as crabs and lobsters [85]. In a study conducted on brown shrimp (*Crangon crangon*) sampled from various locations in the English Channel, synthetic fibers were detected in 63% of the samples with an average value of 0.68 ± 0.55 particles/g (1.23 ± 0.99

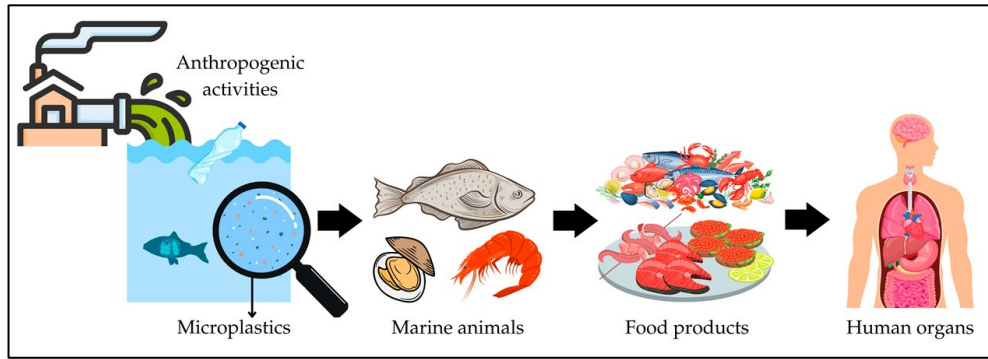
particles/shrimp). In addition, seasonal differences have been reported, with greater absorption of microplastics in October compared to March [54]. Furthermore, in the same study, when comparing whole and peeled shrimp, no particles were detected in the abdominal muscles, which usually represent the edible portion of the shrimp. In fact, by shelling and removing the cephalothorax of the shrimp, the intestinal tract is removed, which contains most of the microplastics.

However, it is not always possible to remove completely the digestive tract from this small shrimp, thus exposing humans to possible ingestion of microplastics. For this reason, the author proposed to reduce the maximum number of microplastics that can potentially be eliminated by shelling and removing the cephalothorax to 90% [54]. In another study on Indo-Pacific shrimp (*Penaeus semisulcatus*) sampled in the Persian Gulf, the authors observed an average of 7.8 particles/crustacean and 1.5 particles/g, indicating the presence of microplastics both on the exoskeleton and in the muscle mass [86]. Other commercially relevant species in which microplastics have been observed include the Chinese mitten crab *Eriocheir sinensis* [87], the shore crab *Carcinus maenas* [88], and the Norway lobster *Nephrops norvegicus* [89]. Here, it is useful to remember that brown shrimps are an important food for a wide range of predators, thus facilitating the potential trophic transfer and accumulation of microplastics at the top of the food chain.

2.4 RISKS FOR HUMAN HEALTH RELATED TO THE ABSORPTION OF MICROPLASTICS

While microplastic contamination is not a new phenomenon, the characterization of risks associated with micro- and nanoplastics is an emerging problem. Risk is generally defined as the product of the hazard represented by an agent multiplied by the sustained exposure. The dangers posed by micro- and nanoplastics include physical characteristics (e.g., size, shape, and surface), additives used intentionally in the plastic manufacturing process, environmental durability, the ability to absorb chemical contaminants and pathogens and to concentrate them along the food chain [14]. Fishery products are known to be an important source of microplastics in the human diet. Therefore, if contaminated, their consumption can pose a threat to human health [90,91] (see also Figure 1).

Figure 1. A model showing how anthropogenic activity cause microplastics to enter the food web, make a path to our food and, ultimately, into our organs.



Considering the recommendations of the EFSA, EUMOFA (European Market Observatory for Fisheries and Aquaculture Products), and NOAA for the consumption of fish by humans at different ages, the intake of microplastics, calculated on the ingestion of three species of fish that are caught and commonly consumed (*Dicentrarchus labrax*, *Trachurus*, and *Scomber colias*), varies from 112 to 842 particles/year according to the EFSA and from 518 to 3078 particles/year/per capita according to the EUMOFA and NOAA [92].

Furthermore, it is relevant to consider that the degree of microplastic pollution and the consumption of bivalves varies considerably from country to country, leading to different levels of microplastic intake per capita in different countries each year [79,81–83]. For example, in European countries with high consumption of bivalves, it has been estimated that consumers ingest up to 11,000 particles/year/per capita, while in European countries with low consumption of bivalves, 1800 particles/year/per capita are ingested on average, which still represents a considerable exposure [79]. In South Korea, on the other hand, this estimate is 283 particles/year/per capita [83]. Furthermore, another study predicted that the consumption of mussels may lead to the ingestion of 123 particles/year/per capita in the UK and up to 4620 particles/year/per capita in the countries that consume the most bivalves (e.g., Spain, France, and Belgium) [56]. In addition, considering the average annual consumption of shrimp in Belgium (0.5 kg/person) and the best-case scenario where 90% of the microplastics are removed by shelling and cephalothorax removal, a quick indicative assessment revealed an ingestion rate between 15 and 175 particles/year for each person [54]. However, the information we currently have is not sufficient to assess the actual amount of microplastic to which humans are exposed, which represents a fundamental parameter for assessing the effects on human health [93].

2.4.1 Toxicity of Microplastics

Microplastics are considered potentially harmful to organisms depending on the degree of exposure and the susceptibility of the individual. Indeed, they can cause oxidative stress, cytotoxicity, and translocation to other tissues [94]. In organisms subjected to prolonged exposure, however, microplastics can lead to chronic flogosis, cell proliferation, necrosis, and impairment of immune cells [93]. In addition, microplastics can release chemicals (organic and inorganic) present in their matrix or previously absorbed by the environment [95] and act as vectors of microorganisms [96].

2.4.1.1 Oxidative Stress and Cytotoxicity

Microplastics can cause oxidative stress due to the release of oxidizing species (for example, metals) that were previously adsorbed on their surfaces and reactive oxygen species (ROS) generated during the inflammatory response [97]. They also contain ROS produced by the polymerization and manufacturing process of plastics [98]. Oxidative stress after exposure to microplastics has been observed in mice [99] and zebrafish (*Danio rerio*) [100]. In humans, however, it has been reported that polypropylene (PP) prostheses induce an inflammatory response with the production of ROS that could lead to rejection [101]. Cytotoxicity is the result of particle toxicity, oxidative stress, and inflammation. Several studies have shown that exposure to microplastics and nanoplastics can potentially cause cytotoxicity. For example, an in vitro study revealed that polystyrene (PS) particles ranging in size from 20 to 40 nm are capable of producing cytotoxic effects on the Caco-2 cell line [102]. However, other in vitro studies have not reported large cytotoxic effects even using high particle concentrations (100–200 µg/mL) [103,104].

2.4.1.2 Metabolic Disorders and Energy Balance

In humans, microplastics could have metabolic effects similar to those observed on mice and marine organisms. They can increase or decrease energy expenditure, reduce nutrient intake, and/or modulate metabolic enzymes. However, in humans the observation of these effects may be limited considering the low exposure concentrations, the greater energy requirements, and the greater complexity of human metabolism compared to the organisms tested [98].

2.4.1.3 Translocation of Microplastics to the Circulatory System and Distant Tissues

After exposure, microplastics can act locally in the intestine or move to other organs through the circulatory system. Once in blood vessels, micro- and nanoplastics can cause a systemic inflammatory response, blood cell cytotoxicity by internalization, pulmonary hypertension, flogosis, and vascular occlusions [105,106].

The translocation of microplastics has been reported in rats, in which the particles, after ingestion and inhalation, reached the liver and spleen via the circulatory system [107]. In another study in mice, fluorescent polystyrene particles with a diameter of 5 μm and 20 μm accumulated in the liver, kidneys, and intestines [99]. Recently, microplastics ranging in size from 5 μm to 10 μm have been detected for the first time in the human placenta, in all its components: maternal, fetal, and amniochorial membranes [108]. Other in vitro studies have shown that PS nanoparticles (44 nm) can accumulate in human renal cortex epithelial cells with no cell effects and no signs of clearance in the first 90 min. However, the continued accumulation of these particles could impair renal function [109]. Furthermore, when transported to distant organs, microplastics can cause chronic inflammation, decreased organ function, and an increased risk of cancer [94].

2.4.1.4 Disorders of the Immune System

After exposure, microplastics can induce local or systemic immune responses depending on their diffusion and the host response [98]. In particular, in genetically susceptible individuals, even just environmental exposure to microplastics is sufficient to interrupt the immune function causing autoimmune diseases or immunosuppression [94]. Furthermore, some studies have found that mussels exposed to microplastics exhibit immunosuppression and modulation of the immune response [105]. Although it has not yet been proven, microplastics could affect the immune system in humans as well. Therefore, further investigations of the effects on the human immune system are needed.

2.4.1.5 Neurotoxicity

In vivo toxicity studies have shown that microplastics can affect neuronal function and behavior. In particular, in the brain of sea bass (*Dicentrarchus labrax*), it has been observed that these particles can cause inhibition of acetylcholinesterase, oxidative stress with increased levels of lipid peroxidation, and induction of anaerobic energy production pathways [76]. The same species also showed a negative impact on swimming performance, which is considered a behavioral indicator [110]. In mice, however, exposure to microplastics debris of polystyrene caused an increase in the

activity of acetylcholinesterase and the serum levels of some metabolites that act as neurotransmitters (threonine, aspartate, and taurine) [99]. At present, however, very little is known about how microplastics could be involved in human neurotoxicity [94].

2.4.2 Effects of Contaminants and Additives Associated with Microplastics

Much of the concern raised by microplastics is due to the effects that could occur with the desorption of contaminants absorbed by the microplastics themselves and the chemical additives present in them [111]. In fact, plastic can concentrate contaminants up to the order of 10^6 [112]. Such contaminants could be released when microplastics come into contact with body surfaces and absorbed, thus reaching the underlying tissues [113].

2.4.2.1 Plastic Additives

On average, 4% of the weight of the plastic produced is made up of additives [33]. However, the percentage of additives can vary significantly, in some cases even reaching half of the total material [114]. Teuten and co-authors [115] conducted a study on the amounts of additives present in microplastics collected from deep waters, coastal waters, and beaches. The results obtained by the authors can be summarized as follows: polybrominated diphenyl ethers (PBDE) from 0.03 nanograms per gram (ng/g) to 50 ng/g, bisphenol A (BPA) from 5 ng/g to 200 ng/g, nonylphenol (NP) from 20 ng/g to 2500 ng/g, and octylphenol (OP) from 0.3 ng/g to 50 ng/g.

Recently, research has also associated endocrine disruptors with various diseases and conditions, including hormonal cancers (breast, prostate, and testicles), reproductive problems (genital malformations and infertility), metabolic disorders (diabetes and obesity), asthma, and neurodevelopmental disorders (learning disorders and autism) [106]. An estimate of the exposure to additives by ingesting microplastics can be obtained as follows. Taking Chinese mussels (*Scapharca subcrenata* and *Alectryonella plicatula*) as an example, which contain on average the highest number of microplastics (4 particles/g) [82] and considering that an adult man generally consumes an average of 225 g of shelled mussels per serving, this meal would lead to ingestion of 900 microplastics. Assuming that these particles are spherical with an average diameter of 25 μm and a density of 0.92 g/cm³ (the density of low-density polyethylene, LDPE), the most common type of polymer of microplastics [116], these 900 microplastics would represent 7 μg of plastic, 4% of

which would be additives. Using BPA as an example, this portion of mussels would contain 0.28 µg BPA. In a conservative scenario, we could assume that BPA is completely released from the microplastic. The EFSA estimated that an average adult BPA exposure from food and non-food sources of 0.19–0.20 µg/kg b.wt. per day [117]. Therefore, a 70 kg adult man would ingest about 14 µg of BPA per day on average. Consequently, bisphenol A from the microplastics of mussels would contribute about 2%, thus representing only a small part of the average daily exposure.

2.4.2.2 Bioaccumulative and Persistent Toxic Compounds (PBTs)

In addition to chemical additives, microplastics in the oceans can also accumulate persistent organic pollutants (POPs), including polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), and organochlorine pesticides such as DDT [71,112]. Due to their hydrophobic properties, POPs accumulate consistently in microplastics present in the marine environment. In fact, high concentrations of the following pollutants have been found in plastic particles taken from oceans, coastal areas, and beaches: non-dioxin-like polychlorinated biphenyls (PCBs) from 0.01 ng/g to 2970 ng/g; polycyclic aromatic hydrocarbons (PAHs) from 4 ng/g to 44,800 ng/g; and DDT and its analogues (DDD and DDE) from 2 ng/g to 2100 ng/g [115,118].

Once absorbed, POPs accumulate in human adipose tissue and their persistent characteristic makes them a serious threat to human health. In fact, exposure to these pollutants is associated with serious health problems such as endocrine disorders, reproductive problems, cancer, cardiovascular disease, obesity, and diabetes. Furthermore, prenatal exposure to POPs not only has negative effects on the health of the mother, but also on the newborn. Indeed, recent studies have shown that prenatal exposure to POPs may be associated with decreased birth weight [119], childhood obesity, increased blood pressure [120], and endocrine disrupting effects [121,122]. An estimate of exposure to POPs through the ingestion of microplastics can be obtained along the lines of that shown for plastic additives, using data on the presence of microplastics in bivalves from Li et al. [82], the amount of microplastics ingested from the consumption of 225 g of Chinese mussels (*Scapharca subcrenata* and *Alectryonella plicatula*), and data on the concentration of POPs in microplastics. In a conservative scenario, the highest organic pollutant concentrations measured in microplastics on a global scale would give a forecast of the maximum expected exposure. The highest POPs concentrations were found in microplastics deposited on beaches: PCBs up to 2750 ng/g and PAHs up to 24,000

ng/g. In this conservative scenario, these microplastics would lead to the ingestion of about 19 pg of PCBs and 170 pg of PAHs [33].

In the latest evaluation of PCB monitoring in food and feed available on the European market [123], the EFSA estimated an average exposure to non-dioxin-like PCBs of 0.3–1.8 µg of PCBs per day (for a human weighing 70 kg).

Regarding PAHs, the EFSA estimated an average exposure of 3.8 µg per day for the average EU consumer [124]. Therefore, even assuming that PCBs and PAHs are completely released from microplastics, consumption of these mussels would only have a small effect on exposure to PCBs (<0.006% increase) and PAHs (<0.004% increase) [33].

2.4.2.3 Potentially Toxic Elements

Potentially toxic elements (PTEs) present in microplastics include Zn, Sb, Al, Br, Cd, Cu, Hg, As, Sn, Pb, Ti, Co, Cr, Ba, and Mn. They can be used as additives (e.g., in dyes, flame retardants, fillers, and stabilizers) during the plastic manufacturing process or they can adsorb in high concentrations to microplastics from the marine environment and, through the food chain, be transferred to aquatic organisms [69,76,125–127] and to humans [85,127,128].

The following metal concentrations were found in the microplastics collected along the south-western coasts of England: a few ng/g of Cd, Ni, and Cr; 7.7 µg/g of Cu; 10.3 µg/g of Pb; 171 µg/g of Al; 290 µg/g of Zn; 308 µg/g of Mn; and 314 µg/g of Fe [129]. Another more recent study, also along the south-west coast of England, found 3390 µg/g of Cd and 5330 µg/g of Pb in microplastics [130].

PTE toxicity depends on many different factors such as dosage, route of exposure, and chemical species, as well as the age, sex, genetics, and nutritional status of the exposed subject. A high concentration of PTEs in humans causes cell and tissue damage, leading to a variety of adverse effects and diseases [131–134]. A recent study has shown that mercury, lead, zinc, copper, and cadmium present on marine microplastics can lead to the co-selection of human pathogens resistant to antibiotics, posing a serious threat to humans when exposed to fishery products and marine environments [135]. Despite this, to date, no studies have been identified that have assessed the relevance of metals adsorbed to microplastics for food [33] and consequently for human health.

2.4.2.4 Microbial Pathogens

Although it has been documented that microplastics can act as substrates for different microbial communities, there is insufficient data on the presence of such

pathogens on microplastics to include them in risk profiling [14]. In summary, therefore, since the data we have available is very incomplete or lacking, it is not possible to carry out a reliable risk analysis to estimate the effects of microplastics on human health. However, with regard to contaminants and additives, the estimated exposure through the ingestion of the microplastics contained in fishery products constitutes only a fraction of the total daily dietary intake of these compounds. Furthermore, considering that the diet is not the only source of contaminants and additives, this exposure is overestimated. It can therefore be concluded that the effect of the consumption of microplastics on the intake of contaminants and additives is most likely negligible [14].

2.5 EUROPEAN REGULATORY AND MITIGATION STRATEGIES

At present, there are no EU maximum levels for microplastics in foods and no product-specific analytical standards for food matrices. Nevertheless, a coherent policy and guidance context has emerged that is relevant to seafood. EFSA's 2016 scientific opinion scoped micro- and nanoplastics in food (with particular focus on seafood), identifying major gaps that still constrain risk assessment: harmonized methods, exposure data (especially for particles <150 µm) and human toxicokinetics, including potential local gastrointestinal effects and in-situ nanoplastic formation. Subsequent EFSA colloquia and updates have reiterated these priorities [33, 136, 137].

Upstream regulatory measures target sources rather than food endpoints. The Single-Use Plastics Directive (EU) 2019/904 addresses key items (including fishing gear) that contribute to marine litter, while Commission Regulation (EU) 2023/2055 under REACH (Registration, Evaluation, Authorization and Restriction of Chemicals) restricts intentionally added microplastics in products (e.g., cosmetics, glitter, detergents), with staged transitional periods. Although not food limits, these instruments are expected to reduce environmental loading and, indirectly, contamination pressure on aquatic foods [138, 139].

On monitoring, the EU Marine Strategy Framework Directive now includes specific guidance for microplastic litter in marine monitoring programs, supporting consistent environmental baselines. In parallel, the Drinking Water Directive (EU) 2020/2184 has led to an EU-adopted harmonized methodology for microplastics in potable water; this is operationally relevant to seafood processing because ice and process water are critical inputs and can be verified under an aligned quality assessment and quality control framework [140, 141].

Food contact materials (FCM) represent another potential entry pathway. EFSA's 2025 literature review concludes that while microplastic release from FCMs during use is documented, many studies likely overestimate migration due to artefacts and non-standardized methods; release is often driven by mechanical abrasion and friction (e.g., wear of plastic surfaces, shedding of fibers), highlighting the importance of handling and materials choices rather than plastic per se. This complements the existing EU framework for plastics and recycled plastics in contact with food [142, 143].

In line with EFSA, WHO (2022) and FAO (2022), the main control gaps are the lack of harmonized, validated analytical methods tailored to food matrices; the inconsistent sampling, digestion, polymer identification, and reporting units (items per individual vs mass/volume), hampering cross-study comparability; the limited data on particles <150 µm and nanoplastics; the scarce human toxicokinetic and dose-response evidence to enable health-based guidance values, and finally the insufficient quality assurance and quality control to prevent laboratory contamination (textile fibers, plastic labware) [144,145].

While methods consolidate, proportionate mitigation can proceed:

1. Upstream/source control: compliance with the Single-Use Plastic Directive and the REACH microplastics restriction reduces environmental inputs; fisheries can adopt gear-loss minimization and port-reception practices aligned with marine-litter guidance [139,140].
2. Pre-harvest: site selection away from known microlitter hotspots; for bivalves, verified depuration/relaying protocols (documented to reduce microplastic burdens, albeit variably); intake water pre-filtration in aquaculture and RAS operations [138,146].
3. Post-harvest/processing: assurance that ice and water meet drinking-water standards (and, where feasible, are checked with the EU harmonized MP method); avoid abrasive handling that increases plastic wear (e.g., substitute worn plastic crates, prefer low-shedding materials, manage brush/pad abrasion); implement fine filtration on process water where practical; strengthen lab and plant QA/QC to prevent microfiber cross-contamination (lint-free textiles, blanks, controlled airflows) [138,144].
4. Product/consumer level: emphasize that evisceration reduces exposure for finfish (MPs concentrate in GI tract), while depuration and shucking are critical for bivalves and small crustaceans typically eaten whole [33, 147, 148].

2.6 CONCLUSIONS

Mass consumption, inadequate waste management, and the durability of plastics have led to a huge accumulation of plastic waste of all sizes in aquatic environments around the world. Current estimates predict that plastic production levels will continue to increase exponentially in the near future. As a result, related marine pollution is also likely to increase.

Microplastics have been detected in all aquatic compartments; hence, they are currently considered an omnipresent contaminant that constitutes a potential risk both for aquatic organisms that ingest them and for humans through the consumption of fishery products.

The ingestion of microplastics by aquatic organisms, including fish species of commercial interest to humans, has been confirmed by laboratory and field studies. The quantities of microplastics observed from the analysis of the intestinal contents of these organisms are generally low. However, data on the presence of microplastics in some species of interest, including small fish, and in tissues outside the digestive tract are still very limited. Furthermore, it is not known whether the negative effects resulting from the ingestion of microplastics by aquatic organisms under experimental conditions may also occur in nature.

There are currently no guidelines for the analysis of microplastics and only limited methods are available for the identification and quantification of these particles. Furthermore, the methods described for the degradation of organic matter all have disadvantages, including the degradation to some extent of certain types of plastics. It is now well documented that microplastics contain additives added during the plastic manufacturing process and that microparticles can efficiently absorb chemical and biological contaminants from the environment. Such contaminants can be transferred to organisms once the microplastics are ingested. However, considering the calculations are based on experimental studies, a critical evaluation of the literature related to the transfer of PBTs from microplastics to marine animals concluded that the ingestion of microplastics by marine organisms is not capable of increasing PBT exposure in these specimens.

Similarly, the transfer of additives from ingested microplastics seems to have a negligible effect on total exposure to the additives, as has been demonstrated for NP and BPA. According to the FAO, the per capita consumption of fish products has increased dramatically, from 9.0 kg in 1961 to 20.5 kg in 2018. However, the amount

of microplastics ingested by humans through the consumption of fishery products is still poorly determined.

As a rule, most fish species are eviscerated before consumption, greatly reducing the risk of ingestion since most of the particles are contained within the gastrointestinal tract. Exceptions to this rule are bivalve mollusks, small crustaceans, and some species of small fish, which are generally eaten whole.

In humans, various potential toxic effects of microplastics have been hypothesized; however, to date, the data we have is not sufficient to make a reliable assessment of the risks to human health. Although it is currently believed that the overall risks to human health from ingesting microplastics present in fishery products are low, it is important to consider that microplastics present in aquatic environments will inevitably increase, due to the degradation of the already present plastic and plastic that will enter in the future.

In the face of all this, it is important and urgent that researchers carry out further studies to fill the current gaps in the knowledge of microplastics. In addition, analytical methods for the detection and quantification of microplastics in aquatic environments and in fishery products should be further developed, standardized, and validated, with a focus on particles smaller than 150 μm .

Furthermore, to make a reliable assessment of human exposure to microplastics it is important to acquire further data on the presence and size of these particles in fishery products, and to understand the effects that food processing could have on microplastics. Finally, further research is needed on the toxicokinetics and toxicodynamic of microplastics to both aquatic organisms and humans, including local effects on the intestinal tract, interactions with the microbiota, and the potential formation of nanoplastics resulting from the degradation of microplastics in the gastrointestinal tract. In this context, more studies must be carried out on microplastics as vectors of pathogens for fishery products and for humans.

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CHAPTER 3 - FIRST METATAXONOMIC CHARACTERIZATION OF GUT MICROBIOTA OF SWORDFISH (*XIPHIAS GLADIUS*)

Truant A., Giacometti, F., Losasso, C., Peruzzo, A., Petrin, S., Zancato, I., Di Leva, V., & Giaccone, V. (2025). First Metataxonomic Characterization of Gut Microbiota of Swordfish (*Xiphias gladius*). *Environmental Microbiology Reports*.

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3.1 INTRODUCTION

Swordfish (*Xiphias gladius*) is a large, silver teleost pelagic fish with a distinctive long, flat rostrum on its upper jaw, forming the typical flat and sharp “sword”, approximately one-third of its body length. It is found in tropical, temperate, and sometimes cold waters of all oceans, including the Mediterranean and its adjacent waters [1]. The swordfish is a valuable fish species, both ecologically, as it is considered a good candidate for monitoring ecosystem changes due to its generalist and opportunistic feeding behaviour [2], and commercially. Indeed, swordfish is commercially important to several European Union (EU) fleets fishing in the Atlantic Ocean and the Mediterranean Sea. With global catches relatively stable at 100-120 kilotonnes in last years [3], EU fleets provide account for nearly one-third of the global supply. Spain is by far the largest producer and, to a lesser extent, Italy, with over 15,000 and 2,000 tonnes landed in 2022, respectively [4]. However, in the EU, the main market for swordfish is Italy, being among the top ten species consumed at household level [5] and showing a nominal value of million euros in 2022 [4]. Its consumption has been growing in an unsustainable way in the recent decades, and the consequent intensive fishing worldwide has compromised swordfish marine stocks. Both local and international policies have been starting to protect this species in the last years [6]. Swordfish populations are subdivided mainly on an ocean-basin scale, with a unique Mediterranean stock distinct from the Atlantic ones, exhibiting different growth and maturity characteristics, and in which two other sub-stocks can be observed, respectively North and South Atlantic [7]. The swordfish is a large, migratory apex predator with a carnivorous diet and occupies a top position in the marine food chain, being an extremely versatile predator capable of exploiting various trophic resources. Indeed, it feeds on a broad spectrum of prey, comprising mainly teleost fish, primarily pelagic fishes at the juvenile stage, and later, as adult predate crustaceans and cephalopods [8], depending on fish size, area and season, and also invertebrates [9]. This species is renowned for achieving substantial dimensions, with adult individuals attaining lengths of up to 3 to 4 meters and weighing over 400 kg, and for crossing long distances in a short time, appearing to prefer different habitats for breeding and feeding [10].

It is well known that the adaptation of vertebrates to their environment is strictly related to their capacity of feeding and that fish have a unique and intimate interaction with their surrounding environment and, in turn, with the microorganisms that co-exist there. The alimentary canal of fish varies greatly with differing life histories, ecology and environmental factors related to fish biology.

Filter feeders, parasites and predators as well as herbivorous and carnivorous fish exist, and each has an appropriately adapted and different digestive system, including digestive organs that may or may not be present [11]. Teleosts's gut, as well as skin mucus and gills, support abundant populations of bacteria that impact the overall health and physiology of the host. Fish intestines, in particular, harbor large and diverse populations of bacteria [12], which are the dominant microbiota of fish intestine [13]. Literature on fish gut microbiota has attempted to provide an understanding of many areas and mainly of the diverse fish species, given that there are approximately 33,700 fish species (only teleosts are more than 28,000 species) on Earth, accounting for over half of the total number of vertebrate species. The term "microbiota" comprises the entire collection of microorganisms in a specific niche, such as the fish gut, and 16S rRNA survey resulted the analytical approach used to taxonomically identify these microorganisms in a specific environment [14]. However, these studies varied considerably in terms of the species investigated, methodologies, and sample collection (for example, fore-, mid-, and high gut), as well as analytical methods (culture-dependent versus culture-independent methods), with consequent difficulties in comparing results and extrapolating the true level of diversity. These conflicting findings were undoubtedly a feature of the diversity that exists among fish, but there was also variability among individuals of the same species. Factors influencing the intra and inter-species diversity found on gut microbiota studies include life stage, diet, season, habitat, captive state, sex and phylogeny [15], even if trophic level, habitat and possibly host phylogeny are the most likely influencers [16]. The influence of diet on gut microbiota is a logical link as well as those with the surrounding water, reflecting diverse environmental compositions [17]. However, microbiota variability among individuals, across a great number of hosts, is still considered not to have been systematically investigated [18].

Worldwide, there is a large body of research on the biology (diet, growth and reproduction), migrations, genetic structure of fish populations, and heavy metal accumulation of swordfish, but only few papers address the microbiological safety of their meat [19-22] and, to our knowledge, to date, no study has yielded a 16S rRNA next generation sequencing analysis in gut this fish species. Herein, this study addresses a metataxonomic analysis of swordfish gut and explores the characterization of the intestinal microbiota of wild individuals in relation with the fish size and the different fishing areas in which they were caught.

3.2 MATERIALS AND METHODS

3.2.1 Fish samplings and gut content collection

A total of 100 swordfish individuals were included in this study. Fish sampling was conducted at a fish processing facility in Venice, Italy, specializing in the marketing (over 40,000 tons of fish annually as wholesalers of fresh, frozen, and blast-chilled fish) as well as the processing and packaging of fish products. Data pertaining to product traceability and labeling (Regulation EC No 178/2002; Regulation EC No 1379/2013) were used to collate the commercial designation and scientific name of the fish species and the area where the fish were caught. The investigated swordfish individuals weighed between 10 and 37 kg and were caught in three different FAO fishing areas: FAO 27 (Atlantic, Northeast), FAO 34 (Atlantic, Eastern Central), and, within FAO 37 (Mediterranean and Black Sea) and the Western Mediterranean subarea (Subarea 37.1), the Balearic division (Division 37.1.1). The swordfish individuals were divided into groups based on fish weight (10–20 kg, 21–30 kg, and >31 kg) and FAO fishing areas (FAO 27, 34, and 37.1.1). Table 1 summarizes the classification of the swordfish individuals.

Table 1. Details of the overall swordfish individuals involved into the study and classified into different groups by the fish weight and the FAO fishing areas.

FAO fishing area	Fish weight (kgs)			Sum
	10-20	21-30	>30	
FAO 27	1	10	14	25
FAO 34	25	7	8	40
FAO 37.1.1	20	8	7	35
Sum	46	25	29	100

The fishes were processed in a timely manner in order to reduce the change in composition of gut microbial communities. Before dissection, according to literature, 70% ethanol was applied to the body surface of the fish samples to decontaminate the skin and scales, not the incision or internal tissues [23-26]. From each swordfish individual, the digestive tract from the stomach to the hindgut was removed intact. Swordfish was kept in a vertical position on the slaughter line; an abdominal incision was performed using sterile knife to remove offal and the whole visceral package. For each swordfish individual, the knife was replaced to ensure the sterility of the procedure. The gut was directly transferred, by gravitational fall, into a sterile bag handled by the laboratory technician, avoiding any contact with non-sterile surfaces and ensuring the traceability of each individual sample. The

entire process took place in a refrigerated environment at 4 °C within the investigated fish processing facility. From each swordfish gut, two separate biological replicates of the fecal content were collected using swabs (FecalSwab™, Copan Diagnostics Inc, Brescia, Italy), transported under chilled conditions (at 4°C) to the Laboratory of Microbial Ecology at the Istituto Zooprofilattico Sperimentale delle Venezie and processed within 36 hours.

3.2.2 DNA extraction and 16S rRNA sequencing

DNA from the gut content samples was extracted starting from 200µL of faecal content with a column-based kit, QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer's instruction. Thermal lysis was carried out for 2 h, and RNaseA (100 mg/mL) was added to each sample to ensure RNA-free preparation. Total DNA was resuspended in 200µL of nuclease-free water and stored at -20°C. The hypervariable V3 and V4 regions of the bacterial 16S rRNA gene of the qualified DNA samples were amplified by the primers Bact341F and Bact785R (Fwd: CCTACGGGNGGCWGCAG and Rev: GACTACHVGGGTATCTAATCC) previously described by Klindworth and co-authors [27] and according to the Illumina 16S Metagenomic sequencing Library Preparation protocol. The thermal cycling profile was as follows: 95 °C for 3 min; 25 cycles of 95 °C for 30 seconds, 56 °C for 30 seconds, 72 °C for 30 seconds; 72 °C for 5 min; hold at 4 °C. PCR clean-up was performed by means of the Agencourt AMPure XP beads (Beckman Coulter Genomics, Indianapolis, IN, USA). Libraries for sequencing were prepared using the Nextera XT DNA Library Prep Kit (Illumina). The concentration of the libraries was determined with the Qubit fluorometric assay, and their quality was assessed with the Agilent 2200 TapeStation. Samples were pooled equimolarly, and the resulting library was sequenced on the Illumina MiSeq platform using a MiSeq 600V3 cartridge (600 cycles, 2x300 bp, paired-end reads).

3.2.3 Bioinformatic and statistical analysis

Raw sequence data were processed using the Quantitative Insights Into Microbial Ecology 2 (QIIME2) version 2020.2 pipeline. Reads were screened, trimmed, quality filtered, and denoised with DADA2 [28] using the following parameters: p-trunc-len-f=284 and p-trunc-len-r=276. Amplicon Sequence Variants (ASVs) were converted in OTUs to reduce computational complexity of the data still ensuring a sufficient level of detail. Operational taxonomic units (OTUs) were defined as those sequences with a similarity $\geq 97\%$ among them. Each OTUs was taxonomically

assigned based on the SILVA database [29], and the obtained OTU-table was then normalized following the Geometric Mean of Pairwise Ratio (GMPR) method [30]. Statistical analyses were conducted by *phyloseq* package [31]. Alpha diversity were performed on the pre-processed count table and measured by means of observed OUT metric, Pielou index and Shannon index to describe the community structure. Beta diversity was evaluated with the Bray-Curtis distance metric and visualized using the Non-metric Multi Dimensional Scaling (NMDS) plot. Kruskal-Wallis non-parametric test was used to compare alpha diversity between different swordfish weight categories and between different FAO fishing areas. If significant, the pairwise comparison Wilcoxon test was performed by adjusting the p-value for multiple comparisons using the false discovery rate (FDR) method [32]. PERMANOVA test was used to compare the beta diversity parameters between groups. Finally, the differential abundance analysis was performed using ANCOM-BC test at Genus level [33]. Graphical outputs were obtained by means of the *ggplot2* package [34]. R version 4.3.2 software [35] was used to perform the above statistical analysis. P-value < 0.05 is considered significant.

3.3 RESULTS

The 16S rRNA metataxonomic sequencing of Swordfish intestinal microbiota produced an output of a total of 21,395,047 reads, yielding an average of 211,227 reads per sample (standard deviation, SD= 48,008). After quality trimming step, 3,297,390 sequences were retained for further analysis with a mean for sample of 329,739 sequences (SD= 10,037) and then sorted into operational taxonomic units (OTUs). The OTUs showed substantial variation across the samples, with counts ranged notably, from 20 OTUs to 190 202 (in sample 65), on average.

Based on the phylogenetic classification using the SILVA database, the microbiota taxonomic composition at the phyla level were Pseudomonadota (92%) and Fusobacteriota (7%) and a small percentage (<1%) was represented by other phyla such as Bacillota, Cyanobacteriota and Mycoplasmatota. In relation to the microbiota taxonomic composition at the genus level, the most abundant genera within the phylum Pseudomonadota were represented by *Photobacterium* (91%), *Pseudoalteromonas* (7%), *Psychrobacter* (1.1%), *Vibrio* (0.3%), *Pseudomonas* (0.3%) and *Shewanella* (0.2%). The most abundant genera within the phylum Fusobacteriota were represented by *Psychrilyobacter* (52%) and *Cetobacterium* (46%), whereas within the phyla Bacillota and Mycoplasmatota respectively the genera *Tyzzzerella* (0.62%) and *Ureaplasma* (0.2%).

The gut microbiota findings showed a variability considering the two investigated categories, namely weight and FAO fishing area. Figure 1 shows the phyla identified in gut Swordfish in terms of relative abundance. Pseudomonadota dominated the gut microbiota across all the weight categories (min 84%, max 99%, mean 91%) and FAO fishing areas (min 86%, max 95%, mean 91%). The differential abundance test revealed that, considering the different weights of the analyzed fish, 10 taxa exhibit a statistically significant higher abundance in fish weighing between 10 and 20 kg, 14 taxa in those weighing between 21 and 30 kg, and 11 taxa in fish weighing over 30 kg. Regarding the different FAO sampling areas, 11 taxa are statistically abundant in fish from FAO area 27, 15 in fish from FAO area 34 and 10 in those from FAO area 37.1.1.

Figure 1. Barplots showing the relative abundance of the most abundant phyla identified in gut Swordfish splitted into the different categories, respectively (A) weight (10-20 kg, 21-30 kg, and over 30 kg) and (B) FAO fishing areas (FAO 27 Atlantic, Northeast, FAO 34 Atlantic, Eastern Central and FAO 37.1.1 Mediterranean and Black Sea, Balearic division).

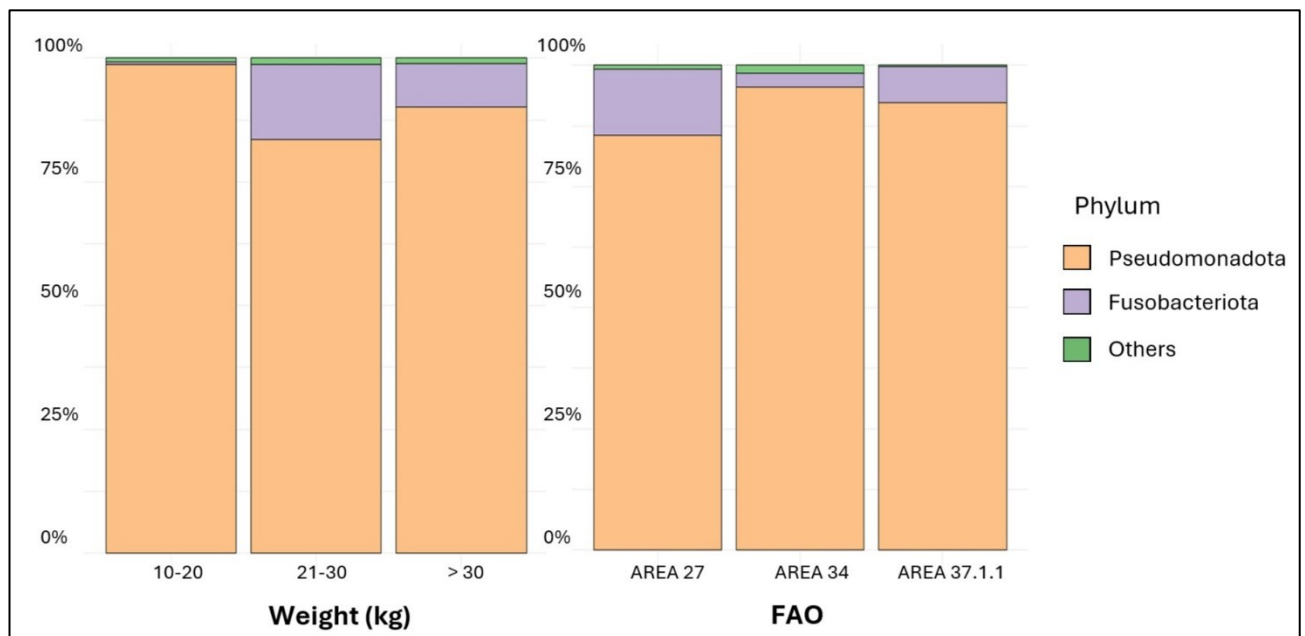
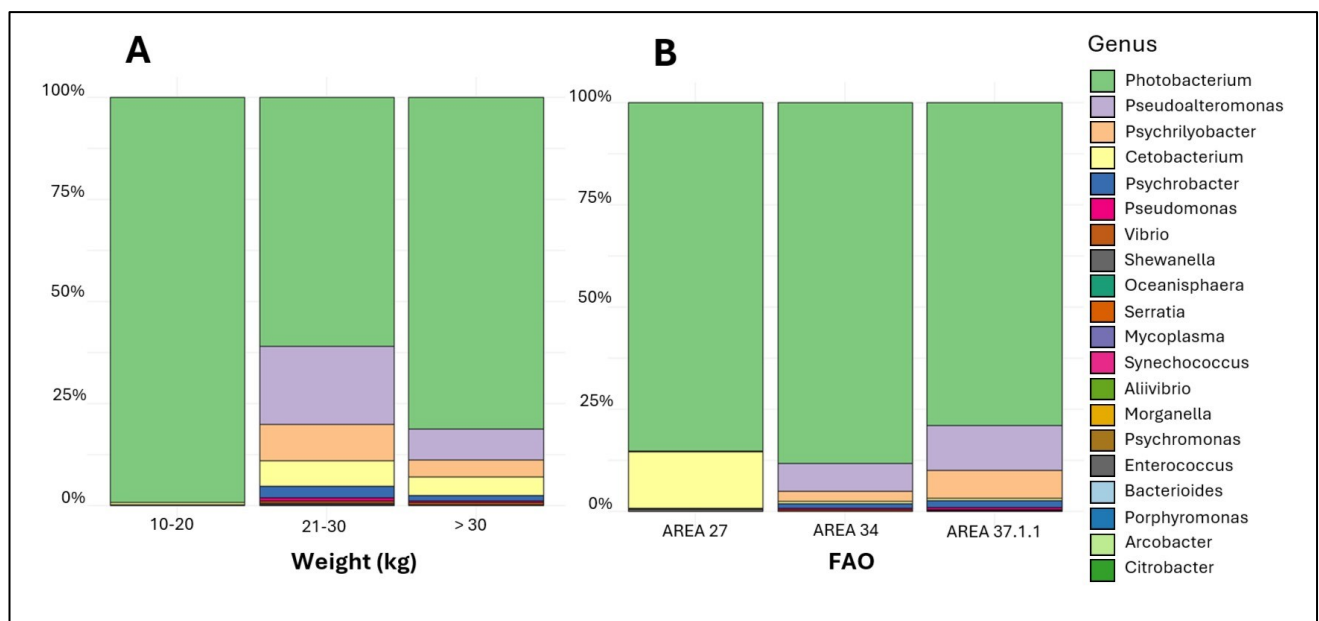


Figure 2 shows the most abundant identified genera in gut Swordfish in terms of relative abundance and their variability within the investigated categories weight and FAO fishing area. The genus *Photobacterium* dominated the gut microbiota across all the weight categories reaching a maximum of 98.5% in smaller fishes (versus 60.2% and 80% in medium and larger fishes) and FAO fishing areas (min 78.6%, max 86.9%). However, among the Pseudomonadota phylum, all the other genera, namely *Pseudoalteromonas*, *Psychrobacter*, *Vibrio*, *Pseudomonas*, show a lower

relative abundance (<0.1 %) only in smaller fishes and in FAO 27 area. Among Fusobacteriota, the presence of *Psychrilyobacter* seems to increase with the weight of the fish (<0.1 % in smaller fishes versus 8.9% and 4.2% in medium and larger fishes) as well as to be more abundant in FAO 34 e 37.1.1 areas (<0.1 % in FAO 27 versus 2.4 % and 6.7% FAO 34 e 37.1.1 areas). Also the presence of *Cetobacterium* seems to increase with the weight of the fish (<1 % in smaller fishes versus 6.2% and 4.6% in medium and larger fishes) whereas, on the contrary, to be more abundant in FAO 27 area (13.7% in FAO 27 versus 0.5% and 0.65% in FAO 34 e 37.1.1 areas).

Figure 2. Barplots showing the relative abundance of the most abundant genera identified in gut Swordfish splitted into the different categories, respectively (A) weight (10-20 kg, 21-30 kg, and over 30 kg) and (B) FAO fishing areas (FAO 27 Atlantic, Northeast, FAO 34 Atlantic, Eastern Central and FAO 37.1.1 Mediterranean and Black Sea, Balearic division).



In addition, alpha diversity metrics unveiled differences among the investigated categories among the overall 100 Swordfish guts, namely weight and FAO fishing area. In more details, among the weight categories, statistical test showed: i) statistically significant lower OTUs count in smaller fishes compared to medium-weight and larger fishes (respectively p-value= 2.3e-7 for 10–20kg vs 21-30kg and 7.1e-5 for 10-20kg vs 31+kg) (see figure 3A); ii) statistically significant lower biodiversity, in term of Shannon index, in smaller fishes compared to medium and large fishes, with the most significant distinction between small and medium fish

groups (10-20 kg vs 21-30 kg p-value= 3.1e-6; 10-20kg vs over 30 kg p-value= 0.0017 and 21-30kg vs over 30kg p-value = 0.04); iii) no statistical differences by the evenness index, revealing a uniform microbial taxa distribution across the three weight categories ($p = 0.5987$) (see figure 4). Among the FAO fishing areas, statistical tests showed: i) no significant difference between the identified OTUs across the samples (p -value = 0.1402) (see figure 3B); ii) only the comparison between fishes from FAO area 34 and FAO area 37.1.1 reveal a significant lower Shannon's index in FAO area 34 than area 37.1.1 (p -value= 0.034); iii) statistically significant differences by the Pielou's index exist between fishes from FAO area 27 and FAO area 37.1.1 (lower in area 27) and between fishes from FAO area 34 and FAO area 37.1.1 (lower in FAO area 34) (respectively p -value= 0.03 and p -value = 8.8e-5) (see figure 5).

Figure 3. Boxplots of the number of identified OTUs in the overall gut samples divided by different weight (A) and different FAO area (B). Outliers are indicated by dots in each boxplot. The significance of differences between the groups is indicated by asterisks: * $p < 0,05$; ** $p < 0,01$; *** $p < 0,001$; p < 0,0001.

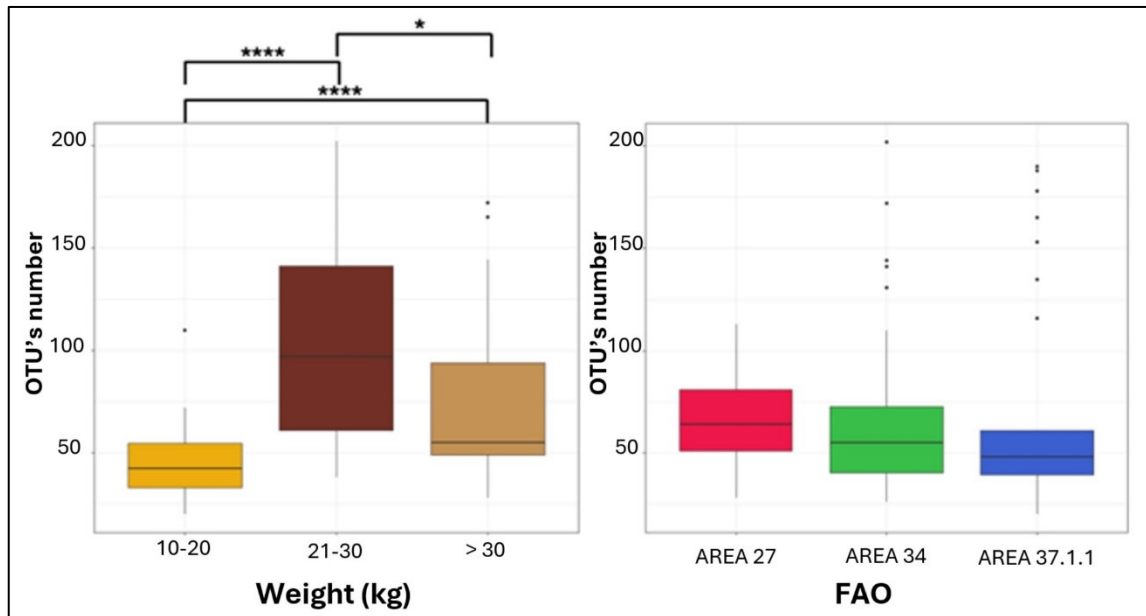


Figure 4. Microbiota diversity in different weight fish categories using Shannon and Pielou diversities indices; the colors of the boxplots represent the different swordfishes' weight categories. Outliers are indicated by dots in each boxplot. The significance of differences between the groups is indicated by asterisks: * $p < 0,05$; ** $p < 0,01$; *** $p < 0,001$; p < 0,0001.

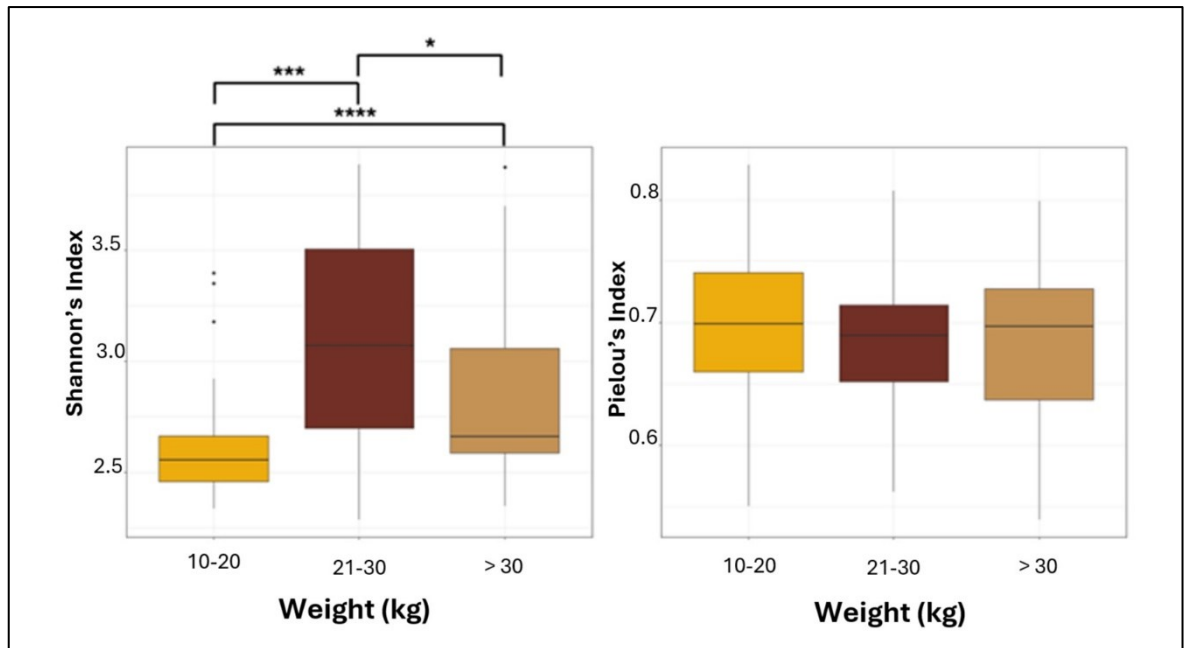
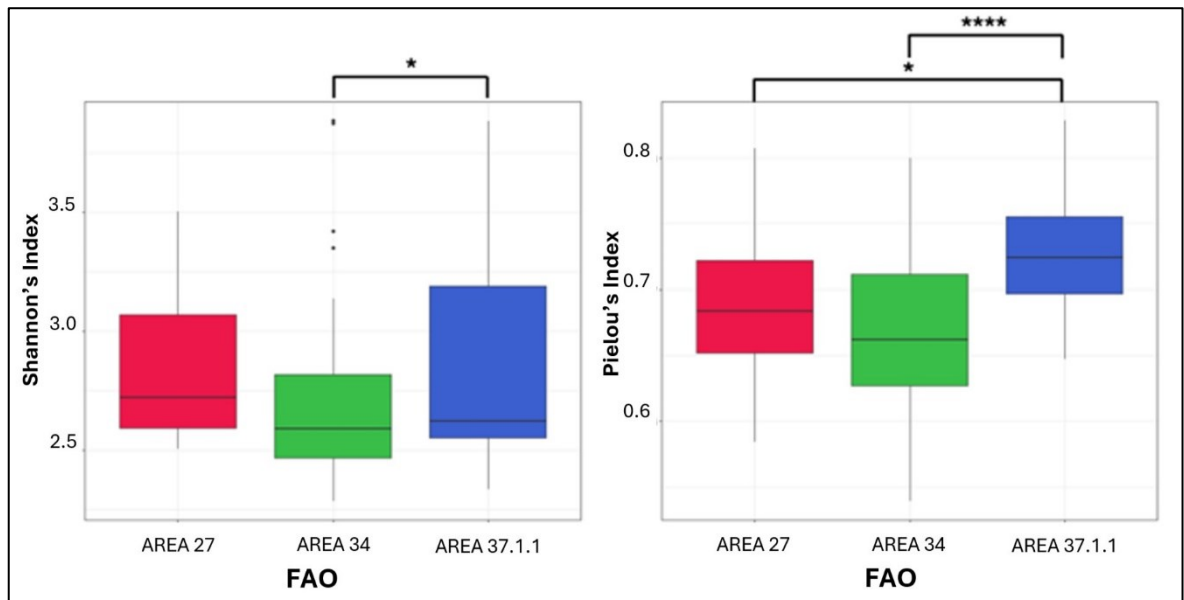


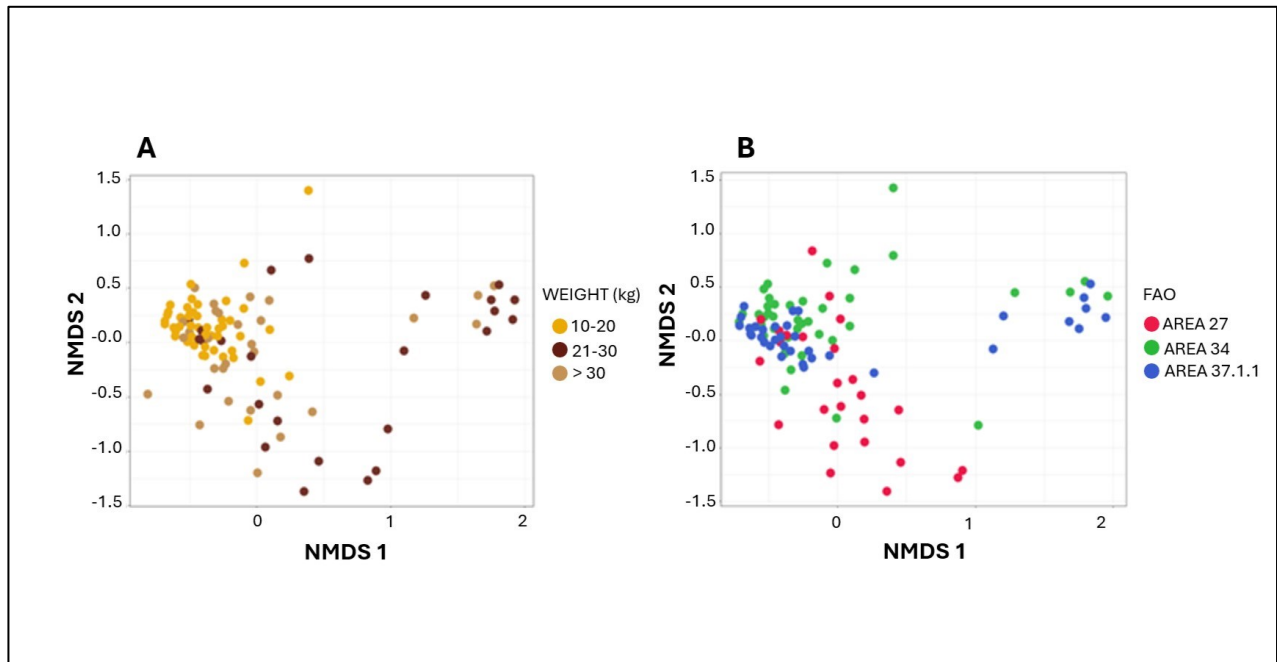
Figure 5. Microbiota diversity in different weight FAO fishing areas using Shannon and Pielou diversities indices; the colors of the boxplots represent the different FAO areas. Outliers are indicated by dots in each boxplot. The significance of differences between the groups is indicated by asterisks: * $p < 0,05$; ** $p < 0,01$; *** $p < 0,001$; **** $p < 0,0001$.



The beta-diversity analysis revealed no significant differences in the taxonomic structure of the microbial community among the three weight categories and FAO fishing areas under consideration. Figure 6 shows the NMDS plot displays samples labeled by weight category and FAO fishing areas, with no discernible clusters observed. Nevertheless, the NMDS plot (Figure 6) shows that samples from small fish (yellow dots) tend to cluster more closely together compared to the other two

weight categories, indicating a tighter grouping pattern despite the absence of statistically tested differences.

Figure 6. NMDS plots showing the weight category (A) and FAO fishing areas (B).



3.4 DISCUSSION

While the literature has begun to elucidate the microbial characteristics of many fish species, available data on gut microbiota are still lacking for *Xiphias gladius*. This study identified distinctions in swordfish gut microbiota between individuals of different sizes and those caught in different FAO fishing areas, elucidating intraspecies microbiota diversity. Given that gut microbiota structure is primarily influenced by trophic level, habitat and host phylogeny [16], it is of clear interest to understand the differences occurring in an apex predator and a highly migratory meso-pelagic fish widely distributed in the Atlantic Ocean and Mediterranean Sea. Therefore, although swordfish are carnivorous fish, their habitat and diet cannot be considered uniform. The investigated swordfish were categorized into small, medium and large individuals based on the the minimum conservation reference size and the average weight data reported for Mediterranean Swordfish. Specifically, Regulation (EU) 2019/1154 [36] on a multiannual recovery plan for Mediterranean Swordfish, and namely Section 2, Article 11 details the minimum conservation reference size for Mediterranean swordfish. It stipulates that, by way of derogation from Article 15(1) of Regulation (EU) No 1380/2013, it is prohibited to catch, retain on board, transship, land, transport, store, sell, or display or offer for

sale Mediterranean swordfish, including in recreational fisheries, measuring less than 100 cm lower jaw to fork length or weighing less than 11.4 kg of live weight, or 10.2 kg of gilled and gutted weight. However, in the case of incidental catches below the minimum conservation reference size, exceptions are allowed only if such catches do not exceed 5 % by weight or number of specimens of the total Mediterranean Swordfish catch of the fishing vessels concerned. Furthermore, the weight categorization reflects the swordfish size distribution recently observed in several areas of the Mediterranean Sea. Among 987 swordfish caught during the 2021 and 2022 fishing seasons, total round weights ranging from 5 to 128 Kg, with a mean value of 33 kg [6,37]. Based on 2019 catches, the size-weight classes between 10 and 35 Kg accounted for 63.40 % of the total catches. Classes above 35 Kg all contributed to the remaining 36.60 % of the total catches, particularly for classes above 80 Kg, where the yield was very low [6,38]. Similarly, based on swordfish catches in the Sicily Region, Italy, between 1986 and 2010, the frequency distributions of the raw individual weight data was skewed but generally peaked in the size range 20–40 kg (min 20, max 79, geometric mean of 40 kg), with most of the individually caught swordfish aged 2–4 years [39]. Additionally, given the weight-at-age and maturity-at-age relationships (50% probability of maturity at age 3.5 years and 37 kg), the captured swordfishes were a mix of larger juveniles and adults, with approximately half of the individuals being mature. However, the true probability of maturity-at-size or -age of swordfish in the Mediterranean is uncertain and likely differs throughout the region [39]. Therefore, the three investigated weight categories (small, medium, and large fish) reflect the real-world scenarios in which swordfish are sold at fish markets, as well as the differentiation between juveniles (small fish) and adults (medium and large fish). Also the three investigated FAO fishing areas have some peculiarities that differently characterized the Swordfish habitats. Mediterranean swordfish have different biological characteristics, compared to the Atlantic stock, with different growth parameters and sexual maturity reached at younger ages than in the Atlantic.

Overall, consistent with previous studies performed in the gut of marine and non-herbivorous fish [11,40], Proteobacteria, now Pseudomonadota Corrig. Phyl. Nov [41] resulted the dominant phylum, followed by Firmicutes, now Fusobacteriota, which constitutes the second most abundant phylum in Swordfish. The abundance of the phylum Proteobacteria in the guts of carnivorous fish seems to have a role in the digestion and absorption of proteins [42]. In literature there is no gut microbiota data to compare our findings in Swordfish, but, in Swordfish cut fishes the study of Indio and Colleagues [19] also reported Proteobacteria and Firmicutes as prevalent

phyla observed by shotgun metagenomic. Instead, unlike literature findings on gut microbiota of carnivorous fish reporting facultative anaerobes, comprising *Vibrio*, *Pseudomonas*, *Acinetobacter*, *Corynebacterium*, *Alteromonas*, *Flavobacterium* and *Micrococcus* as the predominant intestinal microbes [15], in our study *Photobacterium* dominated the gut microbiota across all the weight categories and FAO fishing areas. This finding is in agreement with the comparison on dominant gut bacteria in 16 carnivorous fish species, in which *Photobacterium* were the second most frequently genus reported [11] or highly abundant in Mackerel Icefish [26] and Atlantic cod [43]. *Photobacterium* are Gram-negative, aerobic members of the Vibrionaceae family and has been identified in fish gut microbiota as mutualistic bacteria that might aid chitin digestion of foods [11,26,42] and produce chitinases [11] and harmful enzymes such as neuraminidases [44]. In general, *Photobacteria* occur free-living in seawater and sediments or in interaction with marine animals [45] but certain species could be considered pathogenic for a broad range of marine hosts as well as a considerable problem in the fish food industry, representing specific spoilage organisms in chilled fish and seafood products [46]. The spoilage processes by *Photobacterium* involve production of quantities of trimethylamine and biogenic amines such as histamine [47,48] given several species (*P. aquimaris*, *P. angustum*, *P. kishitanii*, *P. damsela*, and *P. phosphoreum*) are known as histamine producers. Considering Swordfish in terms of microbiological safety, *Photobacterium* resulted the most abundant genera among the microbial populations identified by shotgun metagenomic in the Swordfish flesh, significantly higher in Swordfish in comparison to Tuna and also Salmon [19]. In addition, Swordfish cuts appeared the richest in terms of histamine producing bacteria, even if histamine and other biogenic amines were not quantified [19]. Even though the *Xiphidae* family is not included among the families associated with a high amount of histidine by Regulation (EC) 2073/2005 for the food safety criterium histamine, two incidents of histamine outbreaks are described due to the consumption of imported smoked swordfish and swordfish fillets [21,22].

In the observed Swordfish gut microbiota belonging to adults, namely medium and larger individuals, the abundance of the genera *Pseudoalteromonas*, *Cetobacterium*, *Psychrilyobacter* and *Psychrobacter* increases with the weight of the fish, conversely from juveniles in which these genera are always <1%. This means that the gut microbiota of juveniles (smaller fish) are less rich and diverse than adults (medium and larger fishes). Also the FAO fishing areas seem to affect the gut microbiota composition: the relative abundance of *Pseudoalteromonas*, *Psychrilyobacter* and *Psychrobacter* genera was higher in FAO 34 and 37.1.1 areas whereas *Cetobacterium*

was higher only in FAO 27 area. Due to their unique biological characteristics, we expected the Mediterranean and Atlantic Swordfish stocks to have a distinct gut microbiota but our findings give more evidence that the fish gut microbiota is primarily determined by the fish environment, rather than by genetic factors, as underlined by Kim and Colleagues [40]. In literature on fish gut microbiota, *Cetobacterium* is reported to be a major candidate in *Oncorhynchus mykiss* [49] and in five out of the six healthy edible fish species from intensive freshwater aquaculture and to play a beneficial role in biochemical processes in the fish gut [50]. In addition it is the dominant commensal bacterium in many fish species and their metabolites are rich in vitamin B12, which is inferred to promote the health of fish [51]. Also *Psychrilyobacter* is reported as a key member genus with high relative abundance in guts of marine invertebrates and animals, being present over 20% of the intestines of European abalone [52]. *Psychrobacter* can be recovered in high abundance from the guts of different marine fishes, namely four carnivorous coral reef fishes [53], *Salvelinus alpinus* [54], *Gadus morhua* [17] and *Oncorhynchus mykiss* [49]. *Pseudoalteromonas*, *Cetobacterium*, *Psychrilyobacter* and *Psychrobacter* are all Gram negative bacteria of which *Pseudoalteromonas* and *Psychrobacter* are aerobic, whereas *Cetobacterium* and *Psychrilyobacter* are obligately anaerobic bacteria. They are all described in literature as seafood spoilage microbiota producing volatile organic compounds causing musty off-odours [55].

In the end, the fish gut microbiota has become a frontier research field. Culture-independent high-throughput sequencing of 16S rRNA genes has greatly facilitated studies exploring microbial communities, such as the fish gut microbial compositions, generating large-scale data sets that describe the microbial composition of a certain niche, data that could be used to understand and describe microbial diversity. But certain critical aspects need to be highlighted. Essential features of a bacterial community in a certain niche are characterized by the number of species present and their numerical composition, namely the bacterial diversity [56]. This bacterial diversity is based on OTUs measurement which can be defined at different level of resolution, from phylum to genus and species and can be quantified at two levels, namely the alpha (within-sample) and beta (between-sample) diversity. Alpha diversity metrics summarize the structure of a microbial community with respect to its richness (number of taxonomic groups in a sample), evenness (distribution of abundances of the groups), or both. Beta diversity metrics summarize which samples differ from one another by considering sequence abundances or considering only the presence or absence of sequences [57]. However, both diversity indices have specific biases and therefore it is essential to

pinpoint that diversity is rather a simple statistic with which to describe the complexity of this microbial ecosystem. Studies employing a range of diversity measures reveal that the differences between them can be considerable, influencing the significance of results. This emphasizes the need for a cautious approach when drawing conclusions from any one diversity index [58]. Due to the complexity of fish gut microbiota, the structure and function of the Swordfish gut microbiota have not been investigated in depth. Collectively, these findings improve the understanding of this valuable fish and provide a reference for future studies of Swordfish gut microbiota. Furthermore, considering that the gut is a primary source of bacterial contamination during animal slaughter and processing, leading to the potential presence of spoilage microorganisms and foodborne pathogens in food, a deeper characterization of Swordfish gut microbiota will guide also a better understanding of this valuable fish in terms of food safety.

3.5 CONCLUSIONS

This study provides the first characterization of the gut microbiota of *Xiphias gladius* using next-generation sequencing. In summary, our results offer a comprehensive view of the swordfish gut microbiota, derived from a significant sample size of 100 individuals caught in different FAO fishing areas which were objects of this study (FAO 27, 34, 37.1.1 areas) and belonging to various size classes, enabling a detailed investigation of intra-species diversity. Similarly with other marine carnivorous fish species, Pseudomonadota phylum dominated the gut microbiota, followed by far by Fusobacteriota, but, differently from literature, *Photobacterium* resulted the most abundant genus. Differences in the alpha diversity were observed primarily between different weight categories rather than FAO fishing areas. Specifically, within-species variability was more pronounced across size categories: smaller fishes exhibited a less rich and diverse gut microbiota with an almost exclusive presence of *Photobacterium* (98.5%) compared to medium and larger fishes, in which the genera *Pseudoalteromonas*, *Psychrilyobacter*, *Cetobacterium* and *Psychrobacter* appeared to increase with fish weight. Although *Photobacterium* was dominant across all FAO fishing areas, distinctive microbial community compositions were found, namely the *Cetobacterium* in FAO 27 and *Pseudoalteromonas* in the other areas. Since swordfish capture quota from both Atlantic ocean and Mediterranean sea is less than the 30% respect to the total [59], it would be interesting to study gut microbiota of swordfish deriving from other FAO fishing areas, such as Pacific and Indian oceans, which together contribute to more than the 70% of the global capture.

Moreover in these areas fishing techniques, timing of stocking fish inside the boats before landing, production, conservation and packing techniques in the factories are very different from the studied areas and it could be interesting to verify if they influence the gut microbiota and, in case, the contamination of fish meat and of production environments.

The findings of this study enhance our understanding of the swordfish, a valuable fish much appreciated by EU consumers but also more vulnerable than other fish with regards to food safety. Further research is necessary to clarify the gut microbiota further and to understand the complete picture of the gut and flesh microbiota in swordfish.

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CHAPTER 4 - OVERVIEW OF PATAGONIAN RED OCTOPUS (*ENTEROCTOPUS MEGALOCYATHUS*) FISHERIES IN CHILEAN REGIONS AND THEIR FOOD SAFETY ASPECTS

Truant, A., Giacometti, F., Hernández, J., Espinoza, V., Farías, A., Uriarte, I., Godoy, C., Miotti Scapin, R., Alberghini, L., Catellani, P., & Giaccone, V. (2025). Overview of Patagonian Red Octopus (*Enteroctopus megalocyathus*) Fisheries in Chilean Regions and Their Food Safety Aspects. *Animals*, 15(10), 1464.

<https://doi.org/10.3390/ani15101464>

4.1 INTRODUCTION

A growing global concern is related to the overexploitation of marine resources and consequent impact on marine ecology as well as the availability of high nutritional protein sources for human consumption [1]. On the one hand, the global overexploitation and depletion of many finfish species over the last few decades has led to an increase in the commercial importance of other marine resources, such as cephalopods (squids, cuttlefishes, and octopuses) [2]. On the other hand, the negative trend of overexploitation refers to different fish species and fishing areas, including marine carnivorous mollusks, namely cephalopods [3]. Among cephalopod landings, the squid fishery represents nearly 80% of the worldwide catches, whereas octopuses and cuttlefishes represent only about 10% each [4]. Octopus fisheries are likely to continue to grow in importance and magnitude as many finfish stocks are either fully or overexploited [4]. Fishery stocks that are scientifically assessed are in better condition, either already under sustainable exploitation or rebuilding, than stocks that are unassessed [5]. The vast majority of stocks are unassessed [6], especially stocks exploited by small-scale fisheries.

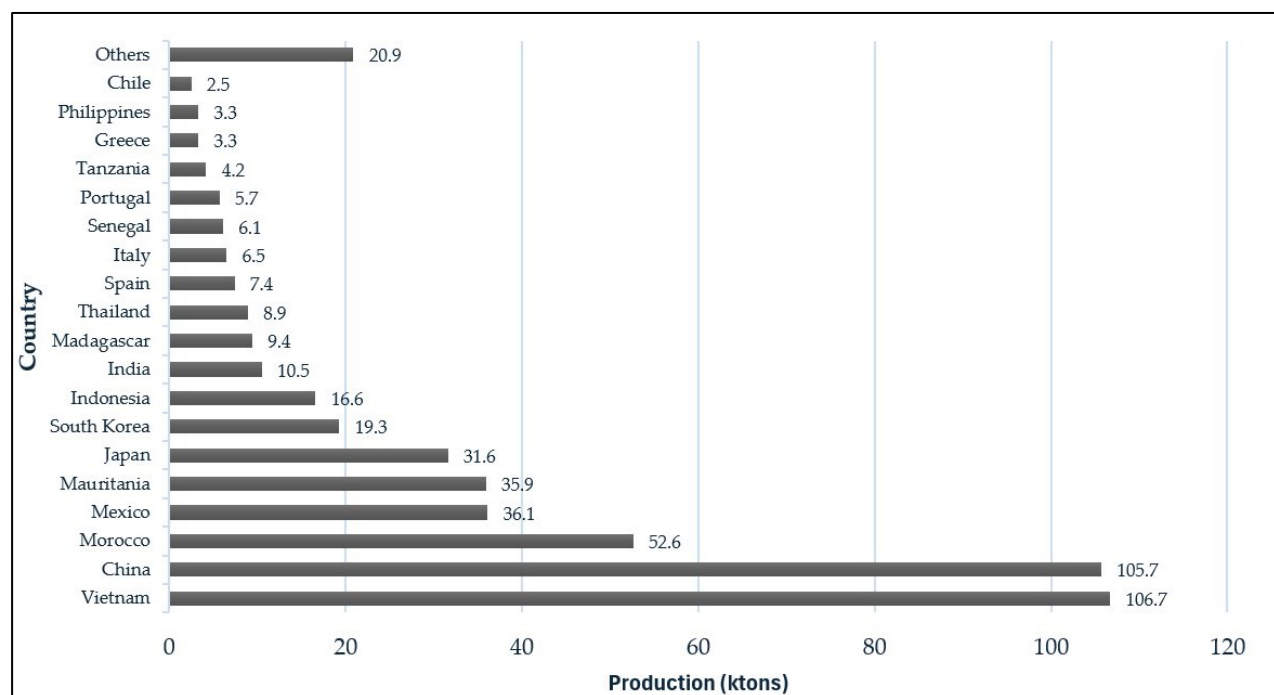
In the specific case of Latin America, octopus fisheries represent only about 8% of cephalopod world catches (see Scheme 1 for more details). This is mainly due to the recent development and the continued dominance of artisanal, small-scale, and low-productivity fishing activities (except for the well-developed octopus fishing sector in the Campeche Bank of the Yucatan Peninsula) [7]. Contributing factors include limited investment in fisheries research and development, limited technical development of artisanal fleets, a lack of specific public policies for the sustainable management of the resource, and weak integration of regional markets into international cephalopod value chains. Furthermore, many Latin American countries prioritize other, more established fisheries (such as anchovy, shrimp, and hake), relegating octopus to a secondary role. Additional challenges include logistics, traceability, and compliance with international regulations that limit its commercial expansion. Nevertheless, there are emerging initiatives led by local communities, research institutions, and certain government bodies aimed at enhancing the ecological and socioeconomic value of octopus resources, potentially paving the way for greater participation in global markets in the future [8-10]. Octopus catches in the Eastern Pacific are mainly taken by Chile, Peru, and Mexico, but Octopus is also common in the Southeast Pacific coast with a wide oceanic and coastal distribution [7]. In the southern tip of South America (Argentina and Chile), the octopus fishery is largely based on *Enteroctopus megalocyathus*, the Patagonian

red octopus, a merobenthic species that inhabits the Pacific and Atlantic coasts, from 42° S to the Strait of Magallanes, including the Malvinas/Falkland Islands [11]. Specifically, in southern Chile, *E. megalocyathus* is an important resource representing a high value product with significative importance for local economies in several areas where small-scale fisheries, namely artisanal fisheries, are still in practice today. This phenomenon can be observed in southern Chilean regions, such as the Los Lagos region, where local communities of fishers (36,556 fishers recorded in 2023) base their familiar economy on artisanal fishing activities and for which local octopus species play an important role [12]. *Robsonella fontaniana*, commonly referred to as “pulpito” or “baby octopus”, is another species frequently caught in the intertidal waters of the Los Lagos region due to its overlapping geographical distribution with *E. megalocyathus*. This species exhibits a broad distribution range, extending from approximately 41° S along the Pacific and Atlantic coasts to 56° S near Cape Horn, Chile [13]. Economically, it remains a marginal species with no support for targeted commercial exploitation, and to date, no official landing records are available [4].

Studies on *E. megalocyathus* from the Atlantic and Pacific oceans have increased notably in the last 20 years, mostly driven by its relevance as a fishery resource and growing interest as a culture species [14]. However, important biological and ecological aspects of this species remain to be investigated, as well as food safety aspects for consumers. Comprehensive investigations and a deeper understanding of the structure and dynamics of natural populations are needed, particularly during the early developmental stages, where knowledge of paralarval ecology remains limited. Clarifying the spatial and temporal occurrence of early life stages, and elucidating their interactions with biotic and abiotic factors, is critical for advancing our understanding of paralarval dispersal, recruitment processes, and the associated interannual variability in cephalopod population biomass [15]. Promoting research focused on habitat selection and utilization throughout the ontogeny of the red octopus, as well as assessing physiological and ecological responses to environmental drivers, is essential, particularly under ongoing climate change scenarios. Additionally, detailed insights into the trophic ecology of the red octopus and its functional role within marine food webs are still lacking, representing a key knowledge gap that should be addressed.

This paper describes the *E. megalocyathus* fisheries in Chilean waters, providing an update review on fisheries, including artisanal fisheries, in the Los Lagos region, with the aim of detailing general food safety aspects in octopus fisheries and products, based on the available literature and public domain data.

Scheme 1. Average global octopus production (2019-2021, adapted from Di Todaro [16]).



4.2 BIOLOGY AND FISHERY EXPLOITATION OF THE PATAGONIAN RED OCTOPUS (*ENTEROCTOPUS MEGALOCYATHUS*)

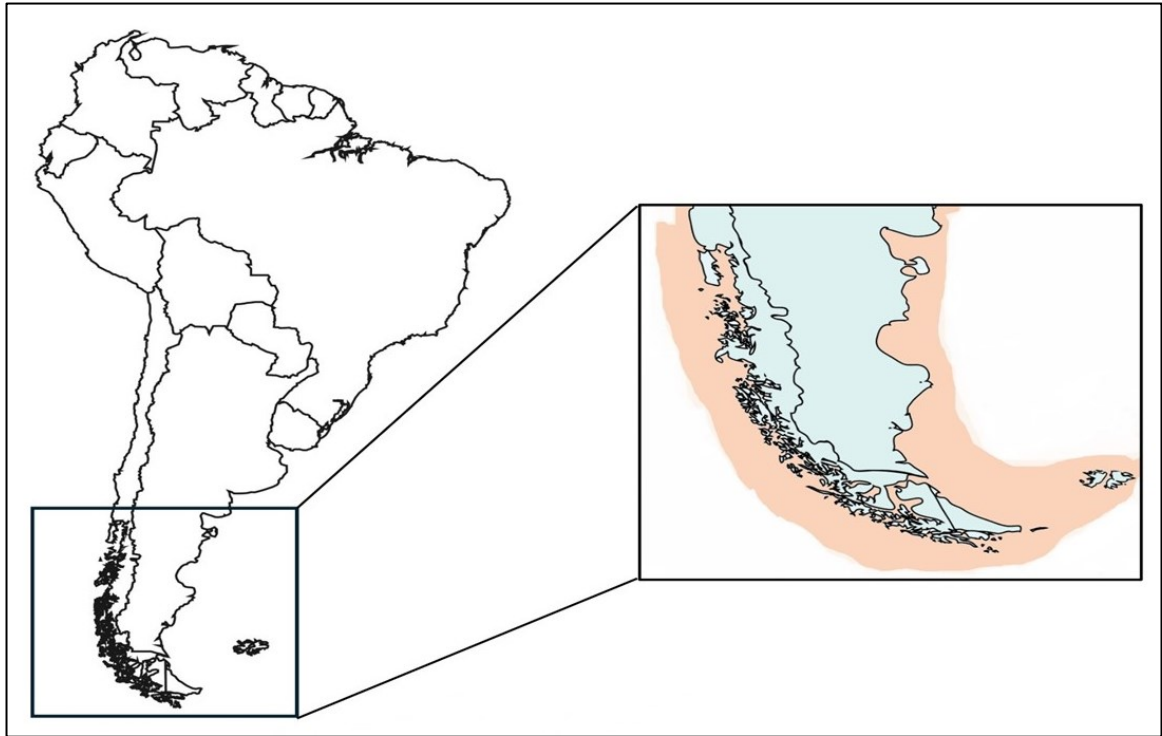
The Patagonian red octopus (*E. megalocyathus*) represents a species of considerable ecological and socioeconomic importance across the southern coastal ecosystems of Chile and Argentina. As a key component of benthic communities and a valuable target for artisanal fisheries, *E. megalocyathus* plays a pivotal role in sustaining both biodiversity and local livelihoods. However, comprehensive understanding of its biology, life history traits, and ecological interactions remains limited, particularly with respect to early developmental stages, habitat preferences, and trophic dynamics. Increasing fishing pressure over the past decades has raised significant concerns regarding stock sustainability, leading to the implementation of management measures such as seasonal closures and the establishment of territorial use rights in fisheries (TURFs). A thorough characterization of the species' biological and ecological attributes, alongside an analysis of artisanal fishing practices and regulatory frameworks, is thus essential to inform effective conservation strategies and promote the long-term sustainability of *E. megalocyathus* fisheries. The following sections provide an integrated overview of these aspects, highlighting both current knowledge and persisting gaps that require further research.

4.2.1 Distribution, Life Cycle and Capture

4.2.1.1 Geographical Distribution

The Patagonian red octopus, *E. megalocyathus* (Couthoy in Gould, 1852), is one of the giant octopuses (genus *Enteroctopus*, Family *Enteroctopodidae*) and can reach up to 7 kg wet weight, over 30 cm mantle length, a total length of 1.3 m [17], and a lifespan between 1.9 and 2.5 years [18]. It is the main octopus species present in the southern Chilean and Argentinean coasts. In more detail, *E. megalocyathus* is distributed from the Los Lagos region (41°S, 72°00'W) throughout the Patagonian Shelf around the southern coast of South America from the Magellan biogeographic province, from Chiloe Island in the Southeast Pacific, via Cape Horn, to the San Matias Gulf in the Southwest Atlantic. It includes the Beagle Channel, Malvinas/Falkland Islands, and Namuncura (Burdwood) Bank [17,19,20] (Figure 1). The Pacific coast, from north of Chiloe Island to Cape Horn, is described as a large insular system (the Chilean Archipelago) characterized by numerous gulfs, fjords, channels, small river basins, and many conspicuous glaciers, which are the product of tectonic processes and ice ages. This makes it a complex landscape with difficult access both by land and sea [21]. The Patagonian red octopus occupies holes and crevices in rocky substrates from the lower intertidal zone down to a depth of 170 m off Argentina and down to a depth of 220 m off Chile [17]. Within its distribution, there are two genetically distinct *E. megalocyathus* populations, one on the Pacific coast and the other on the Atlantic coast, with low genetic diversity in contrast to the high variability found in other octopus species (*Octopus variabilis*, *Octopus minor*, *Octopus vulgaris*, and *Amphioctopus ovulum*) [17].

Figure 1. *Enteroctopus megalocyathus* geographical distribution (authors' own elaboration).

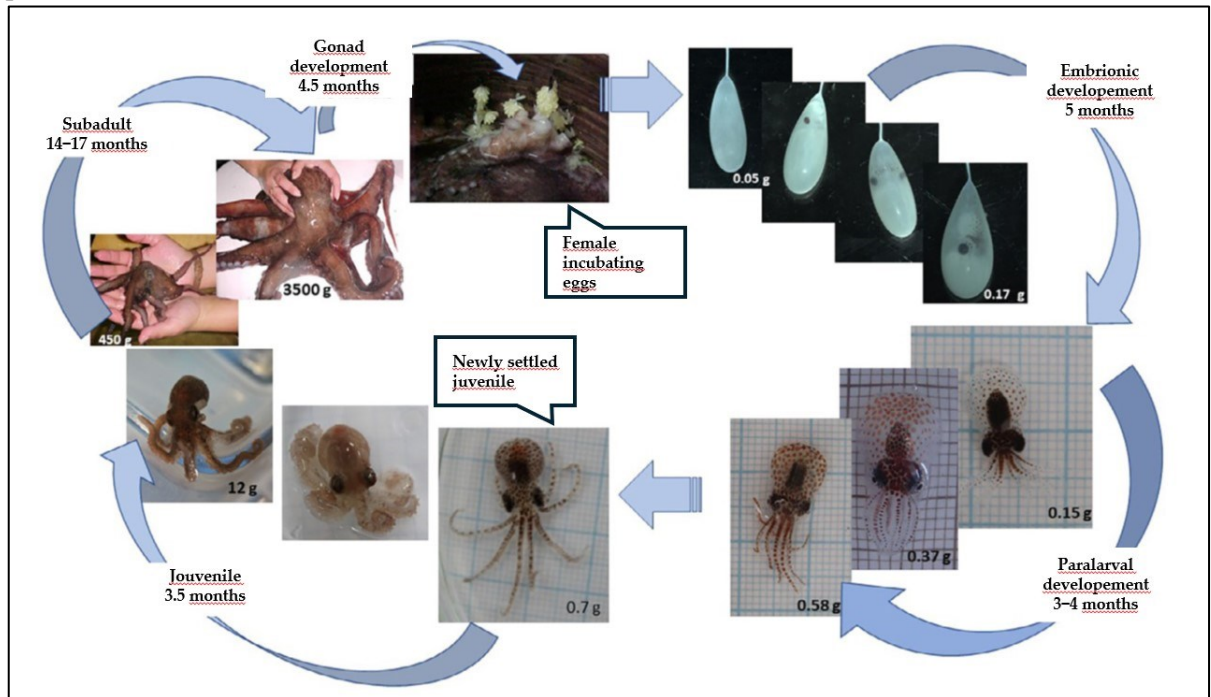


4.2.1.2 Life Cycle and Trophic Ecology

Like other cephalopods, it is a short-living semelparous organism with a seasonal breeding activity. Individuals exhibit separate sexes with marked sexual dimorphism, with males displaying a modified arm called the hectocotylus, which is used for spermatophore transfer to females (a single case of hermaphroditism has been reported in this species) [22,23]. The sexual maturity of individuals starts in winter, reaching its maximum in spring, with the peak of reproductive activity in summer, between December and January [20]. Sexual maturity is mainly influenced by individual size. After mating and breeding activities, which usually takes places in deeper locations than fishing areas [24], females lay egg strings, sticking them in the roofs of rock holes from 6 to 14 m depth [25].

The eggs are highly vulnerable to predation, and the female provides continuous protection, cleaning, and ventilation throughout the entire developmental period, at the end of which they will die [26]. After 5 months, the paralarvae of Patagonian red octopuses will hatch [11]. This phase is characterized by planktonic paralarvae that feed on different planktonic microorganisms, which lasts about 90 to 114 days, before moving to the benthic phase as juveniles (Figure 2).

Figure 2. *Enteroctopus megalocyathus* life cycle (figure adapted from Ortiz et al. [17] with permission from the authors).



It is not known if, in this life stage, paralarvae remain in coastal areas or migrate into the open ocean, transported by sea currents (as suggested for other species by Roura et al. [27]), until, after 3–4 months, they reach the juvenile stage, adopting the classical benthic behavior [28]. Concerning bathymetric distribution, adult *E. megalocyathus* can be found at different depths depending on environmental temperatures. With water temperatures between 12 and 13 °C, individuals are usually found between 15 and 30 m depth along the Pacific coast, whereas they occur at depths of less than 20 m along the Atlantic coast [18]. When higher temperatures (>13 °C) are present, adult benthic individuals have been found at depths of up to 170 m [24], with the record at 220 m depth [29]. A study conducted by the Chilean Instituto de Fomento Pesquero (IFOP) reported environmental parameters characterizing the habitat of *E. megalocyathus* along the coasts of Chiloé Island, highlighting water temperatures between 9.8 and 13.5 °C, salinity ranging from 32.1 to 33.4 PSU, and oxygen concentrations between 8.4 and 9.2 mL-L⁻¹ (with saturation levels reaching up to 100%) [23].

E. megalocyathus is an opportunistic predator with a broad prey spectrum. Although it presents wide dietary variability, for both the Pacific and Atlantic populations, the diet is predominantly based on decapod crustaceans, mainly from the Order Brachyura (*Homalaspis plana*, *Cancer setosus*, and *Cancer coronatus*) and the Order Anomura (*Petrolisthes* sp., *Munida subrugosa*, and *Peltarion spinosulum*) [18,30,31].

Several studies have suggested that octopus predation may play a significant role in shaping benthic community structure [32,33]. With high voracity, broad dietary spectrum, and substantial population density, *E. megalocyathus* likely acts as a key predator in both intertidal and subtidal ecosystems, potentially exerting a notable influence on community composition and trophic interactions [17]. Cannibalistic behavior has also been reported, particularly during early developmental stages when paralarvae density is high and/or prey availability is low [34]. On the other hand, the main predators of the red octopus include Peale's dolphin (*Lagenorhynchus australis*) [35], spiny dogfish (*Squalus acanthias*) [36,37], South American sea lion (*Otaria flavescens*) [38], western rockhopper penguin (*Eudyptes chrysocome*) [39,40], gentoo penguin (*Pygoscelis papua*) [41], and humans (fishers). A more comprehensive overview of the principal red octopus preys and predators is presented in Table 1. All of these environmental factors affect the octopus life cycle, thus affecting population dynamics and densities in certain areas throughout the different seasons.

Table 1. Main preys and predators of the Patagonian red octopus, *Enterocyathus megalocyathus* (Adapted from Ortiz et al. [17]).

Main Preys [20,42]	Main Predators
Squat lobster, <i>Munida subrugosa</i>	South American sea lion, <i>Otaria flavescens</i> [38,43]
Cod icefish species, <i>Patagonotothen</i> sp.	South American fur seal, <i>Arctocephalus australis</i> [44]
Tractor crab, <i>Peltarion spinosulum</i>	Bottlenose dolphin, <i>Tursiops truncatus</i> [45]
Cunningham's triplefin, <i>Helocogrammoides cunninghami</i>	Dusky dolphin, <i>Lagenorhynchus obscurus</i> [46,47]
<i>Ribeiroclinus eigenmanni</i>	Peale's dolphin, <i>Lagenorhynchus australis</i> [35]
Rock crab species, <i>Cyrtograpsus</i> sp.	Commerson's dolphin, <i>Cephalorhynchus commersonii commersonii</i> [48]
True limpet species, <i>Nacella</i> sp.	Beaked skate, <i>Dipturus chilensis</i> [49]
Crab eggs	Spiny dogfish, <i>Squalus acanthias</i> [36,37]
Octopus eggs	Narrowmouth catshark, <i>Schroederichtys biviatus</i> [36]
Conspecifics	Red cod, <i>Salilota australis</i> [50]
	Sandperch, <i>Pseudoperca semifasciata</i> [51,52]
	Argentine sea bass, <i>Acanthistius patachonicus</i> [53]
	Southern rockhopper penguin, <i>Eudyptes chrysocome chrysocome</i> [39,40]
	Gentoo penguin, <i>Pygoscelis papua</i> [41]
	Magellanic penguin, <i>Spheniscus magellanicus</i> [53]
	Imperial shag, <i>Leucocarbo atriceps</i> [54–56]
	Magellan cormorant, <i>Leucocarbo magellanicus</i> [57]
	King cormorant, <i>Leucocarbo albiventer</i> [57]
	Striated caracara's, <i>Phalcoboenus australis</i> [58]

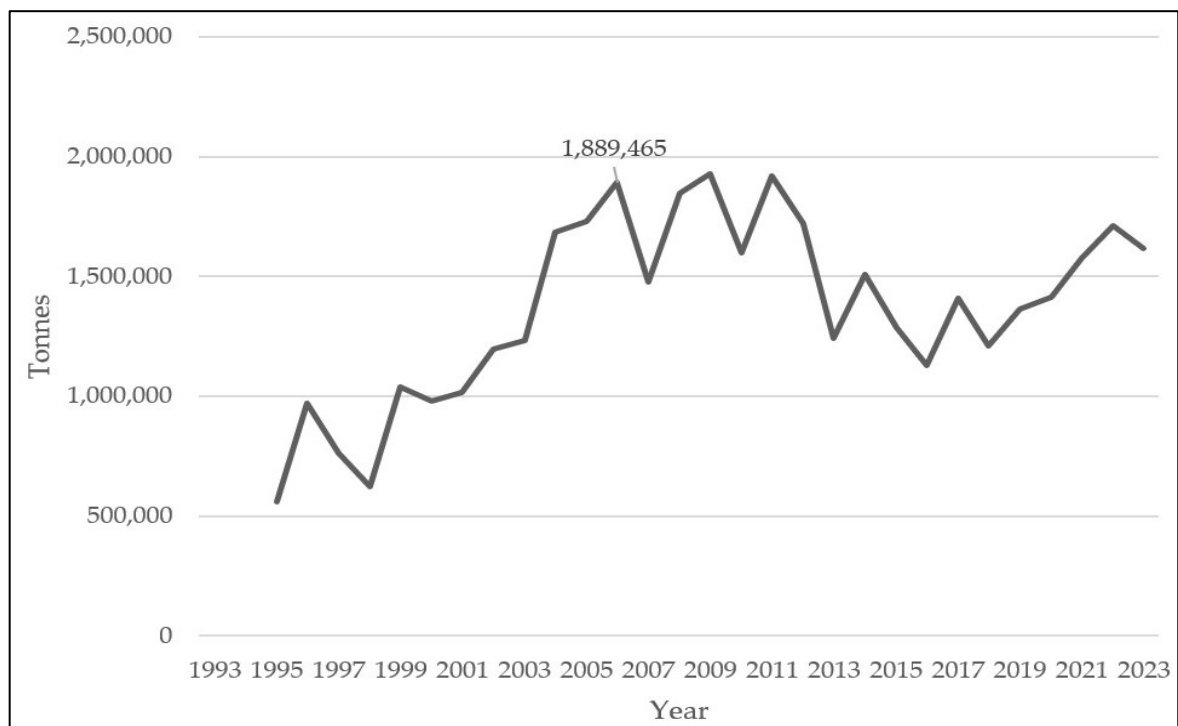
4.2.1.3 Landings and Fishery Relevance

In relation to abundance, the total reported global production of octopuses over the past three decades indicates a relatively steady increase in catch, almost doubling from 179,042 tonnes in 1980 to 355,239 tonnes in 2014 [4]. In Chile, *E. megalocyathus* is a native and merobenthic species; considering its high nutritional, organoleptic value and massive presence in coastal areas, an intensive local exploitation for food consumption of this marine resource is reported. *E. megalocyathus* was determined to be the second most important octopus species after *Octopus mimus*, although its abundance data were not clear and difficult to compare. Reconstructed data reported an average of 700 tonnes for landings by year, with a catch peak at 1700 tonnes in 2008, whereas octopus catches by other countries in the region are trivial [7]. For 2008, an average landing of 500 tonnes/year of *E. megalocyathus* in Chilean water is always reported [20]. Between 2009 and 2011, a complete fishing ban was imposed on this species due to overexploitation. Subsequently, a seasonal fishing ban was implemented, restricting fishing activities from 15 October to 15 March each year, to support the species' reproductive cycle, along with a decrease in the minimum fishing weight of 1 kg (Supreme Decree No. 137/1985) [59]. In 2016, a total of 540 tonnes (equivalent to 0.7% of the global octopus production) of the Patagonian red octopus were landed in Chile [60]. *E. megalocyathus* accounts for 25-40% of Chilean octopus landings [17]. In 2023, an annual landing of red octopus of 571 tonnes was reported in the last available SERNAPESCA report record as “Pulpo del Sur” [13]. Almost 100% of *E. megalocyathus* catch is landed in the Los Lagos region. It reaches its highest population densities and optimal commercial sizes in the cool-temperate waters of the Los Lagos region (Region X) and Aysen (Region XI), where it finds ideal habitats such as rocky bottoms and coastal caves. The water temperature in this region remains within a favorable range for the growth and reproduction of *E. megalocyathus*. These habitats are easily accessible for small-scale fishing (with apnea or traps), without the need for expensive vessels or extensive technology. In the Los Lagos region, there is also a large concentration of artisanal fishers organized in management areas, which promotes a more planned and legal use of the resource.

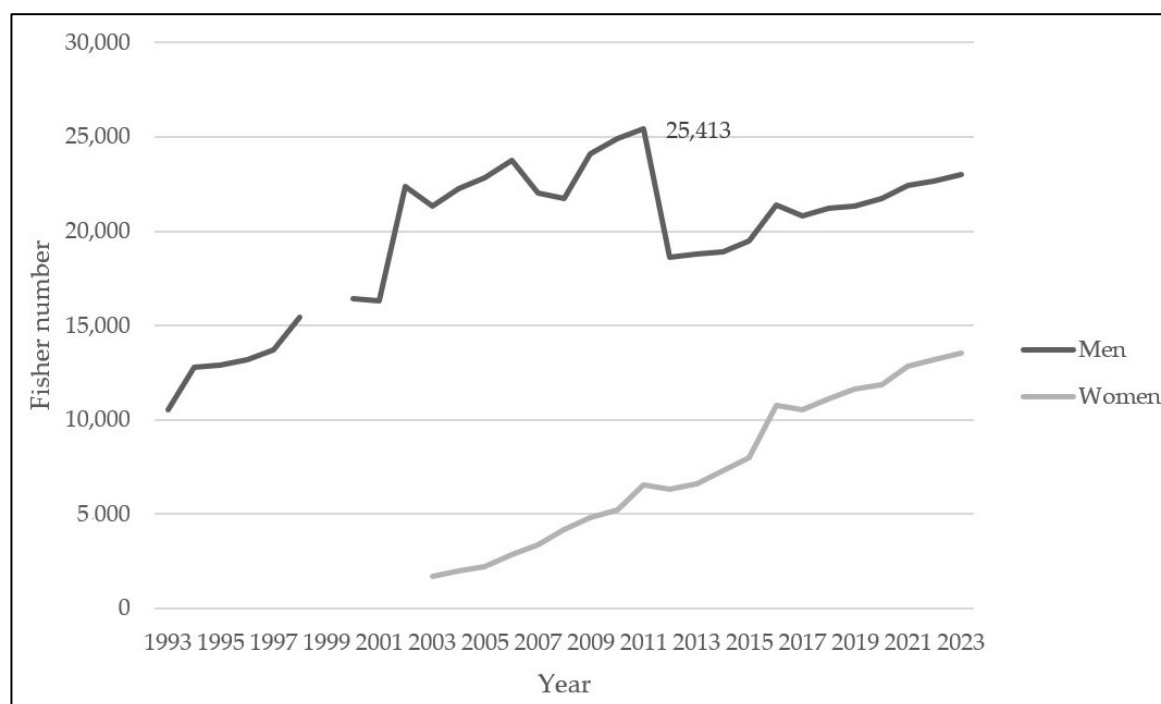
In Chile, a total of 375 small-scale fisheries are targeting invertebrates and macroalgae. Total artisanal fishing landings and number of artisanal fishers showed an increasing trend over recent years (Schemes 2; Scheme 3 for Los Lagos Region). The catch of *E. megalocyathus* is carried out by divers that travel to numerous fishing grounds in small, motorized boats. The octopus season extends from March to October. The data for stock assessment and total annual landings were collected in

Chile by the “Servicio Nacional de Pesca y Acuicultura” (SERNAPESCA), which carries out a census of all fishing trips to evaluate the magnitude of the catch for all large-scale fisheries and for the most important small-scale fisheries. In the case of the red octopus of the Inner Sea of Chiloé, fishers have not yet learned about the sustainable exploitation rates because the fishery is too young, and thus they entered a regime of overfishing by the time of the end of the study reported in [5]. Indeed, as clearly described by Markaida and Gilly [7], in southern Chile, an overfishing of *E. megalocyathus* has occurred and, hitherto, small scale octopus fisheries are experiencing different challenges. In fact, even small-scale fisheries like those for *E. megalocyathus* have been interrupted or banned for some years due to overfishing concerns [11,19]. Open access to the fishery by non-fisher divers with hooks and use of gear such as pots that retain spawning females are current concerns in the octopus fisheries; moreover, improper identification of commercial species is still a common problem [61]. In addition, management measurements such as minimum size or gear restrictions are difficult to enforce in these fisheries. Therefore, there are still areas of opportunity, such as developing methods of octopus fisheries management [7].

Scheme 2. Total artisanal landings in Chile (1995-2003, authors’ own elaboration based on public domain data from SERNAPESCA).



Scheme 3. Number of fishers of Los Lagos Region registered in the Artisanal Fishery Register (Registro Pesquero Artesanal, RPA; authors' own elaboration based on public domain data from SERNAPESCA).



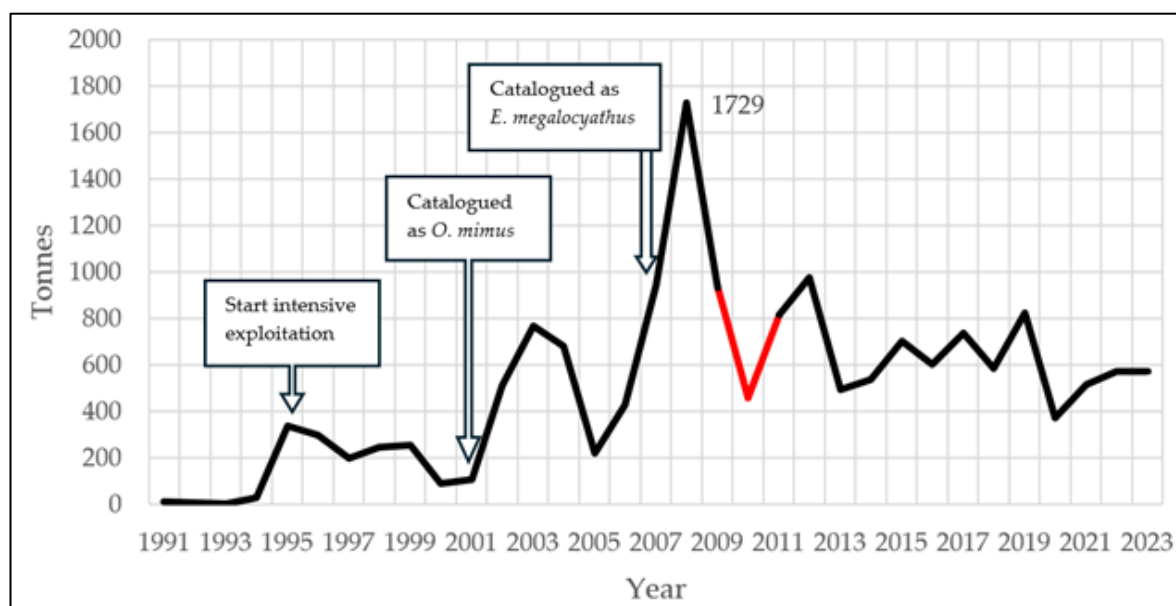
4.3 MANAGEMENT STRATEGIES FOR THE PATAGONIAN RED OCTOPUS (*ENTEROCTOPUS MEGALOCYATHUS*)

4.3.1 Artisanal Fishing Laws in Chile

Article No. 28 of Chilean fishing Law No. 18892/1989 (Ley General de Pesca y Acuicultura) [62] defines artisanal fishing as the extractive fishing activity carried out by natural persons in a personal, direct, and habitual way, and in the case of territorial use rights in fisheries (TURFs), by legal entities composed exclusively of artisanal fishers registered as such. Employees on fishing boats used for artisanal fishing are considered artisanal fishers. This definition also includes extractive fishing divers, shore collectors, and algae collectors (Article No. 29 of Chilean fishing Law No. 18892) [62]. This means that artisanal fishing is characterized by a wide spectrum of different activities, which can range from the recollection of costal resources to scuba diving, with different techniques, different boats, and different roles of the fishers, and it accounts for a substantial share (39% in 2023) of Chile's total fisheries output (considering both fishing landings and aquaculture production) [63]. For local fishers, artisanal fishing is a traditional and hereditary activity with a strong socio-cultural component. Artisanal fisheries provide fundamental socioeconomic support to rural coastal communities in Chile. Small-

scale fishing activities represent a primary source of employment, income, and food security, generating significant revenues for fishers [64]. The legal requirement to become an artisanal fisher is inscription in the National Fishing Register, which shows an annual increasing trend of new inscriptions (Registro Pesquero Artesanal, RPA). A total of, respectively, 58,873, 81,157 (+37.8%), and 89,697 (+10.5%) fishers were registered in 2000, 2010, and 2018 [65–67]. At the end of 2023, a total of 103,017 artisanal fishers (26% of which are female) were reported [68]. All of these fishers are organized into different associations, like the local fisher organizations, namely the National Confederation of Artisanal Fishermen of Chile (Confederación Nacional de Pescadores Artesanales de Chile), the National Confederation of Artisanal Fishermen Federations (Confederación Nacional de Federaciones de Pescadores Artesanales), and the National Council for the Protection of Fish Stocks (Consejo Nacional por la Defensa del Patrimonio Pesquero). At the end of 2023, in the Artisanal Organization Register (Registro de Organizaciones Artesanales), a total of 1850 different fisher associations (considering federations, confederations, syndicate, trade associations, and indigenous communities) were registered. The creation of fisher associations gives social, political, and economic strength to artisanal fishers, obtaining better results in the relationship with institutions. Improper identification of commercial species is still a common problem. In southern Chile, this has led to the overfishing of *E. megalocyathus*. A ban had to be implemented, and Chilean statistics now discriminate octopus landings by species (since 2010). This emphasizes the need to accurately identify any species being landed [7]. In the Los Lagos region, almost 571 tons of Patagonian red octopus were landed in 2023 [13] (Scheme 4).

Scheme 4. *Enteroctopus megalocyathus* artisanal landings in the Los Lagos region (authors' own elaboration based on public domain data from SERNAPESCA). The red line indicates the fishing ban period (2009–2011). The reported catches came from an exceptional derogation of the fishing ban.



According to official reports from SERNAPESCA, *E. megalocyathus* and *O. mimus* were historically recorded as a single fishery resource until 2007. Beginning in 2008, official statistics began to differentiate between the two species, listed, respectively, as “Pulpo del Sur” (southern octopus) and “Pulpo del Norte” (northern octopus). *O. mimus* is distributed along the northern coast of Chile (2.2–27.7° S), with no geographic overlap with *E. megalocyathus* [69]. Based on this, landing data from the Los Lagos region in SERNAPESCA reports are interpreted as pertaining exclusively to *E. megalocyathus*. Given the historical absence of a systematic identification protocol, a comparative table is provided to highlight the diagnostic features useful for species differentiation (Table 2).

Table 2. Key anatomical features of *Enterocotopus megalocyathus* and *Octopus mimus* [69].

Anatomical Characteristic	<i>Enterocotopus megalocyathus</i>	<i>Octopus mimus</i>
Mantle length (max)	Up to 190 mm	Up to 190 mm
Total length (max)	Up to ~1.0 m	Up to ~1.2 m
Body weight (max)	Up to ~7 kg	Up to ~4 kg
Arm length (relative to mantle)	3.5–5× mantle length	4–6× mantle length
Arm formula ¹	2>3>1>4	2>3>4>1
Web depth ²	18–23% of arm length, equal on lateral & dorsal	18–27% of arm length, deeper on lateral arms
Suckers per arm (adult)	~180–210	Up to ~330
Enlarged suckers (male)	8–10 on all arms, starting ~14th sucker (both sexes)	2–4 enlarged suckers on 2 nd and 3 rd arms (males)

Gills (lamellae per demibranch)	11–13	7–8
Funnel organ	W-shaped, outer limbs shorter	W-shaped, limbs equal
Radula	9 elements (7 rows of teeth + marginal plates)	9 elements (7 rows of teeth + marginal plates)
Hectocotylized arm	Right third arm, 70–90% length of opposite arm	Right third arm, ~77% length of opposite arm
Ligula length (% of hectocotylized arm)	11–22%	0.7–1.8%
Calamus length (% of ligula)	10–15%	30–60%
Spermatophore length / number	Up to 3× mantle lengths (~3 produced)	32–40% of mantle lengths / high number
Egg size/number	~17 mm/few (~1000-5000)	2–3 mm/numerous (~400,000)
Skin texture	Loose, longitudinal folds or flaps	Rugose, coarse papillae, 2–3 large over eyes
Color (live)	Reddish purple dorsally, pale ventrally	Orange to red-purple
False eye spots (ocelli)	Absent	Absent (some dark spot may appear)
Habitat depth range	5–25 m (migrates deeper in breeding season or warmer months)	0–30 m
Geographic distribution	Chilean and Argentinean Patagonia	Northern Peru to Northern Chile

¹ Comparative length of the four arm pairs expressed numerically in decreasing order. In octopods, the nonhectocotylized arm 3 (right or left) is used in this formula. ² Triangular section of the interbrachial web, which is the membranous skin connecting adjacent arms.

4.3.2 Territorial Use Rights in Fisheries (TURFs), the Management Areas for Benthic Resource Exploitation

Article No. 47 of Chilean fishing Law No. 18892/1989 [62] identifies the first 5 miles from the coast as an exclusive area for artisanal fishing activities (“Area de reserva para la pesca artesanal”, ARPA), while industrial fishing activities are allowed in the open sea. Artisanal fishing activities are carried out mainly in coves, called “Caletas”, which are the productive, economic, social, and cultural centers in which this fishing sector originates. At the end of 2023, a total of 558 active “Caletas” were reported all along the Chilean coast [70]. In this area, Article No. 55 of Law No. 18892/1989 gives the possibility to legal fisher associations to apply for the creation of territorial use rights in fisheries (TURFs) (“Areas de manejo y explotacion de recursos bentonicos, AMERB”, textually translated as “Management areas for the exploitation of marine benthic resources”). This is an administrative and management measure to give exploitation rights of benthic resources to local artisanal fisher associations. In these TURFs, artisanal fishers can extract resources from the sea bottom, which are identified as main species in Chilean law. All the

extracted products are part of fishing quotas related to management plans of that specific coastal area, which must be approved by Undersecretariat of Fisheries and Aquaculture (“Subsecretaria de Pesca y Acuicultura”, SUBPESCA), which reports to the Chilean Ministry of Economy, Development and Tourism. The main targets of this management practice are the conservation of natural benthic resources while maintaining and promoting their productivity and also promoting the sustainability of artisanal fishing activities by involving local fishers in active participative management. Moreover, in these TURFs, artisanal fishers can implement small-scale aquaculture activities. According to the last reports, up to October 2024, along the Chilean coasts, a total of 499 fisher organizations with recognized TURFs were reported [70], reaching a benthic resources production of 17,100 tonnes [71]. The main products extracted in these areas are algae and mollusks. Most artisanal fishing activities are reported in the Los Lagos region, in the south of Chile, with the community reaching more than 36,000 artisanal fishers at the end of 2023 [68]. Along the coast of this region, there are almost 296 TURFs [72]. For a long period, *E. megalocyathus* was confused with *O. mimus*, which lives in the coasts of Northern Chile, thus confusing the real landing values [64]. Rocha and Vega report that *E. megalocyathus* is fished south of the Nuble region (36°00' lat S) [73]. According to the last available data from SERNAPESCA, in the Los Lagos region, almost 571 tons of red octopus were landed in 2023 [13]. The institution of TURFs was first proposed in response to the overexploitation problem of the muricide gastropod *Concholepas concholepas*, locally known as “Loco” and worldwide as “Chilean abalone”, which represents one of the most valuable marine products of Chilean coasts. Fishers realized the rapid decline in “Loco” populations in 1990, and in the same year, Chilean legislation instituted TURFs to apply a new experimental modality of rational and sustainable management of benthic marine resources. Galarza and Kamiche Zegarra [74] report how fishing often exemplifies the so-known “Tragedy of the Commons” [75], where unrestricted access to a natural resource leads to its overexploitation and potential depletion, as individual fishers use it without awareness of being part of a system and prioritize immediate gains over long-term sustainability, ultimately threatening long-term viability of fish stocks. To overcome this problem, in 1990, Ostrom proposed increasing the capacities and responsibilities of individuals to promote cooperation and new solutions that lead to resource preservation [76]. The mandatory management plans for TURFs are based on this fundamental principle. Since the establishment of the first TURF in 1997, a consistent upward trend has resulted in the current total of 673 TURFs [72].

Early evidence of the effectiveness of this organizational structure was observed in the restoration of “Loco” wild populations, which had been overexploited for extended periods [77]. The establishment of TURFs has significantly contributed to increasing the “Loco” population within managed areas, and over the years of experience, other species have been added, such as the Patagonian red octopus *E. megalocyathus*. Other species, such as the *Gracilaria* sp. algae, have also benefited from the implementation of TURFs, although their effectiveness is not uniform across all benthic resources. Pascual-Fernandez et al. report the effectiveness of participative governance in small-scale fisheries in Spain, highlighting how localized management plans for the octopus fishery, based on fish stock assessments, have helped to improve the sustainability of this sector [78].

4.3.3 Internal Governance, Decision-Making Process, and Conflict Resolution

In Chile, TURFs grant exclusive seabed access to legally constituted artisanal fishing organizations for the sustainable exploitation of benthic resources such as sea urchins, “Loco”, and octopuses. Local governance is exercised by artisanal unions, cooperatives, or associations that hold tenure over specific coastal areas, mainly composed of male members. Decision-making is typically conducted through the Partners’ Assembly, which is the primary deliberative body of each organization, where resolutions are approved by a simple majority (50% + 1). This assembly determines key operational elements, including diver participation, harvest schedules, and pricing strategies. A Board of Directors or Management Committee, elected from among the members, is responsible for implementing the management plan, coordinating harvest activities, and securing permits. The core management tool is the management plan, which is a technical document that must be approved by SERNAPESCA and SUBPESCA. It defines target species, harvest methods, extraction seasons, and allowable quotas. The effectiveness of the plan is assessed annually or biannually through in situ monitoring conducted by external consulting entities (typically universities or environmental firms) hired by the organization, which must be recognized by the fisheries authority and be part of a registry approved by this entity. These technical monitoring reports are mandated by Article No. 55B of Chilean General Fishing Law (Ley General de Pesca y Acuicultura No. 18892/1989) [62]. Internal and external conflicts are common in TURFs. Internally, disputes may arise over income distribution, diver schedules, or member inclusion. Externally, tensions often involve unauthorized fishing and third-party intrusion into managed areas. To address these challenges, many organizations have adopted governance tools, such as internal regulations (clearly defining rights, roles, and

sanctions) and conflict mediation through union or board meetings. In more complex cases, external institutions such as IFOP, SERNAPESCA, or INDESPA (formally “Instituto Nacional de Desarrollo Sustentable de la Pesca Artesanal y de la Acuicultura de Pequeña Escala”) may provide technical or legal support. In certain circumstances, especially where conflicts involve public order, the Ministry of the Interior may also be involved. In areas with multiple TURFs, inter-organizational coordination is often facilitated through territorial committees or collaborative networks, sometimes with municipal support acting as a neutral facilitator or notary. Oversight of TURF management involves a tripartite system including state agencies, the fishing organizations themselves, and external partners. Resource status is periodically assessed through technical monitoring—conducted jointly by the organization and professional consultancy groups—as a prerequisite for harvest authorization. SERNAPESCA plays a central role in overseeing plan compliance, auditing logbooks, controlling landings, and detecting unauthorized extraction. At the community level, many unions organize voluntary patrols or contract private security to prevent illegal fishing, requiring a high investment that is deducted from their profits. Additionally, some organizations implement traceability mechanisms, such as product labeling or digital dispatch guides, to ensure legal origin and maintain the integrity of supply chains [74,77,78]. A summary of institutional organizations is provided in Table 3.

Table 3. Institutional hierarchical organizations regulating the artisanal fishing sector and TURFs (elaborated by the authors).

Level	Institution	Main Functions (Artisanal Fisheries Focus)
1	Ministry of Economy, Development and Tourism	Defines national strategies for sustainable resource use; issues decrees; allocates budgets; oversees institutional structure.
2	SUBPESCA (Undersecretariat for Fisheries and Aquaculture)	Issues special fishing permits, sets regulations, manages the Artisanal Fishers Register and AMERBs, defines fishing quotas and authorized species.
3	SERNAPESCA (National Fisheries and Aquaculture Service)	Maintains the Artisanal Fishers Register, oversees sanitary and traceability programs, controls landings, and ensures food safety for export.
4	IFOP (Fisheries Development Institute) INDESPA (Instituto de Desarrollo de la Pesca Artesanal y Acuicultura a Pequeña Escala)	Provides scientific data, technical assessments, and supports monitoring programs and stock assessments. Supports artisanal fisheries via funding, training, infrastructure, organizational strengthening, and policy implementation.
5	Advisory Councils (National and Zonal) Scientific and Technical Committees	Provide technical input on fisheries management plans, TURFs, and policy reforms. Include artisanal representatives. Advise on methodologies, sanitary standards, and policy development; use IFOP data.

6	National Artisanal Fisheries Register	Official record of artisanal fishers, vessels, and organizations authorized to operate.
7	Artisanal Fisher Organizations	Hold rights to AMERBs, co-manage resources, represent fishers, and implement local governance structures.
8	Individual Fishers	Must be registered; operate within legal norms; can be members of organizations.

4.3.4 Positive Outcomes and Strengths of TURF Implementation in Chile

In recent years, numerous studies have analyzed the implementation and effectiveness of TURFs in Chile, particularly in relation to the regulation of benthic fisheries and the underlying political objectives. The establishment of management and exploitation areas for benthic resources (AMERBs) has proven to be an efficient tool to counteract overexploitation, leading to significant increases in both the abundance and size of managed species compared to adjacent open-access areas. More importantly, AMERBs appear to generate a spillover effect that may facilitate the natural repopulation not only of the protected species themselves but also of other associated marine organisms [79-81]. This has resulted in higher yields, improved product quality [64], reduced navigation and search times, and consequently increased overall economic returns and strengthened community support [79]. Furthermore, management areas offer fishers greater flexibility by allowing them to schedule their harvests to align with optimal market conditions, thus maximizing economic gains [82]. Beyond economic aspects, artisanal fishing activities are deeply embedded in local communities, preserving traditional knowledge, cultural identity, and social cohesion [64].

The implementation of TURFs has empowered fishing associations to sustainably manage benthic resources, fostering participatory governance and enhancing community self-organization [81,83]. This participatory governance structure cultivates a sense of ownership and responsibility for sustainable resource use. Collaboration with professionals from various fields (biologists, sociologists, etc.) has further improved local understanding of stock assessment and resource management practices. Communal ownership encourages cooperation among fishers, strengthens stewardship of fishing grounds, and reduces illegal extraction by outsiders, with fishers actively contributing to the enforcement and surveillance of regulations. Authors reported how the local ecological knowledge of artisanal fishers is crucial for acquiring valuable ecological and fishing information on the poorly studied sea species, effectively complementing biological data to understand their distribution and characteristics, especially given that there are infrequently captured species with lack of commercial value [84].

From an ecological perspective, fishers engaged in TURF management have reported a shift in environmental awareness, recognizing the conservation benefits derived from these practices [81,85,86]. Additionally, artisanal fisheries provide diversification opportunities through small-scale aquaculture and marine ecotourism, contributing to economic stability and reducing vulnerability to external shocks [87]. Overall, artisanal fisheries sustain local economies while simultaneously strengthening social-ecological resilience by integrating economic, cultural, and environmental dimensions.

4.3.5 Challenges and Limitations of TURF Implementation

Despite the positive outcomes and intentions behind TURFs, their implementation has faced persistent challenges. One of the most critical issues that still remains is illegal fishing. Although regulations exist, effective oversight is often lacking, and local fishing organizations must allocate significant portions of their income to surveillance activities. These are typically managed through rotational efforts among members, as stipulated in their internal operating regulations [88]. Conflicts have also arisen over access and use, particularly in areas where TURFs overlap with the interests of other economic sectors such as tourism and mining or indigenous communities. These conflicts have complicated the enforcement of regulations, necessitating the formation of local working groups to manage coastal resource use [88]. Effective control throughout the entire production chain could be a way to reduce the impacts of illegal fishing, which would be complemented by social and community-based oversight. The effectiveness of TURFs in conserving benthic resources has been variable. While some areas have shown signs of ecological recovery and evidence of seed dispersal to adjacent open-access zones, others continue to face overfishing and lack of access control [89]. Furthermore, although policy efforts aim to support local socioeconomic development, not all communities have observed tangible improvements in income or resource access. In fact, some have struggled to adapt to new restrictions imposed by TURFs, resulting in resistance or low participation. Co-management models, where communities are involved in decision-making processes, have yielded more positive results in terms of acceptance and participation. However, the case study of the Patagonian red octopus in the Inner Sea of Chiloe highlights the ongoing risk of overexploitation. Roa-Ureta et al. [5] attribute this to a lack of fisher cooperation and the complexity and size of management areas, which impede effective implementation. The study also notes the need to consider species-specific biological factors, such as the lower fecundity of *E. megalocyathus* compared to other

managed species. In summary, while the political intention to regulate benthic fisheries through TURFs in Chile has been largely positive, their real-world effectiveness varies. Key obstacles include insufficient monitoring, illegal fishing, and socio-territorial conflicts. To improve the impact of TURFs, particularly AMERBs, greater investment in oversight, deeper community engagement, and expanded research efforts are essential to bridge the gap between policy and practice.

4.4 OCTOPUS FISHING PRACTICES AND THE ARTISANAL PRODUCTIVE CHAIN IN CHILE

Different methods for fishing octopus are used by local artisanal fishers. The most effective and used method in Chile is the hook method, which is carried out by inserting a harpoon or hook inside rock crevices in intertidal and subtidal areas (the octopus's main refuge) to carefully extract Patagonian red octopus (*E. megalocyathus*). This is a very selective fishing method that avoids by-catch of other species, but it does not allow for differentiation of laying female octopus from male individuals, thus affecting the reproductive capacity of the species and the survival of eggs. The eggs are susceptible to microbial infections that adhere to their bodies, resulting in death [11,90,91]. Artisanal fishers usually exploit red octopus between 1 and 48 m depth [92]. Other fishing techniques are implemented around the world, depending on various factors, including the target octopus species, environmental conditions, and local traditions and regulations. The hand recollection method, which is prevalent in tropical and subtropical areas, is practiced globally. Fishers collect octopuses by hand from flat beaches and lagoons, often using a spear or harpoon to assist in extracting the animals from their shelters [4]. Pot and trap fishing methods are employed for catching octopuses on a smaller commercial scale [93]. Pots can be constructed from various materials, including terracotta, concrete, plastic pipes, and tires. Japanese fishers utilize pots with live crab as bait and a trapdoor mechanism to capture octopuses. Pots can be used for both targeted octopus fishing and for incidental catches of the animals attempting to prey on target species in other types of fishing [4,94,95]. Trawl nets represent the second most important fishing method for octopuses [96]. However, this method yields a mixed catch of lower quality and value. Large mesh trawl nets are dragged across the seabed by a vessel, capturing octopuses in their path, with a consequent high environmental impact [4]. Fyke nets are used in some areas of the world for octopus fishing. This method is mainly used for fishing *O. vulgaris* in the eastern Mediterranean, specifically in the region of Kavala and Limenas in Greece. In these

areas, fyke nets, composed of two or three net chambers and hoops, are placed in shallow coastal waters, at depths ranging from 8 to 30 m [4]. Like fyke nets, baited traps are used by artisanal fishers in the Canary Islands for *O. vulgaris* fishing practice [97,98]. The barrel longline method is primarily used for fishing Giant Pacific Octopus (*Enteroctopus dofleini*) and consists of a series of floating barrels connected to a mainline with traps or pots hanging from it. Octopuses are attracted to the bait or shelter offered by the traps and become entangled within [4]. Another method is the use of boats armed with fishing lines equipped with multiple hooks at the end. The fishing lines are baited with lures such as live or dead crabs, bivalve shells, colored stones, white plastic bags, or artificial rubber lures, which are used from the shore or small boats in shallow waters [4,15]. Finally, the use of towed rakes is a traditional method employed in some areas of Japan, where rakes are dragged across the seabed by small boats to catch octopuses hiding amongst rocks or in the sand. Together with the trawl net method, towed rakes are a technique with a high environmental impact [4]. The different fishing methods are summarized in Table 4.

Moreover, aside from the artisanal fishers (registered in RPA, as natural or juridic persons), the remaining links of the value chain include intermediaries, processors, and marketing companies, which interact directly with artisanal fishers and their organizations. These actors serve as the primary conduit through which artisanal fishery products are integrated and subsequently delivered to national and international markets (Figure 3).

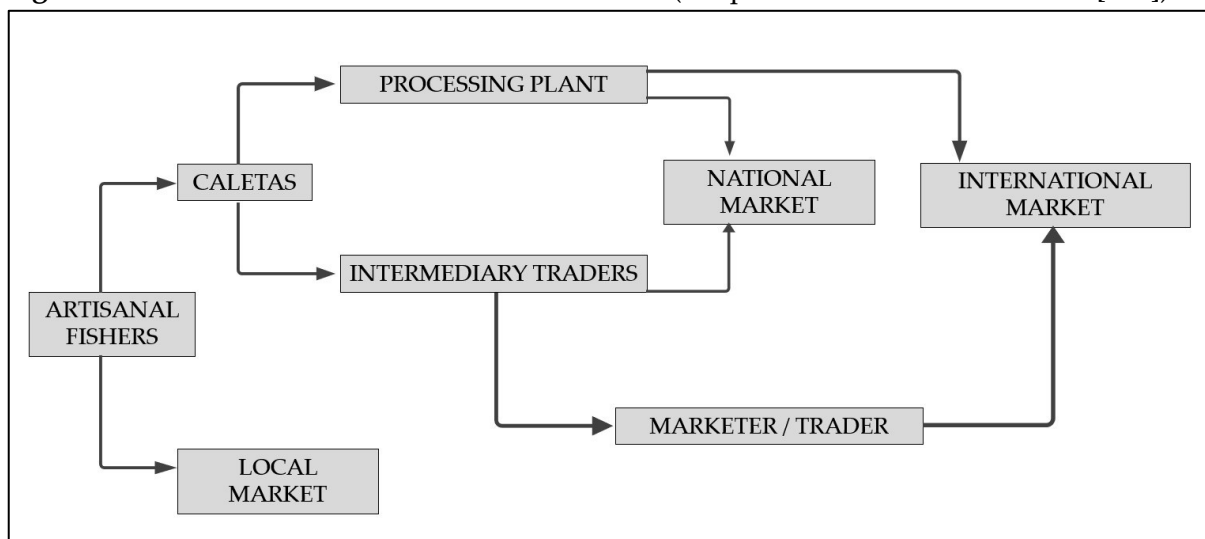
Table 4. Description of the main fishing methods in world octopus fisheries (elaborated by the authors).

FISHING METHOD	DESCRIPTION	IMPACT	TARGET OCTOPUS SPECIES	GEOGRAPHICAL AREA	REFERENCES
Hand recollection	Operations on foot, snorkeling or small boats Intertidal areas (1-30 m depth) Traditional technique No specialized equipment	Very selective and low impact method	See "Hook method"	See "Hook method"	[4,15,99]
Hook method	Harpoon or hook inside rock crevices (1-30 m depth) Intertidal areas	Very selective method Difficult differentiation of laying females Poor quality of raw materials	<i>O. cyanea</i> <i>O. hubbsorum</i> <i>O. insularis</i> <i>O. maya</i> <i>O. mimus</i> <i>C. nocturnus</i> <i>O. tehuelchus</i>	SW Indian Ocean NE Pacific SE Atlantic Western Central Atlantic SE Pacific SW Pacific SE Atlantic	[11,73, 100,101,102, 103,104,105,106,107]
Hookah diving	Same as hook method, but with air supply from a compressor on the fishing vessel (1-40 m depth)	Very selective method Difficult differentiation of laying females, higher catches	<i>O. mimus</i> <i>O. hubbsorum</i>	Ecuador Peru Chile Mexico	[108,109,110]
Pots	Pots of different materials that mimic refugees for octopus in sandy/muddy sea bottom. Checked every 2- days. (3-20 m depth)	Size selective for adults, avoid by-catch. Low incidental mortalities and environmental impact. Possible plastic pollution for bad management	<i>O. vulgaris</i> <i>O. insularis</i> <i>O. pallidus</i> <i>O. sinensis</i> <i>A. fangsiao</i> <i>O. tetricus</i> <i>O. minor</i> <i>E. dofleini</i>	Mediterranean NE Atlantic Eastern Atlantic South Africa SE Atlantic Tasmania Japan Japan Western Australia Corea Alaska	[4,94,95,103, 111,112, 113,114,115, 116,117,118,119]
Trawl nets	Broad-mesh net dragged by cables from fishing vessel. Usually from multispecies fishing or by-catch (60-500 m depth depending on	High impact on seabed and benthic shelf and lope communities	<i>O. vulgaris</i> <i>E. moschata</i> <i>E. cirrhosa</i> <i>Amphioctopus</i> sp.	NW Africa (Saharan Bank) NE Atlantic NE Atlantic Gulf of Thailand	[120,121, 122,123,124, 125,126,127]

	boat dimensions)				
Fyke nets / Baited traps	2-3 cylindrical/cone-shaped chambers, placed in shallow coastal waters (8-30 m depth)	Efficient and selective method. Can cause occasional by-catch. Low environmental impact. Possible ghost-fishing when traps are lost	<i>O. vulgaris</i> <i>O. minor</i> <i>E. dofleini</i>	Eastern Mediterranean (mainly Greece) Canary Island NW Pacific NW Pacific	[4,95, 96,97,98, 127,128,129]
Barrel longlines	Baited barrels suspended from a long line attract and trap octopus seeking shelter (20-100 m depth)	High selective method, low by-catch Low environmental impact	<i>E. dofleini</i> <i>O. conispadiceus</i>	Japan	[4,127]
Fishing lines	Fishing lines with hook-equipped end, baited with different lures (10-80 m depth)		<i>E. dofleini</i> <i>O. maya</i>	Japan Mexico (typical “ <i>Al garete</i> ” fishing lines)	[104,130]
Towed rakes	Rakes dragged over seabed to catch sand-hidden octopus (10-30 m depth)	High environmental impact with strong seabed disturbance	<i>E. dofleini</i>	Japan	[127]

Higher levels of product processing are generally associated with increased income for artisanal fishers. In cases where such value-added processing occurs, it is typically carried out by family members or individuals from the same community or fishing organization, thereby enhancing household income. It is estimated that approximately 32.5% of registered fishers engage in joint marketing activities to increase revenues, while the remaining 67.5% sell their catch to intermediaries [131].

Figure 3. Market chain of artisanal fisheries in Chile (adapted from Benitez and Nava [131]).



4.5 FOOD SAFETY IN THE *E. MEGALOCYATHUS* ARTISANAL PRODUCTION CHAIN

Octopus, as a seafood product, is a significant source of high biological value protein, but it is highly perishable if appropriate safety measures are not taken. The composition of the microbial community found in the gut of octopus, which includes psychrotrophic bacteria, is influenced by the surrounding aquatic environment and may reflect its overall contamination levels [132]. As with other fishery products, cephalopods can develop different hygiene hazards during the various stages of the production chain, with possible negative repercussions for the final consumer. Contamination can occur during both the pre-harvest and post-harvest stages of seafood processing, including general food safety risks coming from bacterial contamination, such as *Salmonella* spp., *Listeria monocytogenes*, and *Aeromonas hydrophila*, spoilage and histamine-producing bacteria (*Morganella morganii*, *Klebsiella pneumoniae*, *Hafnia alvei*, *Morganella psychrotolerans*, *Acinetobacter lwoffii*, *Plesiomonas shigelloides*, *Pseudomonas* spp., *Photobacterium* spp., *Aeromonas* spp., *Vibrio* spp., *Clostridium* spp., *Proteus* spp., *Serratia* spp., *Enterobacter* spp., and *Staphylococcus* spp.) [133,134], and chemical contamination (heavy metals like mercury, cadmium, arsenic, and lead, and organic contaminants like polycyclic aromatic hydrocarbons and organochlorine pesticides) [133]. The colonization of harmful microorganisms or the presence of microbial toxins are significant health risks for consumers and can decrease seafood product quality and nutritional value. In seafood processing, the main hygiene hazards come from microbial contamination caused by poor hygienic conditions, personnel manipulation (e.g., cutting), cleaning practices, or air circulation [135]. In living or recently fished

octopus, the main sources of bacterial contamination are the internal organs (mainly the digestive tract), skin, gills, and associated mucus [132]. Since the most used fishing method in artisanal fishing is harpoon, this usually leads to big wounds in the body of the octopus, which enhances the potential for contamination with environmental and gut bacteria. Evisceration has been shown to reduce microbial contamination and significantly extend the shelf life of octopus, with eviscerated specimens remaining acceptable for consumption for up to 20 days under refrigerated storage, compared to non-eviscerated specimens, which were rejected after only 10 days for *Octopus insularis* [136].

4.5.1 Pre-Harvest Algal Toxin Contamination

Pre-harvest contamination can include pathogenic bacteria such as *Escherichia coli*, *Salmonella* spp., and *Vibrio* spp., and in some cases, algal toxins, which can be produced during certain algal blooms. The Los Lagos region, located within the Chilean Patagonian fjords, is recognized as a major hotspot significantly impacted by harmful algal blooms (HABs), with a growing trend in their temporal frequency in southern Chile and a concurrent shift of these events toward more northern regions of the country [137,138]. It hosts the highest diversity of identified HAB species in Chile, encompassing 29 species relevant to aquaculture areas [137]. Toxic HABs in this region are a paramount concern due to their capacity to transfer toxins through the food web, posing risks to human health and impacting the coastal economy, particularly aquaculture, fisheries, and tourism [139,140]. Key toxic species include the dinoflagellate *Alexandrium catenella*, responsible for Paralytic Shellfish Poisoning (PSP); various species of the genus *Dinophysis* sp. and *Protoceratium reticulatum*, associated with Diarrhetic Shellfish Poisoning (DSP); and the diatoms *Pseudo-nitzschia australis* and *P. pseudodelicatissima*, which produce domoic acid causing Amnesic Shellfish Poisoning (ASP) [141-143]. The spatial and temporal patterns of these events are intricate and often correlated with hydroclimatic conditions [144,145]. Significant PSP outbreaks attributed to *A. catenella* have occurred in the province of Chiloé. In 2002, an event tragically resulted in 50 human intoxications and three fatalities, alongside substantial economic losses [139,146]. Northward expansions of *A. catenella* from the Aysen region have led to outbreaks in the Los Lagos region during the late summer-early autumn of 2002 [146], 2009 [147], 2016 [148], and 2018 [149]. An intense bloom of *A. catenella* was notably recorded for the first time on the oceanic coast of the Los Lagos region in 2016 [150]. Regarding DSP, *Dinophysis* species and *P. reticulatum* are present, with *P. reticulatum* showing a positive correlation with the detection of YTXs (lipophilic

toxins) in shellfish from the Los Lagos and Aysen regions [151-153]. While lipophilic toxin events typically occur in spring-summer, a multispecific toxic bloom in the summer of 2022 in a fjord of NW Patagonia (proximal to Los Lagos) included *P. reticulatum* and *Dinophysis acuminata* [153]. DSP toxins have been detected in the Reloncavi Estuary since 1970, and *Dinophysis acuminata* has been specifically linked to the production of PTX-2 in samples from this estuary. ASP events, primarily driven by *Pseudo-nitzschia australis*, *P. seriata*, and *P. pseudodelicatissima*, exhibit recurrent and often intense occurrences. High concentrations of domoic acid (>300 µg/g) in mussels were documented in the Los Lagos region in November 1999, and a severe event in 2014 necessitated precautionary closures [137]. More recently, the Los Lagos region experienced two prolonged events between December 2020 and January-March 2021 in the Huenquillahue and Chiloé areas. The event in Chiloé, concentrated around Isla de Quinchao, was particularly intense, with recorded values exceeding 130 µg/g of domoic acid and closures lasting 2-3 months [137]. The increasing frequency and intensity of toxic HABs in the region are partially attributed to complex interactions between environmental factors such as stratification and nutrient availability and large-scale climate change drivers [154,155], including hydroclimatic anomalies like those that characterized the summer of 2016, globally modulated by a strong ENSO (El Niño-Southern Oscillation) event, which causes lower precipitation and higher solar radiation. It is important to note that the reporting and identification of HAB events in the Los Lagos region are closely tied to areas of concentrated aquaculture. This is a possible consequence of sea water warming and high organic nutrient loads (mainly coming from nitrogen and phosphorous compounds) of anthropogenic origin, like aquaculture, agriculture, and urban or industrial wastewater [156-159]. Therefore, the total number of blooms, especially those causing minor economic impacts for artisanal fishers, may be underestimated [137]. For a geographical and historical resume of HABs in southern Chilean regions, see Figure 4. Octopus can play a role in bioaccumulation and biomagnification of different toxic substances in the marine trophic chain [160,161]. The main source of accumulation is through the feeding of marine bivalves, which are organisms with a strong bioaccumulation potential [162]. Different authors report accumulation of algal toxins in the internal tissues of octopus (digestive gland, branchial hearts, stomach, salivary gland, and organs with excretory functions as kidneys) [163-165]. Particularly, the risk of toxin bioaccumulation in the digestive gland is high due to the low metabolic rate of excretion and depuration in octopus [166]. However, given that octopus is typically consumed after evisceration, the risk of bioaccumulation poses relatively low

concern for food safety [160]. Relative risk can be posed by misidentification or fraud involving other toxic species [167]. The study of Oyaneder-Terrazas et al. [168] on the bioconcentration of saxitoxins in *E. megalocyathus* demonstrated a toxicity of about 3100 µg SXT-equiv 100 g⁻¹, which is accumulated in fluids and the digestive gland and not detectable in the muscle that is the edible part. However, the authors warn about *E. megalocyathus* as a species that is not considered in the sanitary monitoring of saxitoxins and eventually becomes a high risk to the health of consumers of extreme ages.

Figure 4. Main harmful algal bloom (HAB) events recorded (1970-2020) in southern Chilean coasts, with associated phytoplankton species reported. Different colors indicate water salinity levels (blue for higher salinity; red for lower salinity). Reproduced from [169].



4.5.2 Pre-Harvest Microbial Contamination

Unlike algal toxin contamination, which is a regional event, pre-harvest bacterial contamination tends to be more local, following direct coastal contamination. Fresh octopuses are highly susceptible to microbial contamination, which can impact quality and

nutritional value. The presence of zoonotic pathogens (including *Pasteurella multocida*, *Salmonella enterica* subsp. Typhimurium, *Salmonella enterica* subsp. Enteritidis, *Chlamydia psittaci*, *Coxiella burnetii*, *Bacillus anthracis*, *Streptococcus equi*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Bordetella* spp., *Staphylococcus* spp., and *Clostridium* spp.) in wetlands and water courses flowing into the sea in the Los Lagos region of Chile raises concerns regarding potential contamination of seafood products [170]. The main source of pre-harvest octopus bacterial contamination is from total and fecal coliforms and *Vibrio* species [132,171]. Total and fecal coliforms, originating from warm-blooded animals' gastrointestinal tracts, are recognized indicators of seafood quality and fecal contamination [172,173]. Fecal coliform concentrations in octopus can increase during the rainy season due to anthropogenic impact and favorable conditions for bacterial growth [173]. The increased freshwater flow during rainfall reduces salinity and raises water temperature, creating an ideal environment for bacterial proliferation. These conditions, combined with the presence of zoonotic pathogens in wastewater and wetlands, suggest that contaminating bacteria may flow from wetlands to the sea, contaminating fishery products and posing a risk to human health.

4.5.3 Pre-Harvest Heavy Metal Contamination

Also, the presence of heavy metals could present other health risks because cephalopods, particularly octopus, are known to potentially absorb and accumulate them in body tissues [174]. Octopus is reported as a high accumulator of heavy metals, with mainly mercury, methylmercury, arsenic, zinc, and copper being the most concentrated elements between different seafood species [175]. High arsenic concentrations, up to 48.9 ± 0.15 µg/g of wet weight, have been found in octopus [176], highlighting the concept that benthic species, which inhabit the seabed, may experience greater exposure to environmental contaminants [177]. Moreover, high arsenic concentrations have been found in *O. vulgaris* fished in the Mediterranean Sea, suggesting a relationship with anthropic activities [177], as well as in *O. mimus* from the Peruvian Pacific coasts [178]. In the Chilean austral waters, mining activities and human settlements are typically located far from the coast, suggesting

a potentially low risk of heavy metal contamination in *E. megalocyathus*. However, due to high seismic and volcanic activity, ash from frequent volcanic eruptions may be a potential source of heavy metal contamination in local waters, warranting further studies to evaluate this possibility. The mechanisms of metal release involve the degassing of volatile metal compounds from the magma, physical entrainment within volcanic ash, and subsequent deposition through atmospheric fallout. Once ejected, these metals can be transported over large distances via atmospheric currents before being deposited onto terrestrial and marine ecosystems through wet or dry deposition. In coastal or island volcanic systems, direct runoff from volcanic ash deposits and surface flows after rainfall can rapidly transfer heavy metals into adjacent marine environments. Thus, volcanic activity possibly constitutes an important, although spatially and temporally variable, source of heavy metal contamination in marine ecosystems [179-182].

In cephalopods, traces of these elements are usually found in edible parts like tentacle and mantle with no risk to consumer health, but this can be an important food source to evaluate the daily dietary intake of these metals [183]. Despite this, an overestimation of health risk is common because heavy metal concentration does not reflect the bioaccessibility of these elements, which can show lower absorption during digestive processes [176]. Different authors report a lower bioaccessibility of mercury, specifically of 11% in octopus, which is one of the lowest among seafood products [175,176,184-186], and cadmium [187] in seafood products after cooking procedures, as the process of protein denaturation conditions the accessibility of protein-metal complexes to digestive enzymes [188,189] and leads to the formation of insoluble components [190]. Moreover, higher and potentially toxic levels of heavy metals are usually found in digestive glands [161] and internal organs [191,192], which are systematically removed during animal evisceration, with a consequent drastic reduction in potential contamination in case of human consumption. In some geographical areas, whole octopus is traditionally consumed, posing a potential health risk considering the accumulation of heavy metals in internal organs. Storelli et al. [191] reported a Spanish traditional sauce derived from whole octopus body fermentation. Assuming a weekly whole octopus intake of 100 g (comprising 96 g of mantle and 4 g of hepatopancreas), as observed in certain southern Italian regions, the authors found that high consumers of raw octopus may potentially exceed the EFSA tolerable weekly intake for cadmium, estimated at 2.5 µg/kg body weight (BW), with intake levels ranging between 2.25 and 2.84 µg/kg BW [193,194], underlining the necessity to determine implications for public health [193]. However, considering that total dietary cadmium exposure

typically results from the combined intake of various foods, primarily vegetables and cereals [195-197], any individual risk assessment and mitigation strategy should consider the overall contribution from all relevant dietary sources.

4.5.4 Pre-Harvest Parasitic Contamination

Regarding possible parasite infestations, the presence of nematode larvae attributed to *Hysterothylacium* spp. in *Eledone* spp. (comprising *E. cirrhosa* and *E. moschata*) was reported [198,199]. The presence of *Anisakis* spp. infestation was reported in reared *O. vulgaris* populations in Galician waters, where the source of infestation was untreated, infested marine fish provided as feed (unpublished data) [200]. In their study, Pico-Duran et al. [201] report no incidence of *Anisakis* spp. in Mediterranean *O. vulgaris* from Spain, based on a parasite investigation in commercial cephalopods of the Western Mediterranean Sea. A review of the scientific literature highlighted a significant lack of data regarding parasitic infestation in *E. megalocyathus*. In a study conducted by Garbin et al. [56], pellets from *Phalacrocorax atriceps* were analyzed, revealing the presence of *Pseudoterranova* spp. (L3 larvae) in close association with remains of *E. megalocyathus*, as determined through correspondence analysis. Nonetheless, the authors emphasized the absence of prior records documenting these anisakid larvae parasitizing this particular cephalopod species. Consequently, the role of the red octopus as an intermediate or paratenic host remains uncertain. These findings indicate a low potential health risk associated with parasitosis in octopus derived food products.

4.5.5 Post-Harvest Microbial Contamination

Post-harvest contamination in seafood includes bacteria of public health concern, like *Escherichia coli*, *Salmonella* spp., and *Staphylococcus aureus*, as well as the proliferation of histamine-producing bacteria, including species of *Morganella* spp., *Klebsiella* spp., *Hafnia* spp., and *Photobacterium* spp. [135,202,203]. *E. coli* is an indicator of fecal contamination coming from the environment or directly from personnel manipulation operations, mostly in the last steps of the production chain, but it could be also related to fishing octopus activities in coastal areas, due to the higher water bacterial load as a consequence of major anthropogenic activities and stronger impact of environmental events such as rainy seasons [173,204].

Salmonella spp. is a pathogenic bacterium that can contaminate seafood mainly through unhygienic processing environments, manipulation practices, and transport [132,205], representing a health risk for final consumers [203,205,206].

Another study reported an association between domestic wastewater discharge and a great prevalence of multidrug resistant *Salmonella* spp. in raw octopus products in Campeche, Mexico, where artisanal octopus fishing is still a common practice [203]. *Salmonella* spp. usually reach waterbodies through fecal shedding from domestic wastewater discharge [207] and can survive in the environment months or years after water contamination, depending on biotic and abiotic factors, such as water temperature, organic matter, the presence of other living organisms, and biofilm formation capacity [208]. TURFs are usually implemented far from wastewater discharge and urban activities, thus suggesting a lower risk of fecal bacterial contamination.

Staphylococcus aureus is another pathogen reported in octopus [209]. Given it does not have marine origin, cross-contamination during manipulation is suggested because it is usually associated with human skin and mucosal microflora [210,211]. Vaiyapuri et al. [211] report the presence of Methicillin-Resistant *S. aureus* in the seafood chain because of fish handling, contaminated water, ice, or processing equipment. It is a major foodborne pathogen, which is able to tolerate a temperature range between 7 and 48 °C and minimum water activity of 0.83. This bacterium is a potential toxin producer but needs to reach a minimum concentration of 10⁶ CFU/g, which is reached only under optimum growing conditions of 37 °C and 3.5% Sodium Chloride [212,213]. Although *S. aureus* is able to grow at 7 °C, toxin production starts up to 10 °C and is very low until 20 °C [214], so foodborne illness from octopus consumption is generally low-risk when properly refrigerated [209].

4.5.6 Post-Harvest Biogenic Amine Contamination

Among chemical hazards, biogenic amines (BAs) are another potential health risk posed by octopus consumption. Biogenic amines are produced through the decarboxylation of free amino acids by microorganisms possessing decarboxylase activity, which may include both Gram-negative and Gram-positive bacteria. The formation of BAs is influenced by three principal categories of factors: (1) intrinsic properties of the raw material, such as pH and chemical composition; (2) storage and processing conditions, including whether the product is raw, dried, or cooked, as well as fermentation parameters, hygienic practices during processing, packaging methods, storage temperature, and duration; and (3) microbial contamination, particularly the presence of decarboxylase-positive bacteria [215-218]. Bacterial decarboxylation of free amino acids results in the production of potential toxic biogenic amines (e.g., histamine, cadaverine, putrescine, and tyramine), which are valuable chemical indicators for monitoring fish spoilage, as

suggested by Prester et al. [219] for musky octopus (*Eledone moschata*) and other cephalopods. Psychrophilic histamine-producing bacteria can lead to the accumulation of large quantities of histamine in seafood products, highlighting the important role of *Photobacterium phosphoreum* [220]. Takahashi et al. [221] highlight the risk of histamine food poisoning associated with *Photobacterium iliopiscarium*, reporting histamine production exceeding 500 ppm of histamine even at 5 °C in broth culture trials. Histamine production is highest under acidic pH conditions and starts during the exponential growth phase. The genus *Photobacterium* is reported in *O. vulgaris* [222]. Bacterial growth is significantly influenced by temperature and overall environmental conditions, making the comparison of bacterial contamination in octopuses across geographically diverse regions difficult. The limited scientific research on bacterial contamination in *E. megalocyathus* provides insufficient evidence to fully assess the safety risks associated with this seafood product. Increased funding is essential to enhance our understanding of the health status of the red octopus and its implications for human health. One study suggests that there is a general increase in general biogenic amine content, up to 100 mg/kg, in 4 °C chilled octopus during the storage period, but with a low histamine increase of up to 9 mg/kg in the same storage conditions [223]. Gullian-Klanian et al. [202] highlight the presence of weak histamine-forming bacterial strains in the Mayan red octopus (*Octopus maya*) during refrigerated storage conditions, reporting how histamine formation is present but usually in low concentrations, which is insufficient to cause severe scombroid syndrome but useful to evaluate hygienic quality and spoiling conditions of the product. There is evidence that fresh fish sold in retail markets in Lima, Peru, had higher average histamine levels compared to fresh fish sold in wholesale markets, mainly in the afternoon, highlighting how histamine level is time dependent [224]. Guidelines on seafood histamine content level suggest that histamine concentrations less than 5 mg/100 g of fish meat are safe for consumption, and above 5 mg there are dose-dependent toxic effects [225]. European Commission Regulation n. 2073/2005 [226], based on the Codex Alimentarius guideline “Principles for the Establishment and Application of Microbiological Criteria for Foods (CAC/GL 21-1997)” , establishes that fishery products obtained from species associated with a high histidine content (namely fish species belonging to Scombridae, Clupeidae, Engraulidae, Coryfenidae, Pomatomidae, and Scombrosidae families) must not exceed an average histamine level of 100 mg/kg (m), with a maximum limit of 200 mg/kg (M) for individual sample units, within a sampling plan that allows a maximum of two out of nine samples between the m and M values, and no samples exceeding the M value.

Chilean law follows the indications of Codex Alimentarius, reporting in Article No. 324 a maximum histamine concentration of 200 mg/kg in seafood products [227]. The formation of BAs in seafood products can be effectively mitigated through the adoption of good hygienic practices throughout the entire post-harvest chain. Preventive strategies include rapid chilling of the product immediately after capture, maintaining the integrity of the cold chain (ideally at temperatures below 4 °C), and minimizing the interval of time between harvesting and processing [228]. Proper evisceration of cephalopods can further reduce microbial loads and delay spoilage processes associated with amine formation. Furthermore, the use of clean equipment, sanitized working surfaces, and hygienic handling by trained personnel significantly limits microbial contamination with decarboxylase-positive bacteria [229,230]. Modified atmosphere packaging and the application of mild acidifying agents such as acetic acid (e.g., 0.1% solutions) have also been shown to inhibit bacterial enzymatic activity responsible for amine synthesis [231]. Overall, a comprehensive strategy that combines temperature control, hygienic handling, and appropriate packaging is essential to reduce the risk of excessive BA accumulation in octopus products during storage and distribution. Considering the scarcity of studies on *E. megalocyathus* and the fact that all of these general food safety aspects have been reported in other octopus species, it is worth suggesting the allocation of more research funding to advance the understanding of the health of *E. megalocyathus* and related food safety concerns.

4.6 POSSIBLE IMPROVEMENTS AND SUSTAINABLE PRACTICES

A food handler is defined as any person who handles either food or surfaces that are likely to be in contact with food, such as cutlery, plates, and bowls [232]. Different factors are crucial for ensuring the safety and quality of octopus products for human consumption, particularly regarding handling, ice usage, post-fishing hygiene, and worker training. Inadequate hygiene practices during food handling are a major contributor to the spread of foodborne illnesses. This is often exacerbated by a lack of proper training for food handlers in safe food handling procedures. Once in contact with food, these pathogens can, under favorable conditions, multiply rapidly and reach levels sufficient to cause illness when consumed [233]. Due to the rapid post-mortem deterioration kinetics of octopus, their meat exhibits rapid quality deterioration, characterized by increased TVB-N levels, elevated microbial loads, reduced sensory scores, and reduced water-holding capacity; these factors collectively limit the shelf life to approximately three days. Therefore, the implementation of post-harvest handling treatments is crucial to

ensure the quality and safety of the final product [234,235]. Among these treatments, cooling remains the most widely used and effective method to extend the shelf life of seafood products [236]. Nevertheless, the majority of *E. megalocyathus* fishing sites are located in remote rural areas, far from urban centers. These communities frequently face challenges such as limited access to clean water, inadequate cold chain maintenance, and the use of unsuitable work surfaces and tools [237]; moreover, they do not apply any preservation techniques immediately after capture, aside from subsequent freezing. The absence of immediate post-harvest preservation practices for *E. megalocyathus* has also been documented in Chile, both aboard artisanal fishing vessels and at landing ports [18,23]. The lack of essential infrastructure in remote fishing communities often results in subpar hygienic conditions, which directly impacts the safety and quality of seafood products. Also, proper hand hygiene is crucial in food handling to prevent the transmission of harmful microorganisms from hands to food [238].

Insufficient ice usage and breakdown in the cold chain represent critical challenges in local octopus production. Poor ice management, leading to melting and the accumulation of water at higher temperatures, creates favorable conditions for microbial proliferation and cross-contamination, compromising the safety and quality of the final product. The systematic use of ice, and particularly its implementation directly on fishing vessels using potable water and clean tanks, represents a simple yet effective strategy for mitigating food spoilage in the context of artisanal fisheries and local markets [234,239]. Preserving an uninterrupted cold chain throughout all stages of fishing, processing, and transportation is critical to inhibit bacterial proliferation and ensure the safety and quality of seafood products. Implementing immediate icing upon catch and maintaining consistent refrigeration during storage and transport significantly mitigates the risk of microbial contamination [91]. If on-boat ice production proves difficult to implement, Dima et al. [231] demonstrated that preserving octopus flesh in a 0.1% acetic acid solution effectively slows spoilage processes, extending the shelf life up to five days post-harvest while maintaining microbiological and physicochemical quality comparable to that of ice-preserved octopus. Given the limited availability of ice in octopus fishing areas, the use of a 0.1% acetic acid solution presents a simple, low-cost alternative for ensuring the quality and safety of the Patagonian red octopus, and it may be applicable to other octopus species in resource-limited contexts [231]. Official and mandatory protocols specifically addressing the effective use of ice on artisanal fishing vessels remain limited. Article No. 313 of the Chilean food regulation Decree 977/1996 [227] establishes that seafood products must be stored

at appropriate temperatures to prevent decomposition and microbial growth, specifically requiring maintenance below 5 °C throughout transportation and handling. Although not mandatory, general technical guidelines, such as the Manual de Buenas Practicas Pesqueras [240] and the FAO guide Acuicultura de pequena escala y recursos limitados en America Latina y el Caribe [241], recommend the use of ice on board small artisanal vessels to ensure product quality and safety. Furthermore, following Chilean Law No. 1333/1987 on Requisites of water for different usage (“Requisito de calidad de agua para diferentes usos”) [242], stricter monitoring of water quality and environmental conditions in fishing areas is crucial. Finally, another problem in the octopus fishery is the disposal of evisceration waste, which is expected to be regulated by 2028 [243]. Effective implementation requires subsidies, technical assistance, and economic support from governments and local institutions to fisher associations, providing knowledge, resources, and assistance, to incentivize sustainable practices among artisanal fishers. Training initiatives, such as seminars, manuals, and the formation of skilled personnel within associations to educate and monitor other workers, can contribute to improving and preserving food quality. Courses and classes on food safety risks can significantly enhance good manufacturing and hygiene practices in the octopus production chain. In this context, the implementation of a standardized quality index method for raw Patagonian red octopus within rural artisanal fishing communities is essential for improving product safety, preserving organoleptic properties, and enhancing market access (Table 5). Given the highly perishable nature of cephalopods and the lack of consistent post-harvest handling practices in remote areas, the development of a simple, low-cost, and reproducible QIM can assist fishers in identifying early spoilage indicators—such as alterations in odor, texture, color, or pH—that are often linked to microbial growth and the accumulation of hazardous compounds. Integrating this tool into local training initiatives enables communities to adopt evidence-based decision-making throughout the processing, storage, and marketing phases. This approach not only reduces the risk of foodborne illness but also adds value to the product by fostering adherence to hygiene and quality standards required by national and international markets. Ultimately, this initiative supports food security, economic resilience, and public health under a One Health framework, particularly in geographically isolated regions with limited access to refrigeration and infrastructure.

Table 5. Quality index method adapted for whole raw Patagonian red octopus (*Enteroctopus megalocyathus*) (elaborated by the authors).

No.	Organoleptic Criterion	Description	Grade 1 (First Quality)	Grade 2 (Second Quality)	Grade 3 (Third / Low Quality)
1	External appearance	Shape, physical damage, integrity	Whole octopus, no cuts or damage, vivid and uniform color	Minor damage to arms, slightly dull	Deep cuts, missing parts, dull colour or black spots
2	Flesh texture (raw)	Firmness, consistency to the touch	Firm to the touch, resilient	Slightly soft but maintains shape	Flaccid, falls apart when touched
3	Smell	Freshness, marine aroma	Clean marine/seaweed smell, no strange odors	Slight smell of decomposition or ammonia	Strong, unpleasant or rotten smell
4	Mantle and arm color	Uniformity, color intensity	Bright, uniform red	Pale red or some whitish areas	Intense discoloration, black or greenish spots
5	Mucus presence	Surface cleanliness	Few transparent watery mucus,	Slight mucus	Abundant opalescent viscous mucus or sticky surface
6	Mantle turgor	Elasticity to touch	Turgid, elastic, bounces back when pressed	Slight dent that doesn't fully recover, low elasticity	Deforms easily, wrinkled skin
7	Eyes and suckers	Clarity, firmness	Clear and translucent eyes, black pupil. Firm and well-attached suckers	Slightly opaque eye, dark red/bloody pupil. Soft suckers	Sunken eyes, opaque pupil. Disintegrated or missing suckers
8	Flavor (if cooked)	Taste test (optional)	Sweet, ocean-like taste, firm texture	Mild taste, slightly soft texture	Bitter taste, disintegrated texture

Another way to enhance the One Health approach within this fishing activity is the implementation of small-scale aquaculture for *E. megalocyathus*, which represents a unique opportunity to promote both sustainable production and health aspects of this valuable resource. This initiative involves pioneering land-based rearing in seawater tanks, using natural or artificial balanced diets, and actively engaging members of artisanal fishing communities. Members of the fishing union “Sindicato de Gente de Mar de Chaular” group (Chiloe province, Los Lagos region, Chile) received training on different themes, like octopus zootechnic aspects, aquaculture engineering, octopus feeding, business and management, and coaching with

women and tourism, in other to promote innovative land-based small-scale octopus aquaculture systems [244]. These individuals can act as promoters of change, encouraging a transition from traditional extractive practices to more sustainable fishing methods among other artisanal fishers. Furthermore, in rural communities, the recruitment of women in this innovative activity has the potential to foster their empowerment, leadership, economic independence, social recognition, and visibilization. This is particularly significant in addressing historically entrenched hierarchical and unequal gender relations, which are socially and culturally constructed within the sex-gender system.

4.7 CONCLUSIONS

This study summarized the biology of the Patagonian red octopus (*E. megalocyathus*) and the related fishing laws and artisanal fishery practices in Chile, highlighting the significant socioeconomic role of the species in artisanal fishing communities amidst significant challenges to ecological sustainability and food safety. Different octopus fishing approaches are described. Hook fishing is the most widely practiced fishing method and, although selective, poses a threat to the reproductive success of the species due to the potential capture of brooding females; this practice could be changed by 2028 [242]. Furthermore, the red octopus production chain poses some food safety risks to final consumers. Octopus products are threatened by microbial contamination during pre- and post-harvest handling, bioaccumulation of toxins from algal blooms, and the presence of heavy metals in the marine environment. Evisceration, a common practice in octopus processing, effectively reduces the risk of contamination from toxins and heavy metals. However, inadequate hygiene and insufficient ice usage, particularly on artisanal boats in remote fishing communities, remain critical issues. To improve sustainability of this fishery sector, the positive experience of TURFs in Chile, which have proven effective in promoting the sustainable management of other species, offers a promising model for the Patagonian red octopus fishery. The integration of training programs on post-harvest handling, hygiene practices, and food safety measures within the coastal communities is crucial to ensure the long-term sustainability of this fishery. Implementation and investments in infrastructure, technical assistance, and economic support can improve hygiene and sanitary conditions in the harvesting process, ensuring access to clean water, adequate equipment, resources and training programs for fisher associations, fishing communities, and women, thus providing diversification opportunities in coastal activities. These synergistic actions will contribute to safeguarding consumer health, protecting marine ecosystems, and

ensuring the economic prosperity of the communities that depend on this valuable marine resource.

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CONCLUSIONS

Each chapter in this PhD thesis is "self-conclusive," or designed to be read independently as a thorough exposition of the topic. Therefore, the goal of this general conclusion is to integrate all the studies presented in the body of this thesis and draw general conclusions about their significance in relation to its goals.

Marine food systems are undergoing rapid anthropogenic change (warming, biodiversity loss, chemical inputs/pollution, and intensified exploitation) that alters the interfaces and relationships between environment, animals and human. In response, One Health approach has emerged as the most coherent framework for understanding and managing cross-border threats, by linking human, animal, and environmental health and by insisting on evidence that spans ecosystems, primary production, processing, and retail exposure. This thesis was conceived precisely to operationalize that perspective for seafood: it connects emerged and emerging hazards (AMR and MPs) in seafood species with species-specific microbial baselines (swordfish) and with context-appropriate hygiene guidance for neglected/under-utilized species (Patagonian red octopus), translating integrative evidence into actionable controls along value chains.

The papers presented findings on different topics:

- AMR at retail: phenotypic resistance in psychrotrophic, biofilm-forming *Pseudomonas* spp. from salmon, cod, and plaice fillets.
- MPs: a critical review of occurrence in seafood edible products, exposure pathways, toxicological plausibility, and methodological heterogeneity.
- Swordfish gut microbiota: first metataxonomic baseline to inform spoilage/ARG potential.
- Patagonian red octopus: biology, management, and practical hygiene measures for artisanal small-scale fisheries and landing sites.

Cross-chapter findings converge on several general indications. First, the retail AMR study shows that opportunistic, psychrotrophic *Pseudomonas* (dominant actors in chilled spoilage) frequently exhibit resistance to multiple drug classes, with multidrug resistance notable in *P. fluorescens*. Specifically, an high level of phenotypic resistance to various antimicrobial classes (8 out of 15 antimicrobials tested) has been reported, with about 80% of multi-drug resistant *P. fluorescens* strains. Although these taxa are not classical pathogens, *Pseudomonas* are well recognized as the main SSO of fresh fish fillets and their ecology under refrigeration, ability to form biofilms, and role in cross-contamination position them as reservoirs and vectors of resistance in consumer-level products. In this context, it should be mentioned that, hitherto, the mechanisms of the resistance

of *Pseudomonas* spp. against clindamycin and vancomycin are still missing. For this reason, and considering their widespread contamination in the fish processing environment, *Pseudomonas* spp. should be monitored as possible source of other resistance genes, not only on strains isolated from clinical specimens but also on the strains isolated from food samples.

From a process-control standpoint, being *Pseudomonas* spp. a dominant bacterial genus in food processing facilities and in the microbial community of many fish products, initial *Pseudomonas* loads in fish and time-temperature control remain decisive. The data support practical targets for starting loads in fresh fillets (kept below the range at which spoilage flora rapidly reach sensory thresholds) and justify routine retail monitoring of product deterioration as a process-hygiene indicator alongside validated sanitation focused on anti-biofilm efficacy, verified water/ice quality, and realistic shelf-life limits. FBOs have to control the presence of *Pseudomonas* spp. in whole fresh fish as well as in fresh fillets. Indeed, in spite of the evidence that low number of *Pseudomonas* spp. does not pose a health problem for the consumer, its excessive proliferation could, in one hand, produce histamine (therefore becoming a risk to human health) as well as, in the other, lead to product deterioration by their proteolytic, lipolytic and saccharolytic enzymes as well as the development of anomalous colors, unpleasant off-odors and slime coats, making fish unfit for human consumption. Therefore, the enumeration of *Pseudomonas* loads could be suggested as process hygiene criterion in line with the purpose of pursuant to EC Regulation 2073/2005 in relation to microbiological criteria for foodstuffs. These findings sound good to remind that, also in food hygiene, it is worth evaluating the prevalence of AMR in food and suggesting a continuous and holistic AMR monitoring in different sectors and sources.

The review on MPs documents ubiquitous detection across aquatic compartments and edible taxa (finfish, bivalves, crustaceans), confirming seafood as a relevant exposure route. In more detail, MPs have been found in many species of fish intended for human consumption from the Pacific, Atlantic, and Indian Oceans, and the Mediterranean Sea, both in migratory commercial fish species as *Thunnus thynnus*), seasonal migratory fish (sea bass and *Dicentrarchus labrax*), sedentary fish (plaice and *Pleuronectes platessa*) and fish of great commercial importance such as sardines (*Sardina pilchardus*) and anchovies (*Engraulis encrasicolus*). MPs identification is predominant in the digestive tract and rare in edible tissues, such as muscles. Although direct human exposure to microplastics could be considered negligible, given that most fish species are eviscerated before human consumption, it should be noted that evisceration does not necessarily eliminate the risk of human intake of microplastics. In fact it is clear that, if contaminated, their consumption can pose a threat to human health. Precisely in case of human consumption of small pelagic fish such as sardines, herring, and other small freshwater fish (which are

commonly eaten whole), a greater threat has to be expected than gutted fish. Yet the present evidence base is constrained by heterogeneity in sampling, digestion, identification, and reporting units, which undermines cross-study comparability and precludes reliable dose–response evaluation. Mechanistically, MPs can carry additives and sorbed contaminants and plausibly host microbial communities (including ARG-transporting bacteria), but for several contaminant classes the incremental dietary contribution via MPs in seafood appears small or negligible in current estimates. Hitherto, although microplastic contamination is not a new phenomenon, the characterization of risks associated with micro- and nanoplastics is an emerging and still not-solved problem. In addition, the information we currently have is not sufficient to assess the actual amount of microplastic to which humans are exposed, which represents a fundamental parameter for assessing the effects on human health. Also, the various potential toxic effects of microplastics hypothesized in humans could be classified as not conclusive findings hampering again a reliable assessment of the risks to human health.

In relation to both the need of control this emerging threat as well as to the evidence that MPS in aquatic environments will inevitably increase, it is urgent to identify, standardize and validated analytical methods able to detect and quantify MPs in aquatic environments and in fishery products, with a focus on particles smaller than 150 μm . Also, further studies on the toxicokinetics and toxicodynamic of microplastics to both aquatic organisms and humans, including local effects on the intestinal tract, interactions with the microbiota, and the potential formation of nanoplastics resulting from the degradation of microplastics in the gastrointestinal tract is needed. In this context, the review prioritizes harmonized analytical protocols and standardized reporting, and it recommends integrated surveillance designs that connect occurrence to co-exposure contexts (e.g., metals, POPs, antibiotic residues) and to microbiological endpoints. Risk management can proceed on two fronts while methods consolidate: upstream source reduction and proportionate supply-chain controls (e.g., depuration where applicable, potable water and ice assurance, and prevention of cross-contamination).

The chapter reporting first metataxonomic baseline for swordfish (*Xiphias gladius*) demonstrates a Proteobacteria-dominated gut community with *Photobacterium* consistently prevalent. Notably, microbial biodiversity differences are more pronounced across weight classes than across FAO fishing areas, indicating within-species ecological structuring that likely reflects ontogeny, diet, or physiology. Specifically, smaller fishes exhibited a less rich and diverse gut microbiota with an almost exclusive presence of *Photobacterium* (98.5%) compared to medium and larger fishes, in which the genera *Pseudoalteromonas*, *Psychrilyobacter*, *Cetobacterium* and *Psychrobacter* appeared to increase with fish weight. Although *Photobacterium* was dominant across all FAO fishing areas, distinctive microbial community

compositions were found, namely the *Cetobacterium* in FAO 27 and *Pseudoalteromonas* in the other areas. This baseline is not merely descriptive; it sharpens hypothesis generation for spoilage trajectories (e.g., *Photobacterium*-associated amine formation at low temperatures), ARG carriage, and handling effects, and it enables targeted verification sampling at critical control points in long, multi-node pelagic chains. The results justify follow-up work that resolves gut compartments (mucosa vs. digesta) and integrates functional assays (metagenome/resistome) to translate community composition into risk-relevant traits. Given this is the first metataxonomic characterization of gut microbiota of *Xiphias gladius* using next-generation sequencing, no data are available to verify if fishing techniques, timing of stocking fish inside the boats before landing, production, conservation and packing techniques in the factories could influence the gut microbiota and, in case, the contamination of fish meat and of production environments.

Finally, the Patagonian red octopus case study illustrates how governance and hygiene practices must co-evolve if neglected/under-utilized species (NUS) are to be safely valorized in artisanal value chains. Indeed, a potential valorization of *Enteroctopus megalocyathus* could have the merit to highlight the socioeconomic role of the species in artisanal fishing communities amidst significant challenges to ecological sustainability and food safety. While TURF/co-management frameworks provide institutional scaffolding for traceability and compliance, practical risk reduction hinges on immediate icing, verification of potable water/ice, hygienic evisceration/cleaning, and basic landing-site sanitation adapted to infrastructure constraints. Tissue-specific hazards (e.g., metals in the digestive gland) require product-form-specific advice and verification. Embedding these measures in co-management and training can de-risk market development for NUS while supporting equitable livelihoods, thereby aligning food safety with sustainability goals in small-scale fisheries. These interventions, together with more others, have to be considered crucial for ensuring fishery's long-term viability, protecting consumer health, and supporting local livelihoods under a One Health approach. The need to valorize the potential of NUS has become evident, but, finally, and no less importantly, it is worth noting that NUS still lack a clear and generally accepted definition. The arbitrary use of the terms "NUS" or "underutilized" based solely on limited market visibility may lead seafood suppliers and advocacy groups to stimulate demand in an uncoordinated manner. This can ignore best management practices and ecological context, ultimately increasing fishing pressure on species that may be already overexploited or vulnerable to overfishing.

Synthetically, the thesis argues for four complementary directions. Surveillance should integrate AMR and MPs monitoring across value-chain nodes and pair

culture-based testing with targeted molecular assays for key ARGs and microbiome markers; harmonized metadata (species, origin, product form, thermal history) are crucial to comparability and interpretation. Prevention-first hygiene remains the most effective way to reduce risk: validated sanitation with anti-biofilm intent, strict time–temperature control, and water/ice quality management from vessel to retail are the bedrock of consumer protection for chilled finfish and cephalopods. Species-specific microbiome baselines, such as those established here for swordfish, should be extended where public-health leverage is high, because they enable earlier detection of spoilage or resistance trajectories and sharpen the placement of verification sampling in HACCP plans. Finally, as markets diversify toward NUS, simple, verifiable hygiene practices must be embedded in local governance and training so that safety and livelihood objectives are advanced together.

Several limitations shape a forward agenda. The retail AMR survey would benefit from broader spatio-temporal coverage and systematic inclusion of other opportunistic taxa beyond *Pseudomonas*, ideally linked to resistome data to bridge phenotype and genotype. MP risk assessment requires standardized analytics and exposure metrics that enable dose–response modeling and that integrate co-exposures. The swordfish baseline invites compartment-resolved sampling and multi-omic integration to map specific functions (e.g., decarboxylation potential, ARG carriage) to taxa and conditions. In NUS supply chains, validation of Good Hygiene Practices under small-scale constraints remains a priority, together with investments in potable water/ice access and cold-chain infrastructure at landing sites. These limitations delineate a clear, actionable research trajectory that coheres with One Health priorities.

In conclusion, by weaving AMR phenotypes observed at retail, a critical synthesis of MPs occurrence and exposure, a species-specific microbiome baseline for a high-value pelagic fish, and operational guidance for a neglected cephalopod within artisanal chains, the thesis advances a pragmatic, One Health–oriented seafood-safety agenda. The unifying message is to invest in integrated surveillance, prevention-first hygiene, and species-/context-specific baselines to protect consumers while supporting sustainable and equitable aquatic food systems under environmental change.

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