Alessio Carletti

Effect of marine-derived extracts on mineralogenesis

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Effect of marine-derived extracts on mineralogenesis

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Declaro ser o autor deste trabalho, que è inèdito e original. Autores e trabalhos consultados encontram-se devidamente citados no texto e constam da listagem de referencias incluida.

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(Alessio Carletti)

This PhD thesis is dedicated to my younger self, and to the summer days spent playing with my cousin Giacomo, pretending to be characters from Steven Spielberg's movie *Jurassic Park*.

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Michelangelo Buonarroti, Italian sculptor and painter, once said about one of his most famous works, the *David*:

«*The sculpture is already complete within the marble block, before I start my work. It is already there, I just have to chisel away the superfluous material*».

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ABSTRACT

Bone erosive pathologies are the leading cause of fractures worldwide and represent a pressing medical and economic burden. Diseases like osteoporosis, Paget's disease of bone, hyperparathyroidism, and renal osteodystrophy, have different pathophysiological roots but they all share a common feature: they lead to loss of bone mineral and result into increased bone fragility. This class of disorders are also a compelling pharmaceutical challenge. Currently, there is a limited choice of therapeutic agents available to treat bone loss and they are often characterized by short-timed efficacy and severe side effects. Meanwhile, fish grown in aquaculture, the primary source of seafood for human consumption, typically suffer from skeletal abnormalities that are universally present in all culture conditions and fish species. These skeletal defects appear to be largely caused by factors intrinsically related to the condition of captivity, including the lack of an adequate nutrition. The EU-funded Marie-Curie ITN project BIOMEDAQU, in the scope of which this PhD project is framed, provided a multidisciplinary platform bringing together research in biomedicine and aquaculture (from which Biomed-Aqu) with the objective of creating new knowledge with applications in both research fields.

In this context, marine-based pharmacology, the branch of pharmaceutical research focalized on the screening and characterization of marine natural compounds, can contribute to fulfill knowledge gaps and provide translational applications for both disciplines. Different groups of marine organisms have been studied as sources of "osteoactive" compounds, some of which were described for their highly promising pharmacological and nutraceutical potential. The objective of this PhD project was to screen and characterize extracts obtained from various groups of marine organisms, selected as candidates for the isolation of compounds with potential applications for the biomedical sector, in the development of drugs to treat human bone erosive pathologies, and for the aquaculture industry, to be used as nutraceuticals to be incorporated into fish feeds to ameliorate skeletal health. With this aim, we put in place a medium-scale screening project evaluating about 150 extracts and fractions obtained from different marine organisms that recent pharmacological research has identified as promising sources of bioactive compounds, including holothurians, tunicates, cyanobacteria, marine bacteria, and microalgae. As a result of this screening activity, we identified ethanolic extracts from two microalgal species, *Skeletonema costatum* and *Tetraselmis striata* (CTP4 strain), as the most promising for their pro-osteogenic activities. Then, we further characterized them by

testing using *in vitro* bone-derived cellular models, and *in vivo*, in the model organism zebrafish (*Danio rerio*), used as platform to investigate more in-depth the molecular mechanism of actions of the two extracts. By doing this, we revealed that the ethanolic extract from the microalga *Tetraselmis striata* CTP4 possessed the strongest osteoanabolic properties. We then wanted to provide a proof of concept of possible applications of these two extracts in the aquaculture industry by exploring their potential to be used as dietary supplements for the commercially reared species *Sparus aurata*. Accordingly, we found that *Tetraselmis striata* CTP4 is the most promising extract in this sense, in light of its capacity to promote fish growth, bone mineralization, and reduce the incidence of skeletal anomalies in seabream juveniles. Finally, we decided to dissect the molecular mechanism behind the osteoactivity of the ethanolic extracts from *Skeletonema costatum*, and validate its potential application for the treatment of bone erosive disorders in human patients. We found that the extract has mainly an anti-osteoclastogenic activity, and we put it in the prospect of its previously known anti-inflammatory potential. We also provide evidence that its application can limit bone loss in a medaka model of osteoporosis, and that its anti-osteoclastogenic properties are conserved in a mammalian *in vitro* cell model.

Overall, through this PhD project, by identifying and characterizing these two microalgae extracts in the context of bone mineralization, we have provided the substrate for future research aimed at isolating compounds with potential applications in human medicine and as dietary supplements for aquaculture nutrition.

Keywords

Skeletonema costatum; *Tetraselmis* sp. CTP4; Microalgae; Marine bioactive compounds; Osteoanabolic; Pro-osteogenic; Mineralogenic; Bone metabolic disorders; Osteoporosis; Zebrafish.

RESUMO ESTENDIDO

As patologias erosivas do ósseo são a principal causa de fraturas em todo o mundo e representam um fardo médico e económico com grande impacto financeiro. Doenças como osteoporose, doença de Paget, hiperparatiroidismo e osteodistrofia renal têm diferentes causas fisiopatológicas, mas todos eles partilham uma característica comum: levam à perda de minerais e resultam em maior fragilidade óssea. Essa classe de distúrbios também apresenta um desafio farmacêutico. A escolha muito limitada de opções terapêuticas atualmente disponíveis para tratar distúrbios associados à fragilidade óssea é frequentemente caracterizada por eficácia de curto prazo e efeitos colaterais graves. Por outro lado, peixes provenientes de aquacultura, a principal fonte de recursos marinhos para consumo humano, geralmente sofrem de anomalias esqueléticas que estão universalmente presentes em todas as condições de cultivo e espécies de peixes. Esses defeitos esqueléticos parecem ser em grande parte causados por fatores intrinsecamente relacionados à condição de cativeiro, incluindo a falta de nutrição adequada. O projeto Marie-Curie ITN BIOMEDAQU, financiado pela UE, no âmbito do qual este projeto de doutoramento se enquadra, forneceu uma plataforma multidisciplinar reunindo investigação em biomedicina e aquacultura com o objetivo de criar novos conhecimentos com aplicações em ambas as áreas de investigação. Nesse contexto, a farmacologia de base marinha, ramo da investigação farmacêutica focada na triagem e caracterização de compostos naturais marinhos, pode contribuir para preencher lacunas de conhecimento em ambas as disciplinas.

No **Capítulo 1**, cuja primeira seção está atualmente submetida para publicação, revemos e listamos todos os compostos osteoativos marinhos identificados até o momento. Também discutimos as distribuições taxonómicas de tais compostos e identificamos os grupos de organismos marinhos que parecem promissores para investigações futuras direcionadas para a descoberta de novos fármacos para tratamento de distúrbios ósseos metabólicos. Examinamos também, ainda que brevemente, a disponibilidade de ferramentas de triagem e validação *in vivo* para o estudo de compostos osteoativos de origem marinha. Além disso, também revemos os aspetos nutricionais relacionados com o desenvolvimento de anomalias esqueléticas em peixes de aquacultura e o potencial da suplementação dietética de extratos naturais marinhos ricos em compostos com efeitos promotores da saúde do esqueleto como uma estratégia de custo-benefício para reduzir distúrbios esqueléticos em peixes de cultivo. Na seção final do capítulo, discutimos brevemente as diferentes abordagens que são atualmente aplicadas na bioprospecção ambiental na procura de potenciais moléculas com efeitos terapêuticos e

nutracêuticos, qual o papel que as instituições académicas desempenham na investigação farmacêutica e terminamos com a descrição dos objetivos específicos propostos para esta tese de doutoramento

No **Capítulo 2**, implementamos um projeto de triagem de média escala focado na procura de extratos de vários grupos de organismos marinhos com alto potencial biotecnológico para o isolamento de compostos bioativos. Esses extratos foram depois testados no modelo de peixe-zebra (*Danio rerio*). Bem estabelecido pela sua contribuição para o estudo de patologias do esqueleto devido aos mecanismos de doença altamente conservados e vantagens técnicas sobre modelos tradicionais de roedores, o peixe-zebra é um modelo de escolha para grandes ensaios de triagem fenotípica *in vivo* visando identificar compostos com potencial osteogénico. Assim, na primeira seção do capítulo, que foi publicada na revista *Frontiers in Nutrition* (ver **Capítulo 2.1**), exploramos o potencial bioativo de 14 extratos obtidos de várias espécies de invertebrados marinhos pertencentes aos grupos de pepinos do mar e tunicados, como fontes de compostos antioxidantes, anti-inflamatórios e osteogénicos. Identificamos três extratos etanólicos caracterizados por uma forte atividade osteogénica *in vivo*, que se correlacionou positivamente com o seu potencial anti-inflamatório. Na segunda seção do capítulo (em preparação para submissão até ao fim do primeiro semestre de 2023), realizamos uma atividade de triagem em 133 extratos e frações preparados a partir de vários microrganismos marinhos, incluindo cianobactérias, actinobactérias, planctomicetos e microalgas. Como resultado, identificamos 4 extratos etanólicos de espécies de microalgas marinhas com potente atividade pró-osteogénica.

No **Capítulo 3**, após a triagem realizada no capítulo anterior, o extrato etanólico de *Tetraselmis striata* CTP4 (CTP4) foi selecionado como o melhor candidato para realizar a caracterização química devido à ausência de toxicidade em todas as concentrações testadas no peixe zebra, e à disponibilidade do produtor e parceiro industrial (Necton SA, Olhão, Portugal) para produzir grandes quantidades de biomassa da microalga utilizada para preparar o extrato. Consequentemente, o extrato CTP4 foi processado por meio de um pipeline de identificação guiado por bioensaio com o objetivo de identificar compostos potencialmente responsáveis pelo efeito osteogénico positivo observado em larvas de peixe-zebra. O extrato foi fracionado por cromatografia líquida de alta eficiência, produzindo 15 frações cuja bioatividade foi testada individualmente. Destas, 8 frações apresentaram efeito osteogénico e em concentrações menores quando comparadas às utilizadas com o extrato bruto, indicando que os compostos responsáveis pela ação osteogénica foram enriquecidos após o fracionamento. Duas frações foram selecionadas e processadas por meio de cromatografia líquida acoplada à espectrometria de massas, que evidenciou a complexa composição química das frações, ao revelar a presença

de mais de 8000 compostos. A seleção desses compostos foi realizada aplicando critérios baseados na abundância relativa dentro das duas frações mais bioativas em comparação com as menos bioativas. Como resultado, 15 compostos foram identificados como possíveis candidatos e 7 destes, disponíveis comercialmente, foram adquiridos e avaliados quanto ao potencial osteogénico com o teste do opérculo, mas nenhum deles demonstrou possuir um efeito positivo. Fontes potenciais de variação e identificação incorreta de compostos são discutidas no final do capítulo, bem como possíveis estratégias futuras a serem aplicadas para superar essas limitações.

No **Capítulo 4**, atualmente submetido à revista *Biomolecules* (MDPI), caracterizamos ainda o efeito biológico dos dois melhores extratos selecionados no Capítulo 2, das espécies de microalgas *Skeletonema costatum* e *Tetraselmis striata* CTP4. Foram analisados num sistema *in vitro* derivado de células ósseas de dourada, tendo demonstrado um efeito positivo nos níveis da matriz extracelular (ECM). Ao aplicar linhagens transgénicas de peixe-zebra que marcam diferentes estádios de diferenciação osteoblástica, também descobrimos que elas estimulam a diferenciação osteoblástica e regulam genes envolvidos na formação e mineralização óssea. Ao expor larvas de peixe-zebra aos extratos por suplementação alimentar de longo prazo, confirmamos que ambos, e em particular *T. striata* CTP4, estimulam a mineralização de todos os elementos esqueléticos no peixe-zebra, atuando de maneira osteoanabólicas e também sendo capazes de reduzir a incidência de anomalias esqueléticas.

No **Capítulo 5** (cujo manuscrito está em preparação, e pretendemos submetê-lo à *Aquaculture*), validamos o uso dos dois extratos de microalgas na indústria da aquacultura, testando-os como suplementos alimentares para a espécie de dourada (*Sparus aurata*). As dietas suplementadas com ambos os extratos, foram testadas em diferentes estádios de vida da dourada, incluindo larvas de 30-60 dias após a eclosão (DAH) e juvenis durante a fase de crescimento (5-18g). Além disso, alguns peixes juvenis foram mantidos por mais 6 meses enquanto alimentados com dietas comerciais, a fim de avaliar se o tratamento curto com extratos de microalgas poderia afetar o estado do esqueleto dos peixes até mais tarde no seu crescimento. No geral, descobrimos que a suplementação com extratos de microalgas não afetou a incidência de anomalias esqueléticas em estádios larvais, embora tenham sido capazes de estimular mecanismos moleculares envolvidos no crescimento ósseo e mecanismos antioxidantes. Nos estádios juvenis, no entanto, a suplementação com um dos extratos de microalgas promoveu o crescimento dos peixes, elevou o conteúdo mineral vertebral, reduziu a incidência de anomalias esqueléticas e estimulou os mecanismos anabólicos ósseos. Além disso, após 6 meses, durante os quais os peixes foram alimentados com dietas comerciais, os peixes originalmente

alimentados com um dos extratos ainda eram maiores, menos deformados e com uma forma mais alongada e esbelta em comparação com os peixes controle.

No **Capítulo 6**, cujo manuscrito está em preparação, e pretendemos submetê-lo à revista *Biomedicine & Pharmacotherapy*, exploramos mais profundamente o mecanismo de ação molecular de um dos dois extratos de algas mais promissores, os extratos etanólicos de *Skeletonema costatum*, e fornecemos informação relevante para futura validação do seu potencial para desenvolvimento de fármacos para tratar patologias ósseas humanas. Primeiro, ao testar o extrato num modelo de regeneração óssea baseado na amputação e regeneração da barbatana caudal do peixe-zebra, revelamos que o extrato inibia a bifurcação dos raios e o recrutamento osteoclástico. A aplicação de uma abordagem transcritómica revelou que o extrato suprimiu fortemente os processos inflamatórios e inibiu o recrutamento de macrófagos e a diferenciação osteoclástica nos estádios iniciais da regeneração da barbatana caudal. Em seguida, validamos a capacidade do extrato reduzir a perda óssea num modelo de osteoporose *in vivo* em medaka, mostrando que os peixes expostos aos extratos apresentaram perda óssea menos severa. No final, para explorar se os resultados obtidos nos modelos de peixes poderiam ser consistentes num modelo mamífero *in vitro*, mostramos que os extratos de *S. costatum* inibiram a proliferação e a diferenciação osteoclástica induzida por RANKL em células RAW 264.7 derivadas de murganho (*Mus musculus*). Em conjunto, os resultados obtidos fornecem fortes evidências da presença de compostos anti-reabsortivos em *S. costatum*, possivelmente atuando por um mecanismo anti-inflamatório, destacando o potencial dessa espécie de microalga para o desenvolvimento de fármacos direcionados para tratamento de distúrbios ósseos humanos.

No final, no **Capítulo 7**, fornecemos as principais conclusões sobre os resultados obtidos durante este projeto de doutoramento, enquadrando-os nos objetivos gerais do projeto BIOMEDAQU, salientando igualmente as possíveis contribuições que poderão trazer para as áreas de aquacultura e investigação biomédica.

Palavras-chave

Microalgas; *Skeletonema costatum*; *Tetraselmis striata* CTP4; Compostos bioativos marinhos; Osteoanabólico; Pró-osteogênico; Mineralogénico; Distúrbios metabólicos ósseos; Osteoporose; Peixe-zebra.

LIST OF ABBREVIATIONS AND ACRONYMS

$1,25(OH)_2D_3$	1,25-dihydroxyvitamin D_3
11β-HSD	11β-hydroxysteroid dehydrogenase type 2, protein
$25(OH)D_3$	25-hydroxyvitamin D_3
7-DHC	7-dehydrocholesterol
AA	arachidonic acid
ADO	autosomal dominant osteopetrosis
AFF	atypical femur fracture
ALA	α-linolenic acid
ANOVA	analysis of variance
AR-S	alizarin red S
ARO	autosomal recessive osteopetrosis
bgPCA	between-groups principal component analysis
BIF	bifurcation point
BMD	bone mineral density
BMSCs	bone marrow mesenchymal stem cells
BRONJ	bisphosphonate-related osteonecrosis of the jaw
CaR	extracellular calcium receptor
CFP	cyan fluorescent protein
CKD	chronic kidney disease
CTSs	cathepsins protein family members
CTX	C-terminal telopeptide of type 1 collagen, protein (human)
DAH	days after hatching
DEGs	differentially expressed genes
DHA	docosahexaenoic acid
DMSO	dimethyl sulfoxide
dpa	days post-amputation
dpf	days post-fertilization
ECM	extracellular matrix
EMA	European Medicine Agency
EPA	eicosapentaenoic acid
EtOH	ethanol, ethylic alcohol

EVs	extracellular vehicles
FAO	Food and Agriculture Organization
FBS	fetal bovine serum
FCR	feed conversion rate
FDR	false discovery rate
GFP	green fluorescent protein
GIOP	glucocorticoid-induced osteoporosis
HESI	heated electrospray ionization
hpa	hours post-amputation
hpf	hours post-fertilization
HPLC	high performance liquid chromatography
hPTH 1-34	teriparatide
HR-MS^n	high resolution tandem mass spectrometry
HRTs	hormonal replacement therapies
HSCT	hematopoietic stem cells transplantation
HTS	high-throughput screening
LC-MS	liquid chromatography coupled with mass spectrometry
LDA	linear discriminant analysis
LPS	lipopolysaccharide
MAX Thr	maximum threshold value
MBDs	metabolic bone disorders
MeOH	methanol
MIN Thr	minimum threshold value
MMPs	matrix metalloproteinase protein family members
MNLD	maximum non-lethal dose
MOCs	marine osteoactive compounds
MS-222	tricaine methanesulfonate
MSCs	mesenchymal stem cells
NMR	nuclear magnetic resonance
NPs	natural products
NSAIDs	non-steroidal anti-inflammatory drugs
ONJ	osteonecrosis of the jaw
OP	osteoporosis
OpA	operculum area

OVX	ovariectomized
PANTHER	rotein Analysis THrough Evolutionary Relationships
PBS	phosphate buffered saline
PDB	Paget's disease of bone
PDD	phenotypic drug discovery
PFA	paraformaldehyde
PGE_2	prostaglandin E_2
PPi	inorganic pyrophosphate
PRIs	public-sector research institution
PTH	parathyroid hormone
PTHrP	parathyroid hormone-related peptide
PTHrP 1-34	abaloparatide
PUFAs	polyunsaturated fatty acids
qPCR	quantitative real time PCR
RCTs	randomized controlled trials
RNAseq	RNA sequencing
ROI	region of interest
ROS	reactive oxygen species
SERMs	Selective estrogen receptor modulators
SL	standard length
TDD	target-based drug discovery
TPS	thin plate spline
UHPLC	ultra high performances liquid chromatography
UV	ultraviolet radiation
VD	vitamin D
VK	vitamin K
VK_1	phylloquinone
VK_2	menaquinone
VK_3	menadione
VKDPs	vitamin-K-dependent proteins

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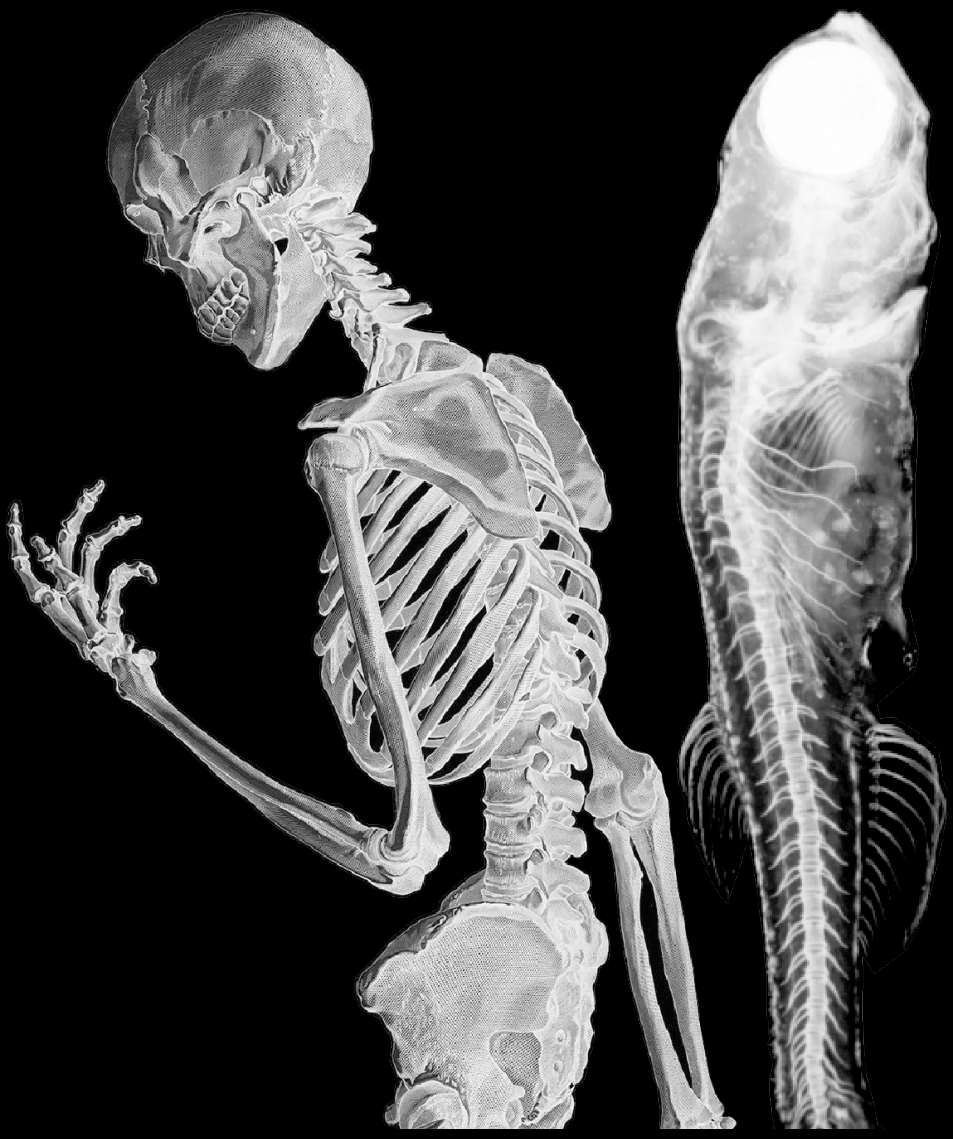
CHAPTER 1.

MARINE BIOACTIVE COMPOUNDS TO TREAT SKELETAL DISORDERS IN HUMANS AND FISH



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(Previous page) This image is an original creation of Alessio Carletti, obtained by modifying the illustration by: Trew, Eisenberger, N. F., & Lichtensteger, G. (1767). D. Christophori Iacobi Trew […] Tabulae osteologicae: seu omnium corporis humani perfecti ossium imagines ad ductum naturae quam in ordinaria connexione secundum habitum suum externum magnitudine naturali sub eiusdem institutione repraesentatae. [s.n.].
Original image obtained from "Historical Anatomies on the web" by the National Institute of health (NIH). (https://www.nlm.nih.gov/exhibition/historicalanatomies/trew_home.html)

CHAPTER OVERVIEW

Metabolic bone disorders are a major cause of disability and mortality, as well as a tremendous economic burden for health systems globally. These disorders, caused by an unbalanced equilibrium between bone anabolic and erosive processes, are characterized by different pathophysiological mechanisms. Nevertheless, the therapeutic strategies implemented to treat patients suffering from different bone metabolopathologies belong to the same limited group of drugs. In addition, these are often poorly effective and associated with undesired side effects. In **Chapter 1.1**, we have briefly reviewed the molecular mechanisms underlying most common metabolic bone disorders, and the availability, efficacy, and limitations of the current therapeutic options.

A potential solution for the unmet need of novel drugs to treat metabolic bone disorders may come from marine-based pharmacology. Many groups of marine organisms are being increasingly studied as a source of natural osteoactive compounds, some of which presenting highly promising bioactivities. In the second part of **Chapter 1.1**, we have reviewed and catalogued all the marine osteoactive compounds identified in the period between 1999-2022. We have then discussed the taxonomic distribution of the organisms from which such compounds were isolated, and identified taxonomic groups that deserve attention of future research aimed at the discovery of drugs for the treatment of metabolic bone disorders.

Finally, we briefly examine the availability of *in vivo* screening and validation tools for the study of marine osteoactives. In addition, skeletal disorders in cultured fish are a major concern for the aquaculture industry, currently the main source of seafood for human consumption. Skeletal anomalies are omnipresent in all species of reared fish, although with different patterns and degrees of severity, hampering the quality of the final product and causing economic losses to the industry. Improper nutrition, together with other causative factors, has been recognized as major cause of these anomalies. In **Chapter 1.2**, we have reviewed the nutritional aspects related to the development of skeletal anomalies in aquaculture fish, and the potential of dietary supplementation with marine natural extracts rich in compounds with health-promoting effects on the skeleton, as a cost-effective strategy to reduce skeletal disorders in farmed fish. In **Chapters 1.3**, we have briefly discussed the different approaches that are applied when bio-prospecting the natural environment in search for potential drugs and nutraceuticals, and what is the role that academic institutions play in the pharmaceutical research.

To conclude this introductory section, in **Chapter 1.4**, we have clearly defined the aims and specific objectives of this PhD thesis.

1.1. MARINE OSTEOACTIVES AS THERAPEUTICS FOR METABOLIC BONE DISORDERS

Abstract

Metabolic bone disorders and associated fragility fractures are major cause of disability and mortality worldwide, and place an important financial burden on the global health systems. These disorders result from an unbalance between bone anabolic and resorptive processes, and are characterized by different pathophysiological mechanism. Drugs are available to treat bone metabolic pathologies but they are either poorly effective or associated with undesired side effects that limit their use. Molecular mechanism underlying the most common metabolic bone disorders, and the availability, efficacy and limitations of therapeutic option currently available are here discussed. A source for the unmet need of novel drugs to treat metabolic bone disorders are marine organisms, which produce natural osteoactive compounds of high pharmaceutical potential. In this review, we have inventoried the marine osteoactive compounds (MOCs) currently identified and spotted the groups of marine organisms with potential for MOC production. Finally, we briefly examine the availability of *in vivo* screening and validation tools for the study of MOCs.

1.1.1. The burden of metabolic bone disorders

In 2019, a meta-analysis of available data from 204 countries and territories reported a global incidence of fragility fractures around 2.3% of the total population and 15.4% of the elderly subpopulation[1]. Bone fragility is a major concern for the global health system, causing severe disability and mortality worldwide[2], and placing an important financial burden on the society. In this regard, the yearly health and social care costs associated with hip fractures was recently estimated at 43,669 USD per patient in the USA[3].

At the origin of fragility fractures there is a series of skeletal disorders that are mostly triggered by the dysregulation of a fundamental homeostatic process: bone remodeling. To maintain mechanical properties and architectural integrity throughout life, bone must renew senescent and damaged structures through a process requiring the concerted resorption and formation of bone mineralized matrix. An unbalance between these two processes will prompt metabolic bone disorders (abbreviated MBDs hereafter)[4,5]. As different pathologies may be characterized by different causing mechanism, we will start this review with a brief description of mineral phenotypes. In this context, the evaluation of the bone mineral density (BMD), defined as “the

amount of mineral per square centimeter of bone"[6], represents the gold-standard in clinical practice to establish a pathological alteration of mineral content and identify patients with MBDs. Low-BMD pathologies are osteomalacia[7], nutritional rickets[8], osteopenia and osteoporosis[9]. High-BMD pathologies are genetic disorders united under the term osteopetrosis[10–12]. Finally, Paget's disease of bone[13-14], primary hyperparathyroidism[15–17] and renal osteodystrophy[18,19] are considered BMD-independent pathologies, as it has been demonstrated that they are not unequivocally diagnosed by a reduced BMD, and several manifestations of these disorders are characterized by locally elevated BMD.

1.1.2. Molecular mechanisms of metabolic bone disorders

For the purpose of this review, we have classified MBDs on the basis of the underlying pathophysiological process, as the therapeutic approach adopted will mostly depends on this factor. As such, MBDs were divided into (i) disorders affecting the mineral homeostasis though the vitamin D (VD)-parathyroid hormone (PTH) regulatory network; (ii) disorders caused by an excessive osteoclast function; and (iii) disorders induced by defective osteoclast function. In the following paragraphs, we will also describe the molecular roots of the most common MBDs, a critical knowledge towards the discovery of novel compounds to treat these disorders.

1.1.2.1. Disorders resulting from an altered mineral homeostasis

Osteomalacia and rickets are primarily caused by calcium or vitamin D (VD) deficiency in adults and children, respectively[20]. Causes of these deficiencies are vast, e.g. reduced dietary intake of VD and calcium or their malabsorption in patients with gastrointestinal or liver disorders, and also increased excretion induced by nephropathologies[4,5]. Low levels of these essential nutrients drive the mineral homeostatic system to change the source of circulating calcium from intestinal absorption to bone resorption. In this situation, PTH stimulates osteoclast differentiation by inducing an overproduction of RANKL and M-CSF by osteoblasts, osteocytes, bone marrow stroma cells and resident lymphocytes[21]. The persistency of this condition leads to osteopenic bones in adults and bended bones in children[5,7,22,23]. Osteomalacia can be rescued in adult upon VD and calcium supplementation, but bone deformities in rachitic children are often irreversible and can only be treated by surgery[4,20,24,25]. Primary hyperparathyroidism is an endocrine disorder characterized by hypercalcemia (elevated blood calcium levels) and inappropriate PTH levels, caused by benign or cancerous tumors in parathyroid glands[26]. Skeletal phenotype is characterized by loss of cortical bone,

reduced BMD leading to osteopenia, and an increase risk of fracture in both vertebral and appendicular sites[26,27]. In the absence of suitable drugs, the only efficient cure is the surgical removal of parathyroid tissue or glands (parathyroidectomy). If surgery is not an option, a blend of calcium regulating agents, bone anabolic and anti-resorptive drugs may be used[26].

Renal osteodystrophy is a condition that covers skeletal disorders in patients suffering from chronic kidney disease (CKD), e.g. osteoporosis, osteomalacia, osteitis fibrosa, and adynamic bone disease[28]. Initially, renal insufficiency triggers a retention of phosphorous and an accumulation of uremic toxins in blood, inducing a state of low bone metabolism known as adynamic bone disease[18,28]. This condition may result from the acquisition of a PTH signaling-resistance by the bone tissue. The persistency of the adynamic bone condition, high level of phosphorous and reduced circulating calcitriol (1,25-hydroxyvitamin D_3) induces hypocalcemia and stimulates parathyroid glands, exacerbating the elevation of serum PTH. Patients eventually develop secondary hyperparathyroidism[18], whose histological landmarks are defined as osteitis fibrosa, and characterized by an increased bone turnover, an increased osteoblast number and activity, woven osteoid, increased osteoclast number and activity, overall increased bone resorption, low BMD, and increased fragility[18,28].

1.1.2.2. Disorders resulting from an excessive osteoclast activity

Osteoporosis *(OP)* and ***Paget's disease of bone*** *(PDB)* are the most common MBDs, with a prevalence of 18.3% and 0.6%, respectively[29,30], and they both result from a dysfunctional and overregulated bone resorption by osteoclasts[4,5].

PDB pathophysiology involves the increased formation of hyper-resorptive osteoclasts during the "osteolytic" and initial phase of the disease. In an attempt to recover the loss of bone mineral, the body increases bone formation, a compensatory mechanism which results in the production of an unorganized and "woven" bone matrix[31]. Typically, pagetic patients show a localized symptomatology (both forms, monostotic, affecting a single bone, and polyostotic, affecting more skeletal elements exist) with a higher number of atypical osteoclasts characterized by a larger size, an increased number of nuclei and an elevated resorptive activity. Osteoclast precursors are generally highly responsive to pro-osteoclastogenic signaling such as RANKL and 1,25-$(OH)_2D_3$ and are resistant to apoptotic signaling[31–33].

Clinical features of PDB include bone pain and increased serum alkaline phosphatase (ALP); Microfractures and increased bone vascularization may also be observed, leading with time to deformations due to the weakened structure[31–33]. Leading causes of PDB are still not fully apprehended, although it appear that bone formation, despite being rapid and unorganized, is

in fact intrinsically normal[34]. Genetic factors associated to the disease include a plethora of mutations and variants in genes associated to osteoclast differentiation and activation, while environmental factors may include epigenetic factors, exposure to certain toxins, infection by several Paramyxoviridae members (measles, canine distemper, and respiratory syncytial viruses)[35].

No cure exists for PDB, and therapeutic strategies currently available to alleviate disease symptoms focus on a set of anti-resorptive drugs, mostly bisphosphonates, targeted at restoring normal levels of bone resorption. Anti-inflammatory drugs may also be implemented, as well as vitamin D and calcium supplementation, in order to prevent possible negative effects of the elevated bone resorption over parathyroid function which may lead to secondary hyperparathyroidism.

Osteoporosis and ***osteopenia*** (commonly considered a pre-osteoporotic form of low BMD disorder) are also characterized by a dysregulated resorptive process. Osteoporosis is characterized by four pathophysiological mechanisms, which may overlap in some patients: postmenopausal osteoporosis, age-related osteoporosis, immobilization-induced osteoporosis, and glucocorticoid-induced osteoporosis (GIOP).

Post-menopausal osteoporosis is a complex and multifactorial condition[36]. In premenopausal women, estrogens participate in bone anabolism by inhibiting osteoblast[37,38] and osteocytes[39,40] apoptosis thus increasing their life spam. Estrogens also prevent bone resorption by inhibiting RANKL-mediated osteoclastogenesis[41], stimulating the production of anti-osteoclastogenic cytokines by regulatory T cells[42], and inducing osteoblast-mediated osteoclast apoptosis in a paracrine manner[43]. Estrogens also excerpt a suppressive effect over thymic function, reducing the population of inflammatory T cells[44]. After the menopause, circulating estrogens are depleted as a result of reduced ovarian synthesis, and the suppressive effect they normally have over thymic function is diminished. As activated T cells produce pro-osteoclastogenic cytokines such as IL-1b and TNF-α[45], which act both through an osteoblast-mediated[46] and an osteoblast-independent manner[47], a chronically elevated bone remodeling is established at menopause, where bone resorption is not compensated by bone formation. This mechanism leads to an overall reduced BMD, increased fragility and fracture risk[48].

Age-related osteoporosis affects both woman and men and initiates after the peak of BMD at adolescence. Rate is similar in both genders but may be intensified in women entering menopause[49]. An hypothesis for a long time[50–52], there is now a growing body of evidence that support the role of an age-related increase in oxidative stress in the age-related diminution of

BMD. In this scenario, reactive oxygen species (ROS) induce bone loss by stimulating osteoclasts differentiation[53,54] and osteoblast apoptosis[55,56].

Immobilization-induced osteoporosis (or disuse osteoporosis) develops in patients that are immobilized for a long period following illness or injuries, but is also observed in astronaut exposed to microgravity[57]. This condition is typically characterized by cortical bone loss, while trabecular bone loss is commonly observed in other osteoporotic conditions, and is the consequence of a reduced mechanical loading on bone, which is mediated by the osteocytes[57], and altered bone remodeling.

Glucocorticoid-induced osteoporosis (GIOP) is a highly prevalent disorder which occurs in patients subjected to a prolonged glucocorticoid treatment for inflammatory diseases[58,59]. Synthetic glucocorticoids impair osteoblast differentiation by dysregulating WNT-β-catenin signaling pathway[60]; they were also shown to stimulate osteoblast apoptosis[61]. Indirectly, glucocorticoids affect osteoblast function by reducing the expression of insulin-like growth factor 1 (IGF-1)[62], which promotes bone formation by mediating the anabolic effects of the parathyroid hormone (PTH)[63]. Glucocorticoids can also stimulate osteoclastogenesis by reducing the production of osteoprotegerin by osteocytes and osteoblast[64].

Therapeutic approaches for osteoporosis comprise a set of bone anabolic and anti-resorptive therapies, which are used with the main objective of preventing bone loss, increasing bone formation, and reducing the fracture risk. The advantages and disadvantages correlated to each of the major groups of pharmacological agents currently implemented will be further discussed in the next section. Importantly, all therapeutics currently approved are characterized by long-term limited efficacy and side effects.

1.1.2.3. Disorders caused by an impaired osteoclast function

They are characterized by a vast group of rare, primary monogenic disorders gathered under the name ***osteopetrosis***, also known as the marble bone disease. There are two prevalent forms of osteopetrosis, which are easily distinguishable based on their inheritance modality. A more prevalent, milder and typically late-onset form (arising late during childhood) known as *autosomal dominant osteopetrosis* (ADO), and a more rare, aggressive and early-onset form (arising early after birth) associated with severe phenotypes and poor prognosis, and known as *autosomal recessive osteopetrosis* (ARO)[65].

Osteopetrosis is characterized by a defective bone resorption, increased bone mass and high BMD, and is associated with bone fragility and an increased risk of fractures, and, in the case of ARO, with defective bone marrow, kidney, and nervous and immune systems[65]. Mutations

in genes that are central to osteoclast function have been associated with the etiology of osteopetrosis, in particular those involved in the acidification of bone microenvironment (*TCIRG1*, *CLCN7*), degradation of the extracellular matrix (*CTSK*), and cell differentiation (*RANK*, *RANKL*, *CSF1R*, *NEMO*, *RELA*)[66,67]. There are currently no pharmaceutics to efficiently treat osteopetrosis, and therapeutic approaches only aimed at managing symptoms and relieve pain, e.g. supplementation of vitamin D and calcium in patients with hypercalcemic seizures, transfusion of red blood cells and platelets in patients with bone marrow failure, transplantation of hematopoietic stem cells in patient suffering from the most severe forms of osteopetrosis[65].

1.1.3. What's on the menu? Current therapeutic strategies, efficacy and limitations

Therapeutic solutions currently available to treat metabolic bone disorders fail to meet the clinical demand. Drugs lack either efficacy or are only effective for a limited time or trigger long-term use-associated side effects that are not compatible with the treatment of life-lasting chronic conditions. In the following sections, we will briefly present therapeutics currently in use, their efficacy and limitations. Figure 1 illustrates examples of main groups of bone erosive disorders, therapeutic approaches, and their molecular targets, currently implemented for the treatment of MBDs.

1.1.3.1. Vitamin D and Calcium supplementation

The central role of calcium[68,69] and vitamin D (VD)[70,71] in bone health is well established, still there is no consensus on the dose that should be recommended to healthy individuals and patients with increased fracture risk[72,73], nor whether benefits accompanying the supplementation of calcium and VD outweigh associated risks[74–76].

Calcium supplementation has little or no effect on the reduction of fracture risk or the increase of BMD in healthy individuals[77,78] but can decrease fracture risks and increase BMD in postmenopausal women[79,80]. It has been associated with an increased risk of cardiovascular disease[81–83], although this association was refuted in a recent meta-analysis of the clinical data[84].

VD supplementation, alone or in combination with calcium, has little or no effect on the reduction of fracture risk or increase of BMD in healthy individuals[85–87] but is associated with a reduced risk of falls in elderly[88,89] and a reduced bone loss in postmenopausal women[90,91].

However, several studies highlighted that the supplementation of vitamin D or calcium alone cannot rescue bone loss once that it has already occurred[92,93].

Calcium supplementation combined with VD supplementation was also not associated with increased risk of cardiovascular disease or mortality[84]. Recently, alfacalcidol [1-α-(OH)D_3], a vitamin D_3 analogue, was found to be more effective for the treatment, rather than the prevention, of postmenopausal osteoporosis, GIOP and osteomalacia, when compared to cholecalciferol[94].

In relation to their application to other diseases than osteoporosis, VD and calcium supplementation represent the primary tool for the prevention and treatment of osteomalacia and nutritional rickets[95], and have demonstrated to be a rapid and effective therapy to restore BMD and serum biomarkers but also to relieve symptoms[95–97]. However, the restoration of bone density and healing of bone fractures may take time (months) and bone loss may be irreversible at some particular sites[98]. VD and calcium supplementation (low doses) is also used in the treatment of primary[26] and secondary[99,100] hyperparathyroidism, to restore plasma levels and prevent the deficiency of both molecules in patients with abnormal PTH production or renal insufficiency. In hyperparathyroidic patients undergoing parathyroidectomy, VD and calcium supplementation is used to prevent post-surgery hypocalcaemia[101]. VD supplementation also finds application in the treatment of Paget's disease of bone, to counteract hypovitaminosis D, which appears to be frequent in pagetic patients[102], but also to prevent flu-like symptoms commonly found in pagetic patients treated with bisphosphonates[103]. Treatment with high doses of calcitriol has been tried to stimulate osteoclast differentiation in patients with congenital malignant osteopetrosis (ARO) but resulted in poor outcomes[104,105]. As such, its use is currently not supported by clinicians[106]. Nowadays calcium and cholecalciferol supplementation is encouraged for osteopetrotic patients to prevent the hypocalcemic seizures that are frequently associated with this condition due to the immobility of calcium from the bone[65,106].

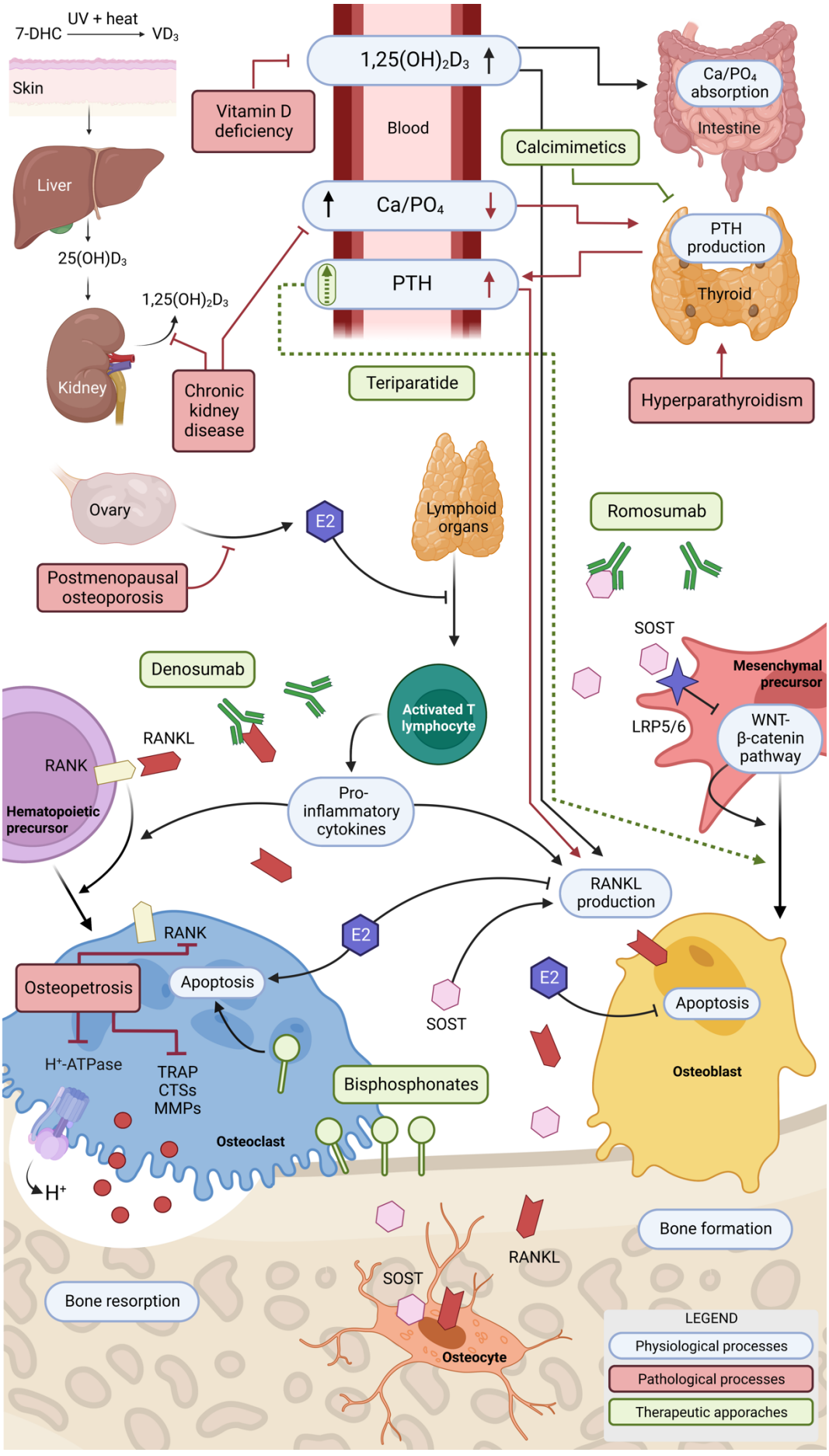

7-DHC
UV + heat
VD3
Skin
Liver
25(OH)D3
1,25(OH)2D3
Kidney
Chronic kidney disease
Vitamin D deficiency
1,25(OH)2D3
Blood
Ca/PO4
PTH
Calcimimetics
Ca/PO4 absorption
Intestine
PTH production
Thyroid
Hyperparathyroidism
Teriparatide
Ovary
Postmenopausal osteoporosis
E2
Lymphoid organs
Romosumab
SOST
Mesenchymal precursor
LRP5/6
WNT-β-catenin pathway
Denosumab
Activated T lymphocyte
RANKL
RANK
Hematopoietic precursor
Pro-inflammatory cytokines
RANKL production
RANK
E2
Osteopetrosis
Apoptosis
H+-ATPase
TRAP
CTSs
MMPs
Osteoclast
H+
SOST
E2
Apoptosis
Osteoblast
Bisphosphonates
Bone formation
RANKL
SOST
Bone resorption
Osteocyte
LEGEND
Physiological processes
Pathological processes
Therapeutic apporaches

Figure 1. Molecular mechanisms of bone metabolic disorders (red boxes and arrows) and therapeutic treatments (green boxes and arrows) currently available. A complex network of organs, tissues, and signals intervein to control bone metabolism and a large number of emerging therapeutic targets are being described. Symbols: Continuous lines with pointed arrowheads indicate process upregulation; Continuous line with blunt arrowheads indicate process downregulation; Dashed lines with pointed arrowheads indicate an intermitted stimulation causing process upregulation. UV, ultraviolet radiation; Ca/PO_4, inorganic calcium and phosphate ions; 7-DHC, 7-dehydrocholesterol; VD_3, vitamin D_3 (also known as cholecalciferol); $25(OH)D_3$, 25-hydroxyvitamin D_3 (also known as calcifediol); $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D_3 (also known as calcitriol); PTH, parathyroid hormone; E2, estradiol; SOST, sclerostin; WNT, canonical Wnt signaling pathway; LRP5/6, low-density lipoprotein receptor-related protein 5/6; RANK, receptor activator of nuclear factor κB; RANKL, RANK ligand; H^+, proton; H^+-ATPase, vacuolar-type proton-ATPase; TRAP, tartrate-resistant acid phosphatase; MMPs, matrix metalloproteinase protein family members; CTSs, Cathepsins protein family members.

1.1.3.2. Vitamin K supplementation

The term vitamin K (VK) collectively designs a group of fat-soluble compounds found in animals and plants and represented by three main forms: phylloquinone (VK_1), menaquinone (VK_2) and menadione (VK_3).

The central role of vitamin K in animal physiology, reviewed by Fusaro et al. (2020)[107], has been largely associated with its function as cofactor of the carboxyglutamyl carboxylase (GGCX), a cytosolic enzyme which catalyzes the carboxylation and functionalization of the so-called vitamin-K-dependent proteins (VKDPs). VKDPs include, among others, proteins important for bone matrix organization and mineralization such as bone gamma-carboxyglutamate protein (Osteocalcin, BGLAP), matrix Gla Protein (MGP), and Gla-rich Protein (UCMA)[107]. Vitamin K also regulates bone metabolism in a GGCX-independent manner by binding the pregnane X receptor (SXR, PXR or NR1l2), which control the expression of genes involved in osteoblastogenesis, osteoclastogenesis, and extracellular matrix formation and mineralization, ultimately affecting bone mechanical properties[107].

Because VK plasma levels in healthy individuals are low and detection is rather difficult, little data is available on the pathology and epidemiology of vitamin K deficiency[107]. VK deficiency has been associated to cardiovascular disorders including neonatal bleeding[108] and vascular calcification in patients suffering chronic kidney disease (CDK)[109]. In patients with end-stage

CDK, VK deficiency is also associated with bone loss in the osteopenic range and an increased fracture risk[110,111].

Other chronic disorders leading to secondary VK deficiency have also been associated with skeletal comorbidities. For instance, patients suffering Crohn's disease have a lower BMD associated with VK deficiency possibly due to intestinal malabsorption[112]. VK deficiency has also been associated with inflammation-related pathologies including knee osteoarthritis[113]. Despite accumulating evidence on the central role of vitamin K in bone health, vitamin K supplementation in postmenopausal and osteoporotic patients did not significantly improved BMD and incidence of fractures[114].

1.1.3.3. Supplementation of n-3 Polyunsaturated Fatty Acids (PUFAs)

Polyunsaturated fatty acids (PUFAs), such as arachidonic acid (AA, 20:4n-6), eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), are important regulators of bone metabolism[115–117]. Eicosanoids and docosanoids, formed upon PUFA oxidation by cyclooxygenases, lipoxygenases, and epoxygenases, act as anti- and pro-inflammatory molecules, respectively, and regulate the equilibrium of bone remodeling[117]. For example, prostaglandin E_2 (PGE_2) act in a both pro-osteoclastogenic and anti-osteoblastogenic manner[117].

PUFAs can also impact directly on bone cells, with n-3 PUFAs inducing proliferation of bone marrow mesenchymal stem cells (BMSCs) and stimulating osteoblast differentiation, and n-6 PUFAs stimulating osteoclastogenesis[117]. PUFAs-derivates are also natural ligands of the peroxisome proliferator-activated receptor gamma (PPARγ), which is an important molecular switch that deviates the fate of MSCs from osteogenesis towards adipogenesis[117]. Multiple animal studies conducted in OVX-rats and mice showed that dietary supplementation of n-3 PUFAs decreased osteoclastogenesis[118], reduced bone loss[119,120], and promoted chondrocyte-to-osteoblast transdifferentiaton[121]. The relative consumption of n-3 and n-6 PUFAs can also regulate the composition of bone cell membranes in fatty acids[122]. In this regard, dietary strategies that reduce the n-6/n-3 ratio have been proposed for the treatment of bone erosive disorders.

Two recent meta-analyses of randomized controlled trials (RCTs) conducted in human patients confirmed that the supplementation of n-3 PUFAs, with α-linolenic acid (ALA) being more potent than EPA and DHA, was able to slightly increase BMD, reduce resorption markers and, in the case of ALA, slightly increase bone formation markers in a short term. A higher effect was also observed in post-menopausal women[123,124]. However, the positive effect of PUFA

supplementation reported in these studies are very low when compared with the effect of pharmaceuticals used to treat osteoporosis.

1.1.3.4. Extracellular calcium receptor modulators

Extracellular calcium receptor (CaR) is a major regulator of PTH secretion by the parathyroid glands in response to variation of calcium levels in the serum of higher vertebrates, and is therefore a key target in drugs discovery for disorders characterized by the dysregulation of calcium mineral homeostasis[125]. CaR activators, also known as calcimimetics, are molecules acting as CaR agonist or allosteric activators. By binding CaR, they inhibit PTH secretion and re-equilibrate parathyroid function in patients suffering primary, secondary, and tertiary hyperparathyroidism. Several calcimimetic drugs are used to treat hyperparathyroidism following parathyroid hyperplasia, parathyroid cancer, chronic kidney disease (CDK), and kidney transplant[125–127].

Among those, cinacalcet has been approved for the treatment of patients with secondary and primary hyperparathyroidism that cannot or refuse to undergo parathyroidectomy. Evidences from case studies and randomized controlled trials highlighted the efficacy of cinacalcet in lowering PTH and serum calcium levels, in accordance with results in mammalian models[127,128]. Cinacalcet also improved bone turnover markers and bone histology but exhibited a poor ability, or none, in increasing BMD[127,128]. Few calcimimetics are currently being evaluated in drug discovery pipelines, mainly because *in vitro* high-throughput technologies are missing and screening is limited to whole animal testing[125].

Calcilytics, allosteric antagonists of CaR stimulating the secretion of PTH by the parathyroid glands, have been proposed to treat patients with osteoporosis after several studies reported the osteoanabolic potential of transient PTH exposure[125]. Despite promising results in ovariectomized rats[129], calcilytics did not confirm their osteoanabolic potential in human and no reasonable advantage over PTH analogues was found. As a results, clinical trials for most of candidate calcilytics were discontinued[125,127].

1.1.3.5. Anti-resorptive agents

Antiresorptive pharmaceuticals are drugs that inhibit bone resorption by osteoclasts either by impairing their differentiation, recruitment or activity, or by promoting their apoptosis[130].

Hormonal replacement therapies (HRTs) were among the first antiresorptive drugs discovered and approved for osteoporosis. Estrogens are potent inhibitors of bone resorption, acting through the downregulation of RANKL-mediated osteoclast differentiation, and estrogen

replacement therapy following menopause could increase BMD and reduce fracture risks[41]. However, estrogen replacement therapy was associated with an increased risk of breast cancer, uterine cancer and cardiovascular diseases, and has progressively slipped out the list of potential treatments for postmenopausal OP[131,132].

Selective estrogen receptor modulators (SERMs) are drugs that can specifically modulate the activity of bone specific isoforms of the estrogen receptor, thus induce the beneficial effect of estrogens over bone without increasing the risk of breast and uterine cancer[130]. Two SERMs currently approved for the treatment of postmenopausal OP, raloxifene and bazedoxifene, have demonstrated a mild positive effect on reducing fracture risk[41]. However, they have also been associated with both mild and rare but severe cardiovascular side effects[41].

Testosterone replacement therapy has proven to be effective in increasing BMD in men with osteopenia and osteoporosis[133], although several studies have associated it with increased risk of cardiovascular diseases[134].

The peptide hormone *calcitonin* is a potent inhibitor of osteoclast activity[135,136], and both human and salmon calcitonin have been used as an antiresorptive treatment for OP, PDB and hypercalcemia, in both injectable and nasal spray forms[137]. However, several studies associated the use of calcitonin with an increased risk of prostate cancer in men[138], prompting the removal of calcitonin from the list of approved therapies for osteoporosis by the European Medicine Agency (EMA) in 2012. Nowadays, calcitonin therapy is limited to patients with PDB and short treatments are recommended[139].

Cathepsin K, a cysteine protease primarily involved in the degradation of bone extracellular matrix and produced in large quantities by active osteoclasts, has also been targeted by antiresorptive drugs. Cathepsin K inhibitor *odanacatib* is assessed in several clinical trials[140,141], and available data indicated a reduction of bone resorption markers and an increase of BMD in a dose-dependent manner[142,143]. However, positive effects quickly disappeared once the treatment was discontinued[144], while odanacatib has been associated with an increased risk of stroke in osteoporotic woman. Consequently, the industrial entity developing the drug discontinued the trials[145].

Bisphosphonates are chemically stable analogues of inorganic pyrophosphate (PPi) with antiresorptive properties, which have been successfully used for nearly 4 decades to treat bone remodeling disorders including post-menopausal OP, age-related and immobility-induced OP, GIOP, PDB, and hyperparathyroidism[26,130,146,147]. Although the implementation of bisphosphonates in clinical practice largely anticipated the full understanding of their mechanism of action, an intense research effort during the last two decades shed some light

over the molecular basis of bisphosphonate action on bone cells. Briefly, bisphosphonates bind to hydroxyapatite crystals at active sites of bone remodeling sites then are incorporated in osteoclasts following bone resorption, where they inhibit the post-translational modification of proteins involved in cell function, ultimately leading to cell death[148]. A link between the effect of bisphosphonates on immune cells such as monocytes, macrophages and T lymphocytes, and their antiresorptive activity has been proposed[149]. Because of their high affinity for calcium, bisphosphonates tend to accumulate in bone, being released by osteoclasts only at active remodeling sites. As a consequence, bisphosphonates are typically administered on a weekly, monthly or even yearly basis. Bisphosphonates commonly used to treat bone related disorders – alendronate, risedronate, ibandronate and zoledronate – are able to decrease bone resorption up to 70%, and reduce the incidence of vertebral and non-vertebral fracture in women with osteoporosis up to 62% and 40%, respectively[130].

Denosumab is a RANKL monoclonal antibody approved for the treatment of postmenopausal OP, age-related OP, and GIOP[150], but also PDB, primary and secondary hyperparathyroidism. Denosumab binds to RANKL with a high affinity, mimicking the activity of the endogenous osteoprotegerin (OPG), and prevents its ligation to RANK receptor at the osteoclast surface, inhibiting the major signaling cascade involved in osteoclast differentiation[151]. Denosumab is a potent inhibitor of bone resorption that can reduce the incidence of vertebral, non-vertebral, and hip fracture in osteoporotic patients of 68%, 20% and 40%, respectively[151], thus has an efficacy similar to that of bisphosphonates and osteoanabolic drug[151].

As for other antiresorptive agents, patients treated with denosumab experience a steep increase in BMD in the first 6-12 months after the beginning of the treatment, but while bisphosphonate treatment has been associated to a steady BMD after this first period, denosumab produces a slow but continuous increase in mineral density[152]. Denosumab has also shown some efficacy in rescuing bone remodeling markers in both old and juvenile pagetic patients[153–155], and in patients with hyperparathyroidism[156,157]. In relation to this latter aspect, denosumab triggered better skeletal improvements, in terms of BMD and bone turnover markers, than parathyroidectomy in osteoporotic patients with PHPT as comorbity[158].

Bisphosphonates and denosumab have been correlated to mild and frequent but also rare and severe side effects, raising concerns among clinicians. Among those more severe but rare, atypical femur fracture (AFF) was reported in 1 patient out of 250 (frequency increases with the duration of the treatment), and osteonecrosis of the jaw (ONJ) was observed in 1 patient every 4000[159,160]. Among those less severe but frequent, upper gastrointestinal side effects, increased risk of esophageal cancer (still uncertain), musculoskeletal pain and flu-like

symptoms were reported for bisphosphonates[153]. Denosumab can induce a reduction in bone turnover, a secondary effect that should be considered when treating CKD patients because of the risk of facilitating the development of adynamic bone disease[153]. Serum levels of calcium and VD must be monitored before and during denosumab treatment due to increased susceptibility to hypocalcaemia[153]. Furthermore, denosumab treatment has been associated to increased risk of adverse effects to infections, presumably due to its immunosuppressive properties[159,161].

Despite their positive effect, last-generation antiresorptive drugs are characterized by a limited long-term efficacy. Indeed, although they can prevent further loss of mineral, they do not rescue the irreversible deficit in bone volume that occurs in degenerative bone disorders[152]. Several authors have proposed that the increase in BMD observed following the treatment with antiresorptive agents may only be an artefact resulting from the secondary mineralization of already-existing mineral matrix, and may not be associated with the deposition of new ECM and increase in bone volume, which are needed for structural improvement and protection against fragility fractures[152]. Furthermore, a discontinuation of antiresorptive therapy is typically associated with a re-increase in bone resorption and subsequent mineral loss. As such, clinicians and researchers are currently evaluating the co-application or the sequential application of antiresorptive and osteoanabolic agents (see below).

1.1.3.6. Osteoanabolic agents

Osteoanabolic drugs have the capacity to impact on the formation and mineralization of the extracellular matrix orchestrated by osteoblasts. It is increasingly admitted that only an osteoanabolic approach can ultimately compensate for the loss of bone volume observed in low-BMD disorders[152]. Yet, there is a surprising scarcity of bone anabolic compounds available to patients.

Among the few drugs used to restore bone mineral density, *strontium ranelate* (SR) was long considered the most promising osteoanabolic compound after several studies reported increased BMD and reduced fracture risk in SR-treated patients[162]. However, its association to increased cardiovascular events and myocardial infarction in postmenopausal women has led to the discontinuation of its production[163], and nowadays its use is not approved any longer by the European Medicines Agency[164].

Two other osteoanabolic drugs are available for osteoporotic patients in Europe: *Teriparatide*, the synthetic analogue of parathyroid hormone (PTH), and *Abaloparatide*, the analogue of parathyroid hormone-related peptide (PTHrP). The dualistic action of PTH on bone

metabolism and the anabolic effect of an intermittent treatment with PTH – rather than the classical catabolic effect associated with the continuous exposure to PTH – is known for a long time[165]. Early studies identified osteoblastic lineage as the primary target for PTH regulation of bone homeostasis[166] and that exposure to low dosage of PTH for short periods indeed triggers the proliferation of osteoblast precursors[167]. Subsequent studies revealed that PTH stimulates osteoblast differentiation by stimulating pro-osteogenic WNT signaling pathway and inhibiting pro-adipogenic PPARγ signaling pathway in mesenchymal stem cells[168,169].

PTH also inhibits apoptosis in osteoblastic cells, contributing to more cells being available for bone formation and mineralization[170]. On the contrary pro-resorptive effect of constantly elevated serum levels of PTH (e.g. during the development of hyperparathyroidism) was attributed to the stage-specific capacity of PTH to induce the expression of RANKL and inhibit OPG expression throughout osteoblast differentiation[171].

PTH synthetic analogue *Teriparatide* (hPTH 1-34) is composed of PTH bioactive region (amino acids 1 to 34). It is currently approved worldwide for the treatment of postmenopausal OP, age related OP, and GIOP, and can reduce up to 80% of vertebral fracture and 50% of non-vertebral fractures in osteoporotic patients, representing one of the most effective treatment currently available[152,172,173]. Teriparatide can also improve bone genetic disorders such as Osteogenesis imperfecta[174]. Despite an excellent short-term efficacy, the long-term use of teriparatide has faced several limitations, e.g. the necessity of parenteral administration (which affect the patient's compliance with the treatment due to side effects related to repetitive injections), and secondary effects such as decreased BMD in the radius, dizziness, leg cramps, headache and hypercalcemia[172].

Due to the dualistic effect of PTH on bone and a short-term efficacy, teriparatide will trigger an osteoanabolic effect for 12-24 months (period known as the anabolic window), then a catabolic effect characterized by increased osteoclast activity and bone resorption. Unfortunately, bone loss will occur even if treatment is discontinued[152,175], thus teriparatide treatment is frequently followed by an antiresorptive therapy[152,175].

When compared to PTH, PTHrP triggers a similar osteoanabolic action but has a milder pro-resorptive effect and a lower tendency to induce hypercalcemia. This could be related to the different affinity of PTH and PTHrP for different conformational status of the receptor PTHR1, influencing the receptor's kinetic with consequence milder stimulation of the downstream signaling cascade[172,176]. Based on the superior performances of PTHrP, the synthetic analogue Abaloparatide (PTHrP 1-34) was recently developed. It is not yet approved for the treatment of osteoporotic patients in Europe but several studies have highlighted the similar effect of

teriparatide and abaloparatide in increasing BMD, and a very similar or higher effect in preventing vertebral and non-vertebral osteoporotic fractures[172]. Abaloparatide is also claimed to have a better anabolic window than teriparatide due to a lower pro-resorptive effect over time[175]. However, this claim is only supported by clinical evidence of a delayed increase in serum resorption marker C-terminal telopeptide of type 1 collagen (CTX) following abaloparatide treatment, and challenged in several studies[152].

It is worth to mention that the administration of teriparatide and abaloparatide to patients with a high risk of cancer, e.g. pagetic patients, is discouraged in the USA as it may favor the development of osteosarcoma, a warning based on studies performed in rats[177,178,179]. Yet, in 35 years of approved clinical use of teriparatide (abaloparatide was only approved in 2017), no concrete evidence of an increased incidence of osteosarcoma in humans was reported[180,181].

1.1.3.7. Co-administration and sequential administration of anabolic and antiresorptive drugs

Because monotherapies have shown some limitations, the efficacy of combinational therapies – i.e. the co-administration or sequential administration of anti-resorptive drugs and osteoanabolic agents – has been evaluated (reviewed in[130,182,183]), and results are contrasted. The co-administration of bisphosphonates and denosumab did not clearly improved outcomes of monotreatments[130,182,183], while the combination bisphosphonate and HRT only resulted in a slight better BMD[182]. A recent meta-analysis of RCTs indicated that patients co-treated with teriparatide and antiresorptive agents showed an improved BMD gain and a reduced risk of fracture[184].

Sequential treatments with antiresorptive agents was only beneficial if the second treatment was done with a more potent antiresorptive; in that case effect of the first treatment could be maintained[182]. Sequential treatments with different types of drugs have proven to be more effective. In this regard, a treatment with bisphosphonates or denosumab following an initial treatment with bone anabolic drug could prevent bone loss commonly observed after monotherapies of osteoanabolic agents, and maintain or further increase gains in BMD[130,182,183].

However, this ideal setup has not been applied yet in clinics, where most patients are typically treated first with an antiresorptive drug, then with another antiresorptive drug or an osteoanabolic agents whenever fracture risk is consistently high. Available evidence shows that the beneficial effect of teriparatide is higher in naïve patients (that never received an anti-resorptive agent before) than in those receiving the treatment following an antiresorptive therapy, suggesting that the reduced rate of bone remodeling induced by antiresorptive may be

blunting the remodeling-based gain in BMD triggered by osteoanabolic drugs[152,183]. However, the substitution of an antiresorptive therapy by an anabolic therapy appears to be overall beneficial to patients, at least regarding gain and maintenance of BMD, although the effect of this therapeutic sequence on fracture risk has yet to be evaluated[130,182,183].

1.1.3.8. Dual-action agents

Romosumab is a human monoclonal anti-sclerostin antibody, whose use was approved in USA and EU in 2019 for osteoporotic patients presenting a high risk of fracture. Sclerostin (SOST) is produced by osteocytes and serves as a master regulator of bone formation through its binding to LRP5/6 receptors and the subsequent inhibition of WNT-β-catenin canonical signaling pathway, which is paramount for osteoblast differentiation and metabolism[185]. Romosumab also increases the expression of osteoprotegerin (OPG) and consequently inhibits osteoclast differentiation[175]. Therefore, romosumab action on sclerostin promotes bone anabolic and antiresorptive effects, which is the rationale for considering romosumab as a dual-action drug.

Clinical trials have demonstrated that romosumab treatment induces a rapid increase in bone formation markers, an increase in BMD and an equally rapid decrease in bone remodeling markers[175]. A number of randomized controlled trials have highlighted the capacity of romosumab to reduce the incidence of fragility fractures to an extent comparable, if not superior, to the effect of bisphosphonates and teriparatide[152,175]. Romosumab is characterized by a short and powerful anabolic window that triggers a rapid increase in bone formation during the first months of treatment.

However, after few months, romosumab anabolic window dissipate and is substituted by a mild antiresorptive mechanism[152,175]. As such, romosumab treatment, similarly to single-action osteoanabolic drugs, needs to be followed by the treatment with antiresorptive agents[186]. Common adverse effects of romosumab include headache, arthralgia and injection site immune reactions. An increased risk of cardiovascular events such as myocardial infarction, stroke, and cardiovascular death have been associated with romosumab treatment[186]. Little is known about romosumab long-term associated side effects.

1.1.3.9. Emerging therapeutic approaches for bone disorders

Our knowledge on the molecular determinants of bone metabolism has greatly improved during the last decades, widening the spectrum of potential druggable targets to treat MBDs. Among the molecular regulators recently identified for the treatment of bone-eroding diseases,

antiresorptive agents such as H^+-ATPase suppressors and Src proto-oncogene inhibitors are promising candidates, as important factors involved in osteoclastic function[187,188].

Novel potential targets for osteoanabolic agents include intermediates of the WNT-β-catenin pathway such as DKK1, GSK-3, and SIRT1, activators of the soluble guanylate cyclase (sGC), and bone morphogenetic proteins (BMPs). Hydrogen sulphide donors (H_2S), kynurenine pathway blockers, and modulators of the osteoblast-osteoclast crosstalk (e.g. compounds impacting RANKL signaling, cell-cell interaction proteins such as semaphorins SEMA3A and SEMA4D, and sphingosine-1-phosphate) are also promising candidates for the development of next-generation dual-action drugs[187].

The identification of crosstalk in cellular signaling pathways central to bone and other tissues and organs, has opened the possibility to implement therapeutic strategies with a more holistic approach. In this regard, drugs targeting muscle, fat and blood vessels are gaining momentum in the treatment of MBDs. For example, activin receptor regulators, a fundal key. component of the extracellular matrix involved in osteoclastic differentiation is being studied in animal models[187].

Myokines, factors produced by skeletal muscles, are being described for having a control over bone metabolism and might represent druggable targets for MBDs[187]. As adipocytes and osteoblast have a common origin, drugs able to shift the equilibrium from adipogenesis to osteogenesis in mesenchymal stem cells (MSCs), such as TGFβ- and PPARγ-modulators, are also being evaluated[187].

Similarly, the existence of a crosstalk between endothelium and bone has shed some light on the possibility for angiogenesis regulators to be targeted by therapeutically approaches for MBDs. Among those, intermediates of the Notch signaling pathway and regulators of bone vascularization such as SLIT3 and SHN3 are being evaluated [187].

A crosstalk between gut microbiome and bone health have been identified and the capacity of probiotics and prebiotics to promote bone health has been evidenced[187,189].

Because oxidative stress and inflammation are important factors in the development of MBDs, antioxidant and anti-inflammatory compounds are increasingly being evaluated for their positive impact on bone health[190–192]. Finally, the interaction between bone and immune system suggests that immunostimulants may also have a beneficial effect on bone[193].

Nowadays, recent advancements in the fields of molecular biotechnologies such as gene therapy, gene silencing, and regenerative medicine, have led to the development of innovative biotechnological approaches for treating metabolic bone disorders. Among those, a recombinant RANKL-based vaccine has shown to be able to prevent osteoporosis in

ovariectomized mice[194]. An adenovirus-delivered, microRNA-based gene silencing method was able to prevent bone loss in a in mice osteoporotic model by silencing *RANK* and *CTSK* expression[195].

Stem cell transplantation technologies can also be applied to the treatment of metabolic bone disorders. In this regard, the transplantation of mesenchymal stems cells (MSCs) has shown promising results in pre-clinical studies, and clinical trials are currently being conducted in osteoporotic patients[196,197]. Mesenchymal stem cells-derived extracellular vehicles (EVs) have also drawn some attention because of their osteogenic potential[198]. Hematopoietic stem cells transplantation (HSCT), a well-established life-saving therapeutic option for malignant infantile osteopetrosis (ARO)[199], has been recently applied to the treatment of patients suffering from the less-severe autosomal dominant form of osteopetrosis (ADO)[200,201]. A combinational strategy based on the transplantation of autologous hematopoietic stem cells (aHSCT) were the disease-causing mutation was previously corrected through gene therapy delivered via lentivirus transformation have been adopted with success in an osteopetrotic mice model[202].

1.1.4. Marine natural products as alternative players in MBD therapeutic strategies

Natural products (NPs) have been central to drug discovery for millennia and are at the origin of many modern pharmaceutics. Although the use of NPs in pharmaceutical research has slow down at the early 1990s due to technical disadvantages related to a poor compatibility with high-throughput screening (HTS) approaches, recent biotechnological advances and the advent of the "omics" has placed them back in screening pipelines for novel drugs[203–206]. In addition, the diversity of the bioactivities found in NPs, but also their chemical novelty, and effectiveness in leading to the discovery of first-in-class medications (i.e. drugs that perform through novel and unique mechanisms of action), are features that have contributed to their leading role in drug discovery. As such, only 24.6% of all drugs approved by FDA in the last four decades were purely synthetic, while the remaining were either fully natural (4.6%), naturally-derived (18.9%), biological (isolated from an organism/cell line or produced in a surrogate host; 18.4%), biologically-produced vaccines (7.5%), natural product mimics or synthetic compounds whose bioactive portion is naturally-derived (25.7%)[207]. In this new era of NP-inspired drugs, the marine environment is increasingly seen as a solid source of bioactives because of its vast yet largely unexplored biodiversity in contrast to the much more explored terrestrial environment[208–210]. In fact, although terrestrial ecosystems are richer (about 4 times) than the marine ecosystems regarding the number of species, 70% of these species belong to a

single taxonomic group, the Insects[211]. Oceans have a lower species diversity, but a much larger taxon diversity that translates in a variety of morphological and physiological features and metabolites, that held a great deal of interest for biotechnological and pharmaceutical applications[212].

1.1.4.1. Animals as first-choice resources in marine pharmacology

While plants (25%) and microorganisms (13%) have historically been the major sources of natural bioactives for terrestrial-derived pharmaceutics[213], animals have been mostly used to fuel the pharmaceutical sector with natural bioactives. A comprehensive review on this topic has estimated that approximately 75% of the marine compounds were isolated from marine invertebrate animals, the major phyla being Porifera (marine sponges) with 32%, and Cnidaria (e.g. corals, jellyfishes, anemones, and sea fans) with 16%, followed by important groups such as Mollusca (mollusks) with 5%, Echinodermata (e.g. starfish, sea urchins, and sea cucumbers) with 5%, and Chordata (e.g. tunicates and vertebrates) with 4%[214]. Although they are largely unexplored and their diversity is very much unknown[215], marine microorganisms have contributed with 22-34% of all-type marine bioactives[214]. Among those, fungi, microalgae and bacteria are gaining momentum as highly promising sources of bioactive[216–219].

1.1.4.2. Marine Osteoactive Compounds (MOCs)

Compounds isolated from marine organisms hold a great potential for the treatment of MBDs[220–223]; still little research effort has been put on the discovery of marine compounds with osteoactive properties. This section will overview the literature data on the isolation of marine osteoactive compounds from 1999 to 2022. Note that only compounds with pharmacological applications will be presented here, i.e. marine-derived biomaterials with applications in bone regeneration, fracture healing and tissue engineering[224–230] will be overlooked. A total of 92 marine osteoactive compounds (MOCs) have been identified through our survey (Figure 2), of which 49 (53.3%) are anti-resorptive, 31 (33.7%) are osteoanabolic, 11 (12.0%) have a dual-action, and 1 (1.0%) are anti-osteonecrotic; they are listed in Table 1.

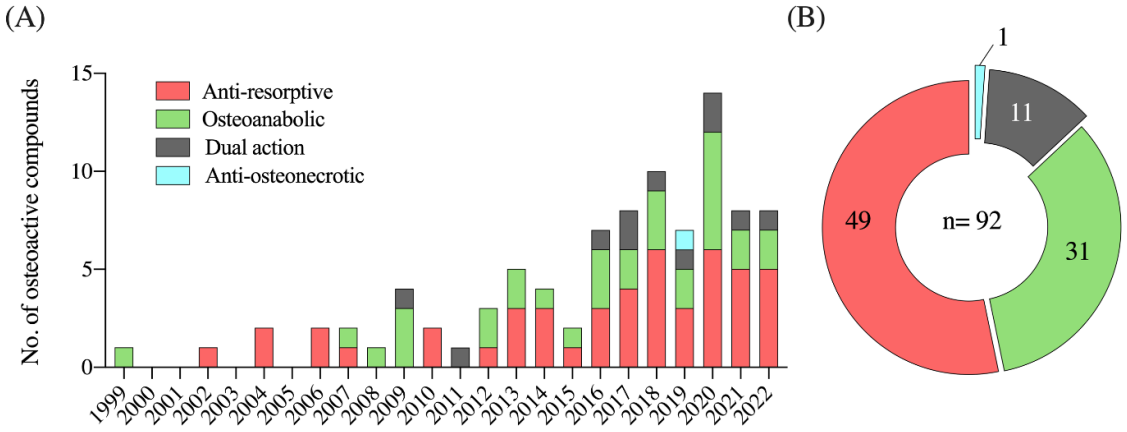


Figure 2. Survey of the literature available in Google Scholar regarding marine osteoactive compounds (MOCs) discovered since 1999 (A), and their distribution based on their mechanism of action on bone (B).

Table 1. Marine osteoactive compounds described in the period 1999-2022 that could be used to treat bone metabolopathologies.

Effect	Compound	Molecular mechanism	Source group	Model used for screening	Tested on a disease model?	Drug discovery stage	Reference
Anti-resorptive	Salinosporamide A	Suppression of RANKL induced osteoclastogenesis	Actinobacteria	Murine RAW 264.7 macrophages	Not tested	Pre-clinical	231
	Biselyngbyaside	Suppression of RANKL-induced osteoclastogenesis through c-Fos and NFATC1 inhibition; Reduction of pit formation; Stimulation of osteoclast apoptosis through induction of Caspase-3 and nuclear condensation	Cyanobacteria	Murine RAW 264.7 macrophages	Not tested	Pre-clinical	232
	Irijimaside (A-E)	Suppression of RANKL-induced osteoclastogenesis (TRAP activity)	Cyanobacteria	Murine RAW 264.7 macrophages	Not tested	Pre-clinical	233,223
	Bromo-honaucin A	Suppression of RANKL-induced osteoclastogenesis via Akt inhibition and ERK activation; Inhibition of osteoclast markers (*Ctsk*, *Mmp9, Dcstamp*); Suppression of pit formation	Cyanobacteria	Murine RAW 264.7 macrophages	Not tested	Pre-clinical	234
	Kalkitoxin	Suppression of RANKL-induced osteoclastogenesis, pit area and actin ring formation; Inhibition of osteoblast markers (*Mmp9*, *Acp5, Dcstamp,* CTSK, NFATC1, FOS); Inhibition of MAPK and AKT pathways; Prevention of bone loss, restoration of BMD and bone microarchitecture in LPS induced osteoporotic mice	Cyanobacteria	Murine bone marrow–derived macrophages	Mouse model of LPS-induced bone loss	Pre-clinical	235
	Symbioimine	Suppression of RANKL-induced osteoclastogenesis	Dinophyceae	Murine RAW 264.7 macrophages	Not tested	Pre-clinical	236
	Sulfated glucurono-rhamnoxylan polysaccharide	Suppression of RANKL-induced osteoclastogenesis; Suppressed TRAP activity, actin ring formation, and expression of MMP9, CTSK, TRAF6, GSN, CA II, ITGB3; Suppressed activation of PTK2, CBL. Increased femur and tibia BMD and OPG/RANKL ratio in OVX-mice	Ulvophyceae	Murine RAW 264.7 macrophages	OVX mouse	Pre-clinical	237
	Fucoxanthin	Suppression of RANKL-induced osteoclastogenesis; Stimulation of osteoclast apoptosis through induction of Caspase-3; Modulation of MAP Kinase and Nrf2 Signaling	Phaeophyceae	Murine RAW 264.7 macrophages; Murine MC3T3-E1 pre-osteoblasts	OVX rat	Pre-clinical	238–240
	Sargachromanol G	Suppression of RANKL-induced osteoclastogenesis; Inhibition of osteoclast markers (*Acp5*, *Ctsk*, *Mmp9*, *Calcr*); Suppression of	Phaeophyceae	Murine RAW 264.7 macrophages	Not tested	Pre-clinical	241

	RANKL-mediated IκBa degradation; Inhibition of RANKL-induced p38, JNK, and ERK phosphorylation (MAPK pathway)					
Mycoepoxydiene	Suppression of RANKL induced osteoclastogenesis; Blockage of TAK1 phosphorylation, thus inhibition of *Nfatc1* expression; Inhibition of NF-κB and ERK1/2 pathways	Sordariomycetes	Murine primary bone marrow cells	OVX mouse	Pre-clinical	242
Stachybotrysin	Suppression of RANKL induced osteoclastogenesis; Downregulation of ERK, JNK, and p38 phosphorylation	Sordariomycetes	Murine bone marrow–derived macrophages	Not tested	Pre-clinical	243
Macrolides (1, 5, 9)	Suppression of RANKL induced osteoclastogenesis differentiation	Sordariomycetes	Murine bone marrow–derived macrophages	Not tested	Pre-clinical	244
Chlovalicin	Suppression of RANKL induced osteoclastogenesis	Sordariomycetes	Murine bone marrow–derived macrophages	Not tested	Pre-clinical	245
Insulicolide A	Suppression of RANKL-induced osteoclastogenesis *in vitro* and bone resorption *in vivo*; Inhibition of IκBa phosphorylation, NF-κB, p65, and RelB nuclear translocation; Reduction of *Dcstamp* expression. Inhibition of RANKL-induced up-regulation of NFATc1, DC-STAMP but not c-Fos *in vitro*	Eurotiomycetes	Murine RAW 264.7 macrophages	Mouse model of LPS-induced osteolysis	Pre-clinical	246
6-epi-Notoamide T	Suppression of RANKL-induced osteoclastogenesis; Downregulation of osteoclasts markers (*Nfatc1*, *Acp5*, *Ctsk*, *Atp6v0d2*, *Dcstamp*, *Ocstamp*)	Eurotiomycetes	Murine RAW 264.7 macrophages	Not tested	Pre-clinical	247
Austalide V	Suppression of RANKL-induced osteoclastogenesis	Eurotiomycetes	Murine bone marrow–derived macrophages	Not tested	Pre-clinical	248
Chlorinated polyketide (2,7)	Inhibition of LPS-induced NF-κB activation in RAW cells; Inhibition of RANKL-induced osteoclastogenesis in bone marrow derived macrophages	Eurotiomycetes	Murine RAW 264.7 and bone marrow–derived macrophages	Not tested	Pre-clinical	249
Taichunins (G, K, N)	Suppression of RANKL-induced osteoclastogenesis	Eurotiomycetes	Murine RAW 264.7 macrophages	Not tested	Pre-clinical	250
Mactanamide	Suppression of RANKL-induced osteoclastogenesis	Eurotiomycetes	Murine bone marrow–derived macrophages	Not tested	Pre-clinical	251
Agelasine D	Suppression of RANKL-induced osteoclastogenesis; Inhibition of osteoclast markers (*Fos*, *Nfatc1*, *Acp5*, *Ctsk*, *Mmp9, Dcstamp*,	Porifera	Murine bone marrow–derived macrophages	Not tested	Pre-clinical	252

	Ocstamp); Inhibition of pre-osteoclast fusion; Inhibition of ERK phosphorylation and NF-κB activation					
Placotylene A	Suppression of RANKL-induced osteoclastogenesis through inhibition of NFATc1 transcription and translation	Porifera	Murine bone marrow–derived macrophages	Not tested	Pre-clinical	253
Halenaquinone	Suppression of RANKL-induced osteoclastogenesis through suppression of IκBa degradation and Akt phosphorylation	Porifera	Murine RAW 264.7 macrophages	Not tested	Pre-clinical	254
Haploscleridamine	Cathepsin K inhibitor (IC_{50} 26 μM)	Porifera	Cathepsin K inhibitory assay	Not tested	Pre-clinical	255
Ceylonamide (A, B)	Suppression of RANKL-induced osteoclastogenesis	Porifera	Murine RAW 264.7 macrophages	Not tested	Pre-clinical	256
Ceylonin A	Suppression of RANKL-induced osteoclastogenesis	Porifera	Murine RAW 264.7 macrophages	Not tested	Pre-clinical	257
Aaptamines	Suppression of RANKL-induced osteoclastogenesis	Porifera	Murine RAW 264.7 macrophages	Not tested	Pre-clinical	258
Neviotine (A, D)	Suppression of RANKL-induced osteoclastogenesis	Porifera	Murine RAW 264.7 macrophages	Not tested	Pre-clinical	259
Amakusamine	Suppression of RANKL-induced osteoclastogenesis through the inhibition of *Nfatc1*	Porifera	Murine RAW 264.7 macrophages	Not tested	Pre-clinical	260
11-Episinulariolide acetate	Anti-inflammatory activity in LPS-stimulated macrophages through inhibition of the protein expression of iNOS and COX2. Attenuation of phenotype histological and anatomical landmarks and suppression of CTSK, MMP-9, ALPL, TNF expression in mouse model of adjuvant-induced arthritis	Cnidaria	Murine RAW 264.7 macrophages	Mouse model of rheumatoid arthritis	Pre-clinical	261
Junceellolide D	Suppression of RANKL-induced osteoclastogenesis via increasing NRF2 stability and nuclear translocation; Abolished RANKL-induced generation of reactive oxygen species (ROS); Inhibition of RANKL-stimulated activation of NF-κB and MAPK signaling pathways	Cnidaria	Murine bone marrow–derived macrophages	Not tested	Pre-clinical	262
Excavatolide B	Suppression of LPS-induced osteoclastogenesis; downregulation of osteoclast markers (TRAP, *Ctsk*, *Mmp9*); Rescue of clinical and histopathological features in rat models of adjuvant-induced arthritis and collagen-induced arthritis. Inhibition of osteoclastogenesis through suppression of NFATc1 signaling in cartilage and synovial tissues	Cnidaria	Murine RAW 264.7 macrophages	Rat models of adjuvant-induced arthritis and collagen-induced arthritis	Pre-clinical	263

	Orsaldechlorin (A, B)	Inhibition of LPS-induced NF-κB activation; Suppression of RANKL-induced osteoclastogenesis	Cnidaria	Murine RAW 264.7 macrophages	Not tested	Pre-clinical	264
	Secosteroids (2, 11, 12)	Suppression of RANKL-induced osteoclastogenesis	Cnidaria	Murine bone marrow–derived macrophages	Not tested	Pre-clinical	265
	Iejimalide (A, B)	Suppression of RANKL-induced osteoclastogenesis via the inhibition of V-ATPase	Chordata	Murine bone marrow–derived macrophages	Not tested	Pre-clinical	266
Osteoanabolic	Largazole	Inhibition of histone deacetylases; Downregulation of *Bmp-2*, 4, 6, 7, and 9; Upregulation of *Runx2*, *Alp*, *Opn* in C2C12 cells. Osteogenic properties during mouse calvaria regeneration and rabbit calvaria fracture healing	Cyanobacteria	Murine C2C12 muscle myoblasts; Mouse calvaria bone formation assay; Rabbit calvaria bone fracture healing assay	Not tested	Pre-clinical	267
	Majusculamide (A, B)	Stimulation of osteoblastogenesis and ALP activity	Cyanobacteria	Murine MC3T3-E1 pre-osteoblasts	Not tested	Pre-clinical	268
	Amphirionin-5	Increase (320%) in proliferation of pre-osteoblastic cells	Dinophyceae	Murine MC3T3-E1 pre-osteoblasts	Not tested	Pre-clinical	269
	Floridoside	Stimulation of osteoblastogenesis and formation of mineralized nodules; Upregulation of *Bmp-2*, *Runx2*, *Sp7*, *Col1a1*, *Alpl*, *Bglap*, *Spp1*; Stimulation of COL1A1 production and ALP activity	Florideophyceae	Murine bone marrow mesenchymal D1 cells	Not tested	Pre-clinical	270
	Dunaliella salina-derived peptide P32 (ALVFQAQH)	Improved craniofacial skeleton development and mineralization in a zebrafish model of glucocorticoid-induced osteoporosis; Upregulation of osteoblast markers (*runx2*, *alpl*, *bglap*) and downregulation of antioxidant response markers (*cat*, *sod1*). Rescue of BMD and microarchitectural parameters in OVX-rats	Chlorophyceae	Zebrafish GIOP model	OVX rat	Pre-clinical	271
	Nannochloropsis oculata-derived tetrameric peptide	Stimulation of osteoblastogenesis; Upregulation of osteoblast markers (ALP activity, *BGLAP COL1A1*, *BMP-2*, *BMP4*); Increase of bone mineralization and phosphorylation of MAPK and SMAD pathway in both human MG-63 and murine D1 cells	Eustigmatophyceae	Human osteosarcoma MG-63 cells; Murine bone marrow mesenchymal D1 cells	Not tested	Pre-clinical	272
	Phlorotannins 1, and 2 (see reference for extended nomenclature)	Stimulation of osteoblastogenesis; Increase of ALP activity and ECM mineralization; Increased of total protein and collagen synthesis	Phaeophyceae	Human osteosarcoma MG-63 cells	Not tested	Pre-clinical	273

Dioxinodehydroeckol	Stimulation of cell proliferation, osteogenic differentiation, and ECM mineralization; Upregulation of osteoblast markers (ALP, BMP2, COL1A1, BGLAP). Stimulation of Smad, ERK, Runx2 pathways	Phaeophyceae	Murine MC3T3-E1 pre-osteoblasts	Not tested	Pre-clinical	274
Sargahydroquinoic and sargaquinoic acids	Stimulation of cell proliferation, osteogenic differentiation and upregulation of osteoblast markers in MC3T3-E1 cells. Inhibition of adipogenic differentiation, lipid accumulation and downregulation of adipocyte markers in 3T3-L1 cells	Phaeophyceae	Murine MC3T3-E1 pre-osteoblasts and 3T3-L1 fibroblasts	Not tested	Pre-clinical	275
Phlorofucofuroeckol A	Stimulation of cell proliferation, osteoblastogenesis and ECM mineralization; Upregulation of osteoblast markers (ALP, BMP-2, BGLAP) through the stimulation of Wnt/β-catenin pathway	Phaeophyceae	Human bone marrow mesenchymal stem cells	Not tested	Pre-clinical	276
Phorbaketal A	Stimulation of osteoblastogenesis through TAZ-mediated RUNX2 activation	Porifera	Murine embryonic C3H10T1/2 fibroblasts	Not tested	Pre-clinical	277
Phorbasone (A, B)	Stimulation of ECM mineralization and osteoblastogenesis; Upregulation of osteoblast markers RUNX2, ALPL, SP7, and PTH/PTHLH	Porifera	Murine embryonic C3H10T1/2 fibroblasts	Not tested	Pre-clinical	278
Aerophobin-1	Increased vertebral bodies mineralization in zebrafish larvae	Porifera	Zebrafish embryo (AB strain)	Not tested	Pre-clinical	279
Norzoanthamine (and truncated form)	Protective effect against collagen I, elastin and BSA degradation; Acceleration of hydroxyapatite crystals formation. Inhibition of nitric oxide (NO) production. Inflammation-suppressive effect via inhibiting MAPK pathway and COX-2 and iNOS expression	Cnidaria	Murine embryonic C3H10T1/2 fibroblasts and MC3T3-E1 pre-osteoblasts	OVX rat; OVX mouse	Pre-clinical	280–283
(-)-7β-hydroxy-8α-methoxydeepoxysarcophytoxide	Stimulation of osteoblastogenesis, collagen content, ALP activity, nodules formation	Cnidaria	Murine MC3T3-E1 pre-osteoblasts	Not tested	Pre-clinical	284
Sarcomilasterol	Stimulation of cell proliferation, ECM-mineralization, and upregulation of osteoblast marker ALP	Cnidaria	Murine MC3T3-E1 pre-osteoblasts	Not tested	Pre-clinical	285
Blue mussel-derived octapeptides FSVVPSPK and PIISVYWK	Stimulation of osteoblastogenesis in human cells via stimulation of Wnt/β-catenin signaling pathway; Stimulation of osteoblastogenesis and ECM mineralization via stimulation of MAPK and BMP pathways	Mollusca	Human and murine bone marrow mesenchymal stem cells;	OVX mouse	Pre-clinical	286,287

	in mice cells. Attenuated cortical bone loss and reduced bone resorption markers in OVX-mice.					
Oyster-derived octapeptide YRGDVVPK	Increased cell proliferation, osteogenic differentiation, and ECM mineralization	Mollusca	Murine MC3T3-E1 pre-osteoblasts	Not tested	Pre-clinical	288
Compound pearl protein polypeptide (CPPP)	Increased cell proliferation, osteogenic differentiation, and ECM mineralization in primary osteoblasts. Increased BMD, Increased E2 and TGF-β1serum levels, downregulation of bone resorption markers (ALP serum level, BGLAP and Ca, P urine contents) in OVX-rats	Mollusca	Rat calvaria primary osteoblasts	OVX rat	Pre-clinical	289
N-acetyl-*D*-glucosamine (NAG, Chitin-derived)	Increase cell proliferation, differentiation and ECM mineralization. Protective effect against H_2O_2-induced oxidative damage. In OVX-rats, reduction of OVX-induced weight gain and uterine coefficient; Increased Ca and ALP serum levels; Improved BMD, bone mechanical properties, tibia microarchitecture and histological features	Arthropoda	Murine MC3T3-E1 pre-osteoblasts	OVX rat	Clinical (other diseases); Pre-clinical (OP)	290
S. japonicus polysaccharide SP-2	Increased osteoblastogenesis and ECM mineralization though activation of BMP signaling pathway	Echinodermata	Murine MC3T3-E1 pre-osteoblasts	Not tested	Pre-clinical	291
Pardaxin	Upregulation of BMP-2 and downstream markers of osteoblastogenesis (*Runx2*, *Sp7*, *Bglap*, Akt and ERK phosphorylation, ALP activity); Increased ECM mineralization in vitro. Increased number of mineralized vertebral bodies and increased mineralization of cranial skeletal structures; Upregulation of RUNX2, MMP-2, SP7 in zebrafish larvae GIOP model	Chordata (Fish)	Murine MC3T3-E1 pre-osteoblasts	Zebrafish model of glucocorticoid-induced osteoporosis	Pre-clinical	292
Fish bone tripeptide (Lys-Ser-Ala)	Stimulation of cell proliferation, osteogenic differentiation, and ECM mineralization. Upregulation of osteoblast markers (BGLAP, SPP1). Stimulation of MAPK and Smad pathways via binding to BMP-2 receptors	Chordata (Fish)	Murine MC3T3-E1 pre-osteoblasts	Not tested	Pre-clinical	293
H-CS/DS-GAGs (glycosaminoglycans	Stimulation of osteoblastogenesis and ECM mineralization	Chordata (Fish)	Murine MC3T3-E1 pre-osteoblasts and calvaria primary osteoblasts	Not tested	Pre-clinical	294

	rich in chondroitin and dermatan sulfates)						
	Ciona intestinalis calcitonin-like peptide	Stimulation of cell proliferation, osteoblastic differentiation, and ECM mineralization though activation of MAPK pathway	Chordata	Murine MC3T3-E1 pre-osteoblasts	Not tested	Pre-clinical	295
Dual-action	Macrolactin F	Inhibition of RANKL-induced osteoclastogenesis, F-actin ring formation, resorption activity and suppression of the activation of Akt, JNK and p38 pathways. Downregulation of osteoclast markers. Promotion of nodule formation in MC3T3-E1 cells; Upregulation of osteoblast markers and ALP activity; Activation of Akt and Smad pathways	Actinobacteria	Murine bone marrow–derived macrophages and MC3T3-E1 pre-osteoblasts	Not tested	Pre-clinical	296
	Macrolactin A	Inhibition of RANKL-induced osteoclastogenesis, bone resorption and actin ring formation; Downregulation of osteoclast markers (CTSK, ACP5, *Mmp2*, MMP9, NFATC1, FOS) through inhibition of MAPK/Akt signaling pathway in bone marrow macrophages. Stimulation of osteoblastogenesis and ECM mineralization; Upregulation of osteoblast markers (ALP activity, RUNX2, BMP-2, SP7, SMAD4, SPP1, expression) though stimulation of MAPK/Akt pathways in pre-osteoblastic cells. Improvement of BMD, bone volume, bone histopathological features, and TRAP+ cells population in LPS-induced osteoporotic mice	Actinobacteria	Murine bone marrow–derived macrophages and MC3T3-E1 pre-osteoblasts	Mouse model of LPS-induced bone loss	Pre-clinical	297
	Fucoidan	Stimulation of osteoblastogenesis and mineralized nodule formation; Upregulation of osteoblast markers (*Alp* levels and ALP activity; BMP-2 and BGLAP expression). Suppression of RANKL-induced osteoclastogenesis; Reduced number of nuclei per osteoclast, bone resorption and downregulation of osteoclast markers (*Acp5*, *Nfatc1*, *Oscar*, *Mmp9*)	Phaeophyceae (Heterokonta)	Human osteosarcoma MG-63 cells; Murine RAW 264.7 macrophages	OVX mouse	Clinical (Other diseases); Pre-clinical (OP)	298,299
	Fucosterol	Increased osteoblast proliferation, and ECM mineralization; Upregulation of osteoblast markers (ALP activity); Decreased RANKL-induced osteoclastogenesis; Downregulation of RANK	Phaeophyceae (Heterokonta)	Human osteosarcoma MG-63 cells; Murine bone marrow–derived macrophages	OVX rat	Pre-clinical	300,301

	receptor; Increased femur BMD, improved bone microarchitecture, increased serum level of osteocalcin and decreased CTX in OVX-rats					
Diphlorethohydroxycarmalol	Suppression of RANKL-induced osteoclastogenesis via inhibition of NF-κB signaling pathway in macrophages; Protection against H_2O_2-induced oxidative cell toxicity and ROS generation; Increased ALP activity and nodules formation; Upregulation of Col1a1, Alpl, Smad1/5, Sp7, Bmp2, and Runx2 in osteoblasts	Phaeophyceae (Heterokonta)	Murine bone marrow–derived macrophages and MC3T3-E1 pre-osteoblasts	Not tested	Pre-clinical	302,303
Alginate oligosaccharide	Increased osteoblast proliferation and mRNA and protein expression of PTH1-84 and vascular endothelial growth factor (VEGF). Increased serum level of PTH1-84 and VEGF, and increased vertebral and femoral BMD in OVX rats. In D-galactose induced osteoporotic mice, increased femur BMD and enlarged trabeculae; downregulated expression of senescence biomarker p53; Inhibition of RANKL/RANK/C-Fos pathway and reduced NF-κB nuclear translocation; decreased serum levels of osteocalcin; Upregulated expression of osteoprotegerin (*Tnfrsf11b*)	Phaeophyceae (Heterokonta)	Human osteosarcoma MG-63 cells	OVX rat; Mouse model of D-galactose-induced bone loss	Clinical (cystic fibrosis); Pre-clinical (OP)	304
Astaxanthin	Suppression of RANKL-induced osteoclastogenesis; downregulation of osteoclast markers (*Nfatc1*, *Acp*5, *Ctsk*, *Dcstamp*) in macrophages. Increased proliferation and osteoblastogenesis in MG-63 cells via aryl hydrocarbon receptor (AhR) pathway; increased expression of osteoblast markers (*CYP1A1*, *BGLAP*, *SPP1*, *COL1A1*, and *RUNX2*). Increased osteoblastogenesis and ECM mineralization through fatty acid metabolism regulation in bone marrow cells. Suppressed serum levels of bone resorption markers (calcium, inorganic phosphorus, alkaline phosphatase, TRAP activity); increased BMD and bone microarchitecture of trabecular bone in tibia and femur in OVX mice	Chlorophyceae (Chlorophyta)	Mouse bone marrow–derived macrophages; Human osteosarcoma MG-63 cells; Rat primary bone marrow mesenchymal stem cells	OVX mouse	Clinical (osteoarthritis, joint inflammation, others)	305–307
Austalide K	Suppression of RANKL-induced osteoclastogenesis in macrophages; Reduction of NFATc1 expression at protein and gene level; Increase in BMP-2-induced osteoblastogenesis in myoblasts; Increased expression of osteogenesis related genes (*Runx2*, *Bglap*, *Spp1*)	Eurotiomycetes (Ascomycota) – Free living	Mouse bone marrow–derived macrophages and mouse C2C12 muscle myoblasts	Mouse model of LPS-induced inflammatory osteolysis	Pre-clinical	308

	Hymenialdisine	Inhibition of RANKL-induced osteoclastogenesis, bone resorption, and osteoclast differentiation markers by blocking NF-κB and MAPK signaling pathways, and NFATc1 expression. Stimulation of osteoblastogenesis and ECM mineralization by activation of the glycogen synthase kinase 3β (GSK-3β)/β-catenin/T-cell factor (TCF)/lymphoid enhancer factor (LEF) signaling pathway, upregulation of *Runx-2*, *Col1a1*, and *Bglap*, and ALP activity	Porifera	Mouse bone marrow–derived macrophages, RAW 264.7 macrophages and MC3T3-E1 pre-osteoblasts	OVX-Mice (phenotype prevented)	Pre-clinical	309
	Blue mussel-derived dodecapeptide (IEELEEELEAER)	Stimulation of osteoblast proliferation and differentiation (ALP activity) and reduction of RANKL-induced osteoclastogenesis (TRAP activity) in mouse cells. Prevention of cortical bone loss in OVX mice	Mollusca	Moue MC3T3-E1 pre-osteoblasts and RAW 264.7 macrophages	OVX mouse	Pre-clinical	310,311
	Compound amino acid-chelated calcium (CAA-Ca)	Increased BMD and bone calcium contend; Ameliorated bone architectural features; Reduced serum and bone tissue levels of bone resorption markers. Increased expression of Wnt signaling pathway	Mollusca	-	OVX rat	Pre-clinical	312
Anti-osteonecrotic	Polydeoxyribonucleotide	Resolved bone necrosis; increased bone vascularization; increased osteoclast population and bone remodeling, Increased bone volume and ameliorated bone microarchitectural features	Chordata (Fish)	-	Rat model of bisphosphonate-related osteonecrosis of the jaw	Clinical (scars, ulcers, sclerotic diseases)	313

Our survey revealed an overall scarcity of studies, with only 81 articles published between 1999-2022 about the isolation of new MOCs. However, the last two decades have seen a steadily increase in these studies (Figure 2A), which is in agreement with the overall increment of all-type marine bioactives reported by previous authors[208]. As such, a much larger research effort on the discovery of osteoactive compounds from marine organism is expected in the upcoming years. The distribution of MOCs on the basis of the taxonomic group they were isolated from is shown in Figure 3.

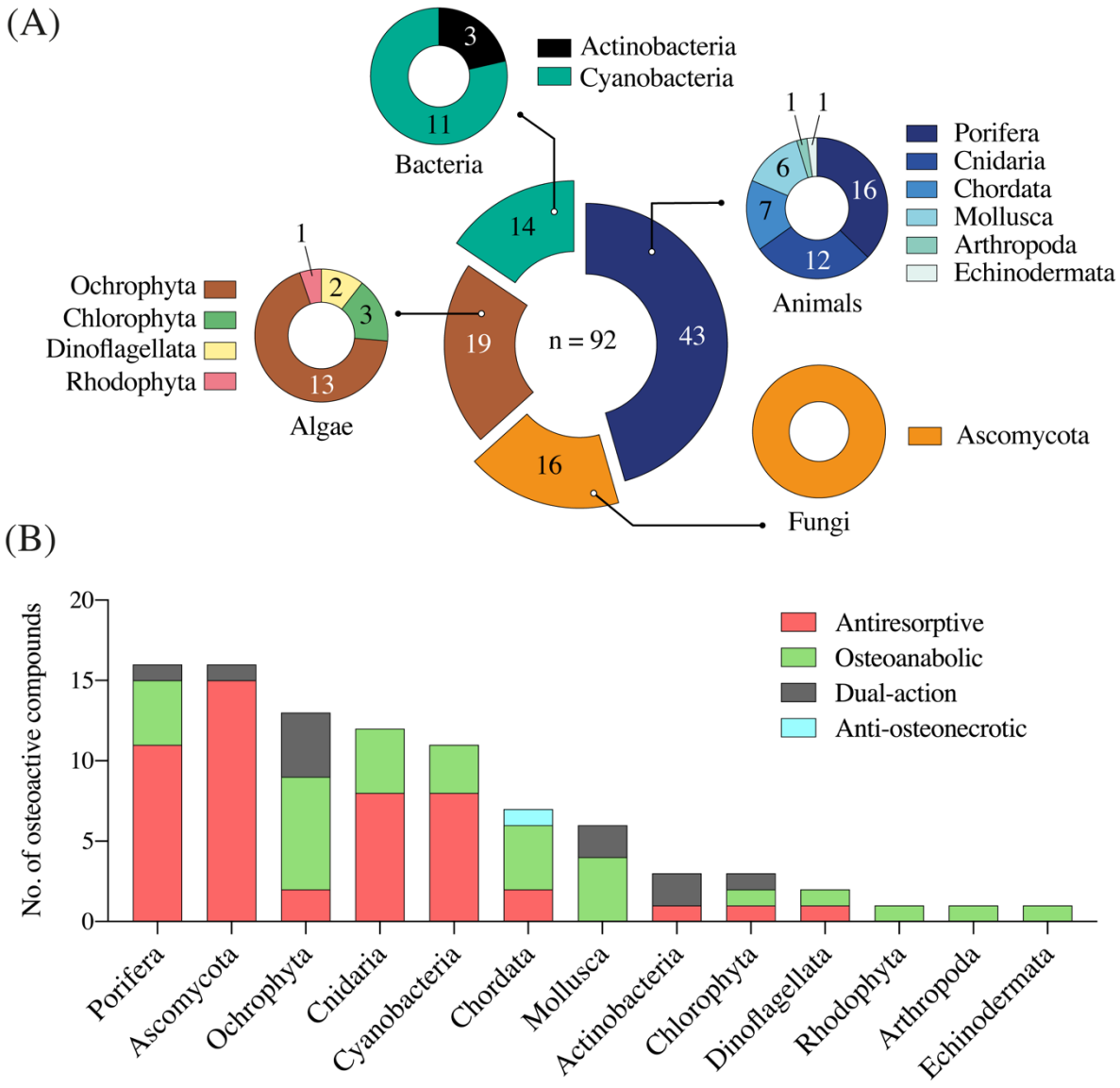


Figure 3. Taxonomic distribution of the species at the origin of the marine osteoactive compounds (MOCs) reported in the literature from 1999 to 2022 (A) and the number and type of MOCs described by Phylum.

Animals (43 compounds), and in particular invertebrates, are the most prolific group of organisms in terms of marine osteoactive compounds (Figure 3A), followed by different algal phyla (19). Fungi (16) and Bacteria (14) similarly contributed to MOC isolation. Beside animals (47%) and microorganisms (39%), large pluricellular brown algae (Ochrophyta, Phaeophyceae) also significantly contributed (14%) to MOC isolation.

No osteoactive compounds have been yet isolated from marine plants. These data, although limited to a reduced set of compounds, provide a proof of concept that marine organisms are valuable sources of natural bioactives for marine pharmacology.

The distribution of MOCs at Phylum level (Figure 3B) revealed that marine sponges (Porifera) and fungi (Ascomycota) provided the highest number of bioactives (16, 17.4%), followed by brown macroalgae (Ochrophyta, 13, 14.1%), corals (Cnidaria, 12, 13.0%), and cyanobacteria (11, 12.0%), followed by Chordata (7, 7.6%) and Mollusca (6, 6.5%). Algal phyla to which belong dinoflagellates, green- and red algae (Dinoflagellata, Chlorophyta, Rhodophyta), and crustaceans (Arthropoda) only accounted for 3% of the remaining MOCs. Interestingly, MOC distribution resemble the tendency previously described for all-type marine bioactives[214], an indication that a similar sampling effort was directed towards these groups. Also of interest, 8 of the fungi related MOCs were isolated from species that live in close symbiotic relationships with marine sponges (4), corals (3) and mangroves (1).

1.1.5. Future perspectives

1.1.5.1. Underexplored groups as promising sources of MOCs

There is increasing evidence that several groups of marine organisms underrepresented in the current screening scenario hold a high potential for the isolation of pharmacologically relevant osteoactives. In this regard, algae biodiversity – estimated to be from 70.000 to 1 million species[314] – has been largely underexplored although it hosts great promises for bioactive compounds[315]. A large number of yet unidentified compounds have been isolated from different groups of marine algae, including some with osteoactive potential, making them an untapped source of possible future drugs.

Among these, commercial food supplement and mineral-rich extracts derived from the red coralline algae *Lithothamnion* spp. showed pro-mineralogenic and anti-inflammatory activity *in vitro*[316,317], while improving osteoporotic phenotype in vivo[318,319]. Extracts from green (*Codium fragile*, *Cladophora rupestris*)[221] and red (*Plocamium cartilagineum* and *Ceramium secundatum*)[320,321] macroalgae also showed pro-mineralogenic activity in fish

osteochondroprogenitor cells and pro-osteogenic activity in zebrafish assays. Other red (*Dichotomaria obtusata*) and brown (*Padina pavonica*) macroalgae have shown pro-osteoblastogenic potential when tested in bone mice marrow mesenchymal stem cells[322] and human primary osteoblasts[323]. Fermentation-derived peptides from the microalga *Pavlova lutheri* showed pro-osteoblastogenic properties in human osteosarcoma cells[324]. Recently, calcium-chelating peptides derived from several species of marine microalgae showed to be able to rescue the osteoporotic phenotype in a zebrafish GIOP model[325].

Beside the promising results obtained in the screening of MOCs, marine algae have the advantage of a well-established and technologically advanced culture industry supporting the large-scale production of algal biomass. Algae and in particular microscopic algae (e.g. microalgae), have been long cultivated for nutritional, biotechnological, and industrial applications and are being used for the production of food, dietary supplements, cosmetics, pharmaceuticals, biofuel, fertilizers, and are implemented in the treatments of wastewaters[326]. Following important biotechnological advancements that improved growth conditions and allowed the establishment of genetically modified strains optimized for growth and compound biosynthesis[327,328], microalgae is expected to become more relevant species for marine pharmacology in the following years.

Beside algae, marine invertebrates have proven to also be promising sources of osteoactive compounds. Among those, mollusks form a highly diverse group of marine organisms and have evolved in a myriad of forms occupying a variety of ecological niches[329]. Different groups of mollusks have sourced a large variety of osteoactive compounds during the latest years.

Among those, bivalves (Bivalvia), such as mussels, oysters, clams and scallops, have originated peptides, polysaccharides, and glycoproteins with antioxidant and anti-inflammatory activity, and lipids and polyunsaturated fatty acids with strong anti-inflammatory and anti-arthritic properties[330]. Both osteoanabolic[310] and anti-resorptive[331] compounds have also been isolated from various bivalves species. The most known of these compounds is probably the mother of pearl, also known as nacre, a hard, iridescent, and composite material present in bivalve inner shell and composed of an organic proteinaceous matrix cementing an inorganic aragonitic component. Beside its long known pro-mineralogenic properties[332–334]. nacre is also a "dual action" agent with osteo-inductive properties towards mesenchymal stem cells[335,336], and anti-resorptive activity through the inhibition of cathepsin K[337]. The potential of nacre as biomaterial for bone graft and tissue regeneration has also raised a strong interest for its use in medical devices[338–340,341] studies. Fermented extracts of the oyster *Crassostrea gigas* have also a dual-action activity, stimulating osteogenic differentiation via WNT and IGF

pathways[342,343] and suppressing osteoclast differentiation, thus preventing OVX-induced bone loss in mice[344]. Aqueous extracts from the bivalve *Pisidium coreanum* showed anti-osteoclastogenic activity and were able to rescue osteoporosis in ovariectomized mice[345].

Among the gastropods (Gastropoda), which account for the vast majority of mollusk biodiversity[329], sea snails and slugs have provided a plethora of bioactive compounds. Among those, methanolic extracts of the brown dwarf turban (*Turbo brunneus*) prevented bone loss in ovariectomized mice[346,347], intestinal digests of abalone (*Haliotis discus*) stimulated MAPK-mediated osteoblastic differentiation[348] and methanolic extracts of the sea snail *Euchelus asper* improved the osteoporotic phenotype of ovariectomized mice[349].

Sea urchins (Echinoidea), starfish (Asteroidea) and sea cucumbers (Holothuroidea) are echinoderms (Echinodermata) at the origin of about 5% of all-type marine bioactives described so far[214,350,351]. Several osteoactive extracts are being derived from echinoderms. Among these, polyhydroxylated naphthoquinones extracted from the sea urchin *Evechinus chloroticus* were able to increase ECM mineralization in human osteosarcoma cells when administered together with calcium chloride, while decreasing it when administered alone[352]. Sea cucumbers hold a great deal of potential with both osteoanabolic[353,354] and antiresorptive[355,356] compounds identified.

Among chordates (Chordata), tunicates and in particular sea squirts (Ascidiacea) are source of natural bioactives with anticancer, antimicrobial, and antioxidant activities, some of which are being currently evaluated in clinical trials[357]. Similar to marine sponges, ascidians are benthic filter-feeders that have evolved a rich set of symbiotic relationships with microorganism. As a result, many bioactives isolated from ascidians are synthetized by their associated microbiome[358]. Several compounds with osteoactive properties have being isolated from ascidians[260,295,359] and recently, extracts with anti-oxidant and anti-inflammatory activities have shown pro-osteogenic properties[360].

In vertebrates, bone-derived gelatin from the Saffron cod (*Eleginus gracilis*) and skin-derived gelatin from the blue shark (*Prionace glauca*) have shown protective properties against bone loss in ovariectomized rats[361–363], while bone powder from tuna (*Thunnus* spp.) could reduce bone loss in a GIOP mouse model though the coregulation of NF-κB and Wnt/β-catenin pathways and modulation of gut microbiota composition and metabolism[364].

Despite being the second largest and most biodiverse group of marine animals, crustaceans have been largely underexplored in relation to bioactive compounds[329]. Chitin and chitosan are polysaccharides of the exoskeleton that have received most attention for their properties as biopolymers, but also for their bioactivities as antimicrobial, antioxidant, antitumoral,

anticoagulant, antidiabetic, and wound healing agents. Interestingly, chitosan and chitosan-derivates find application as naturally-derived scaffolds in bone tissue engineering thanks to their pro-osteogenic and antimicrobial activities as well as other suitable properties as biopolymers[365,366].

Finally, dichloromethane and ethanolic extracts of halophyte plants *Salicornia herbacea* and *Spergularia marina*, respectively, were reported to have anti-adipogenic and pro-osteoblastogenic activities in vitro[367,368]. Recently, polyphenols-rich extracts of *Spartina alterniflora* and *Salicornia fragilis* were found to have pro-mineralogenic activity in fish osteochondroprogenitor cells and pro-osteogenic activity in zebrafish[369].

1.1.5.2. The availability of animal models and screening tools is not fully exploited

The global interest for these underexplored marine organisms as a source of osteoactive compounds has steadily increased in the last two decades following the demonstration that they produce osteoanabolic and antiresorptive compounds. However, the discovery of novel MOCs is only achievable through a coordinated effort that should aim at the fractionation of the extracts, isolation and identification of the osteoactive compounds, together with the validation of their biological activity and the elucidation of their mechanisms of action. In this aspect, animal models are increasingly available for compound validation, thanks to the advancement in animal genetic manipulation technologies[370,371].

Notwithstanding, this availability is not fully exploited yet, as only 28.2% of the compounds listed here were validated in an animal model of bone metabolopathologies (Figure 4), while the vast majority, i.e. 71.8%, were only tested *in vitro*, mainly using rodent cell lines. Of the compounds that were validated using *in vivo* disease models, 23 were tested in animal models of osteoporosis, 3 were tested in mouse models of arthritis, and 1 was tested in a model of bisphosphonate-related osteonecrosis of the jaw (BRONJ). None were tested in animal models of VD-deficiency, hyperparathyroidism, Paget's disease of bone, or osteopetrosis. Of the compounds tested in animal models of osteoporosis, 17 were tested in rodent models of ovariectomy-induced osteoporosis, 4 were tested in mouse models of LPS-induced bone loss, 1 in a mouse model of D-galactose-induced osteoporosis, and 1 in a zebrafish model of glucocorticoid-induced osteoporosis (GIOP).

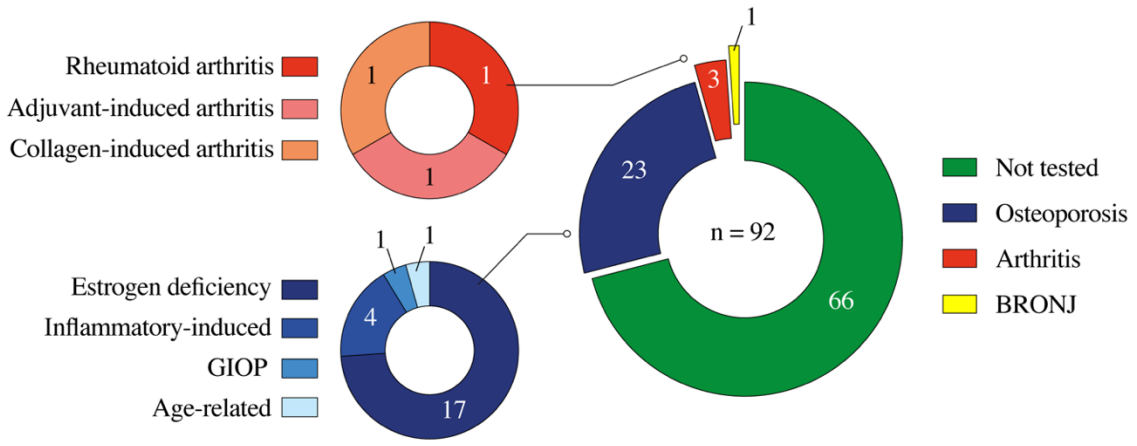


Figure 4. Distribution of marine osteoactive compounds (MOCs) based on the animal disease model they have been validated on. BRONJ (bisphosphonate-related osteonecrosis of the jaw); GIOP (glucocorticoid-induced osteoporosis).

In this context, rodents and in particular the mouse, are the most used animal models in biomedical research due to their genetic similarity with humans, small size, short lifespan and relatively low maintenance cost compared to other mammalian models[372,373]. A large variety of mouse models mimicking skeletal disorders are available. The ovariectomized (OVX) rat and mouse, aim at resembling mechanistically the pathophysiology of postmenopausal osteoporosis and are considered gold-standard *in vivo* models to validate the efficacy of compounds and drugs with anti-osteoporotic potential[374,375]. Mouse models that resemble age-related osteoporosis[376,377], male senile osteoporosis[374,378,379], and GIOP[380,381] are also available to researchers but none of these models have yet been implemented to evaluate the efficacy of emerging MOCs. A rat model of bisphosphonate-related osteonecrosis of the jaw (BRONJ)[382], has been successfully used to validate the anti-necrotic potential of a salmon sperm-derived polydeoxyribonucleotide[313]. Great achievements have also been obtained in the modelling of disorders of mineral homeostasis, including vitamin D deficiency[383–386], primary hyperparathyroidism[387–389], and renal osteodystrophy[390–392] using rodents. Models have also been developed for bone genetic disorders such as PDB[393–395] and osteopetrosis[65,396].
However, rodent models have technical disadvantages that limit the high-throughput of screening pipelines for drug development. When compared to fish and invertebrate models, rodent systems bring the complexity and the genetic proximity that better resemble human, but are also costlier and more time-consuming. As such, they may be more suitable as compound

validation tools, rather than for early screening. Teleost fish and in particular the Zebrafish (*Danio rerio*) and the Japanese medaka (*Oryzias latipes*) are becoming extremely relevant in bone research and modelling of human skeletal diseases[397,398]. These small teleosts offer several technical advantages that make them particularly suitable models for drug screening such as smaller size, cost-effectiveness, shorter life-spam and higher fecundity when compared to mammalian models.

Moreover, the translucency of the embryonic stages of development and the easier and cheaper amenability to gene delivery and genetic engineering systems allowed for the production of a vast array of transgenic and mutant lines that can be used for *in vivo*-cell tracking and disease modelling[399].

Furthermore, teleost ability to regenerate bone and cartilage tissues offer a different approach for evaluating the osteoactivity of drugs and compounds[400]. As such, a large numbers of drug screening tools have been developed in the latest years based on teleost fish[401,402], offering a cost-effective, medium- and high-throughput alternative to mammalian based-systems and at the same time providing a level of biological complexity which cannot be yet achieved by *in vitro* systems.

In addition, several zebrafish and medaka models of human bone disorders are currently available, including osteoporosis[403], osteomalacia[404], osteopetrosis[405], and PDB[406,407]. It is expected, and encouraged, that in the coming years the research community working in the field of marine osteoactive compounds will fill the gap in terms of exploitation of *in vivo* screening and validation tools available.

1.1.6. Conclusions

Metabolic bone disorders and fragility fractures are major cause of suffering and morbidity for human beings, as well as a tremendous sink of resources for the global health systems. Advances in pharmaceutical research have provided an array of therapeutic options that in some cases are characterized by a good efficacy. However, for a large number of bone metabolopathologies there is an unmet need of effective drugs, and all the available therapies have been associated to undesired side effects. Oceans have recently acquired a critical role in pharmaceutical research and drug discovery and may offer a potential solution to solve the lack of suitable drugs. Marine organisms are being more and more studied as a source of osteoactive compounds and some with highly promising bioactivities have already been described. Here, we have reviewed all the marine osteoactive compounds identified and isolated so far. The

taxonomic distribution of marine organism as sources of osteoactive compounds widely resemble the one for all-type compounds, reported by previous authors, with marine invertebrates such as sponges and cnidarian, as well as microorganism including fungi and cyanobacteria being the dominant sources. However, several relatively underexplored groups of marine organisms are demonstrating a great pharmaceutical potential. These includes marine microalgae, for which a growing technological proficiency in culture technologies is expected to expand their future role in pharmaceutical research, but also other groups such as mollusks, holothurians, ascidians and fish. To achieve these goals, a cooperative effort between the chemical characterization of marine-derived compounds and the exploitation of drug screening and validation tools currently available will be necessary.

1.2. MARINE OSTEOACTIVES AS A NUTRITIONAL SUPPLEMENT IN AQUACULTURE

1.2.1. Skeletal anomalies in aquaculture fish: The nutritional aspect

The aquaculture industry, with a global sales value of nearly 260 billion USD, currently represent the major source of fishery products worldwide, having progressively surpassed the extractive fishery in the last decades. Despite the fact that it has been facing a slow-down on annual growth during the first two decades of the third millennium (5.8 and 4.5%, respectively) in comparison with the highs of the 80s and 90s of the last century (10.8 and 9.5%, respectively), it represents a major and fundamental component of the global food supply machinery[408]. Farming of finfish, produced both inland and in marine aquaculture, represent the greater portion (nearly 53%) of this global annual production[408].

The omnipresence of skeletal anomalies in all farmed fish species represent a worldwide burden for the aquaculture industry due to the significant economic losses and animal welfare issues which are double-bounded to it. Several types of skeletal malformations reduce the survival of fish, increase sensitivity to infections, minimize growth performances and are linked with poor health condition[409]. Moreover, the sorting effort and time required to remove malformed animals from the production, slows down the industrial pipeline, increases technical tasks and labour, and risks sorting mistakes in which malformed fish arrive to the costumers thus affecting the perception of the industry and product quality[410].

Skeletal anomalies in farmed fish are a very complex problem due to their intrinsic variable nature. They are the result of the interaction across many causative factors that act synergistically. Different anomalies are present in different species under different rearing conditions and their incidence changes not only among farms and hatcheries, but even within the same batch of eggs reared at different times and/or locations. Calculating the economic damage to the industry is quite challenging, however it has been estimated in 2008 that skeletal anomalies cost the EU approximately 50.000.000 € per year, but it is probably an underestimation of the true value[409].

Skeletal anomalies can affect any part of the skeleton during any life stage[410]. The different types of skeletal anomalies have been extensively described in a previous review[409]. They can be either based on the severity of the malformation or the area of the skeleton affected as well as in which stage of the lifecycle they typically arise. There are several types of anomalies: early developmental anomalies typically affecting the notochord and the primordial fin-fold,

the swim bladder and the yolk-sac, which except for the notochord are not components of the skeletal system; vertebral column anomalies such as severe curvature, dislocation, shortening and twisting of the spine like lordosis, kyphosis and scoliosis; single vertebrae anomalies affecting one or more vertebrae or their articulating elements such as vertebral fusions; anomalies of the fins elements and anomalies of the cranial bones. Many factors have been identified to be related with the development of skeletal anomalies including genetic, gamete quality, aging, rearing conditions such as temperature, salinity, photoperiod, O_2/CO_2 levels, stock density, mechanical stress, oil film on the water surface and even specific tank characteristics (e.g. tank colour and dimensions)[409]. The lack of knowledge regarding the species-specific and life stage-specific nutritional requirements of the different species reared in aquaculture has been recognized as one of the main gaps in knowledge which has led to a high incidence of skeletal deformities, together with the genetic background and improper rearing conditions[409].

Beyond doubt, larval nutrition is one of the key factors which affect the development of the skeleton and therefore plays a role on the onset of skeletal anomalies[411,412]. Broodstock nutrition on the other hand, is known to affect the biochemical composition of the eggs, sperms and consequently of the future larvae[413–415]. The nutrients accumulated in the egg are the unique source of energy and biomolecules for the embryo until its mouth opens, therefore parental nutrition determines the batch quality in terms of hatching rates, survival of the embryos and affect the incidence of larval malformations, as reported by several previous studies[416–419].

Considering that many of the events which will determine the onset of skeletal anomalies in the later stages of the life cycle actually take place during early development, parental nutrition is very likely to strongly affect the processes involved in the onset of skeletal anomalies. Very few studies evaluated the impact of broodstock nutrition on the incidence of skeletal anomalies of the offspring[420] and this aspect needs to be subject of further attention by research in aquaculture nutrition.

Different classes of nutrients have been identified to be necessary for a correct skeletal development in fish. Among those fatty acids, of which the most studied are poly-unsaturated fatty acids (PUFAs)[409,412]. Teleost, and marine species in particular, have a high nutritional requirement of these compounds due to their incapacity to biosynthesize them from their metabolic precursors. Lack of some essential fatty acids such as eicosapentaenoic acid (EPA, 20:5 n-3), docosahexaenoic acid (DHA, 22:6 n-3) and arachidonic acid (ARA, 20:4 n-6), causes severe impairment of the embryogenesis and induce skeletal anomalies[409].

Phospholipids are another fundamental group of nutrients necessary for a correct skeletal development, which fish larvae have low capacity to biosynthesize and therefore need to be provided through the diet[409,421,422]. Lipid oxidation, autocatalytic processes that can occur *in vivo* as well as in the lipidic fraction of the ingredients used for the preparation of fish diets, have been directly linked to high incidence of skeletal anomalies due to their capacity to induce oxidative stress in the bone[423,424].

Other nutrients, if present in unbalanced amount in fish diets, will lead to the rise of skeletal anomalies. Water-soluble vitamins (vitamin B_3 and L-ascorbic acid) have an important role in the correct development of the skeletal system[425,426]. Among the liposoluble vitamins, vitamin A is fundamental for many processes including bone development and morphogenesis. However an excessive dosage of vitamin A (hypervitaminosis A) is typically associated with high rates of skeletal anomalies in fish[426,427]. Vitamin D, similarly to mammals, is essential for calcium and phosphate homeostasis in fish and its deficiency can cause a variety of skeletal problems[426,428]. Vitamin K, beyond being involved in the process of blood coagulation, is essential for the metabolism of the "vitamin K-dependent proteins" present in the bone matrix[429,430]. Vitamin E carries out an important function in protecting the organism from oxidative stress, and therefore is fundamental for a correct skeletogenesis[431].

Among the minerals, the most studied for their effect on skeletal health in fish is phosphorus. This nutrient need to be provided through the diet since both freshwater and seawater contain an insufficient amount of this structural component of the bone matrix[431]. Phosphorous deficiency leads to an increased incidence of skeletal anomalies and even the total lack of mineralized bone tissue has been reported due to phosphorus deficiency, despite this condition was completely reversible by posterior phosphorus supplementation[432]. Calcium, beyond its structural function for bone, serves as cofactor for many enzymes and is fundamental for blood clotting, neuronal transmission, muscle function and osmoregulation[431]. However, calcium deficiency is rather rare in aquaculture fish, due to its abundance in both fresh- and marine waters. Thanks to teleost capacity to internalize calcium via branchial absorption, the role of bone as a calcium reservoir is less prominent in fish than in terrestrial animals such as mammals. Magnesium supplementation appear to be necessary only in freshwater fish, for which a deficiency has been linked to increased incidence of skeletal anomalies[431].

Some trace elements such as manganese, zinc and selenium are necessary for a proper skeletogenesis and even though their daily requirement is very low, a planned supplementation might be necessary[433].

An important factor limiting the advancement of the aquaculture industry is represented by larval nutrition. Although the life cycle has been successfully closed for many species of both freshwater and marine teleosts, larval feeding still represents the main bottleneck for production, being the cause of the generally low larval survival rates reported. In fact, first feeding for most of the species is still based, due to their technical advantages, on few groups of life zooplanktonic species such as rotifers (*Brachionus* spp.) and the brachiopod *Artemia* spp. However, it is an accepted fact that these organisms have a sub-optimal nutritional profile for most of the species reared in aquaculture. As such, they frequently need to be enriched in proteins, fatty acids and vitamins by using either synthetic emulsions or natural sources, such as microalgae[412]. However, enrichment efficacy is limited by the capacity of those organisms to metabolize nutrients[412]. Furthermore, the use of rotifers and *Artemia* spp. for fish larvae feeding is limited to few species, as they are too big for the mouth gapes of the larvae of many marine teleosts[434].

Copepods are the main natural food for wild fish larvae and their nutritional profile is generally considered excellent. Moreover, their life cycle, which include many different naupliar stages, spans a wide range of sizes and provides the perfect fit for the needs of the ongrowing larvae of different fish species[435,436]. Nevertheless, the use copepods as life preys in aquaculture is still sporadic, due to many technical issues related to the scale-up of copepods culture to the industrial level. Long life cycles and generation time, together with the need of culturing copepods at low densities in order to avoid cannibalism, and low fecundity which arise with overcrowding, are the main barrier for the large-scale culture of copepods[435]. The harvest of copepods from the wild on the other hand is strongly discouraged, due to the risks of infection from viruses and other fish parasites, which this practice involves[435].

Recently, a great improvement has been done on the design and implementation of formulated feeds for larval stages, commonly referred to as microdiets. Their use could reduce the need for life prey organisms, which require an intensive culture effort, and would allow to design species-specific formulations with highly controlled balance of nutrients. Up to now, promising results have been achieved at experimental scale following a co-feeding strategy with live prey and microdiets[437–439].

However, a complete replacement of life feed with microdiets with positive result were only reported few times for commercial species[412,440,441]. A recent work carried out with the model species *Danio rerio*, reported the reduction of the incidence of skeletal anomalies and increased growth and reproductive performances[442]. A significant research effort is paramount to improve our knowledge of the nutritional requirements of the different species reared in

aquaculture, in order to find a balance between growth and health, which translate into a low rate of skeletal anomalies[409,412]. In this sense, dietary supplementation and the use of nutraceuticals in aquaculture is becoming an important strategy to obtain better growth performances and ameliorate fish health conditions.

1.2.2. Marine sources of osteoactives for aquaculture nutrition

To date, most studies conducted in the scope of nutritional supplementation for aquaculture fish focused on the establishment of the correct dosage for specific nutrients in formulated diets. A great attention of research in this direction is drawn by the use of alternative ingredients to be used as protein and lipid sources. Plant-derived proteins and lipids are being long-time used to substitute fishmeal and fish oil, with the aim of increasing the sustainability of the aquaculture industry[443,444]. However, plant-based ingredients have frequently demonstrated to contain anti-nutritional compounds that hamper fish growth and health[445]. As a results, animal- and new plant-derived alternative ingredients are actively sought by research in aquaculture nutrition[444]. Other than that, compounds with bioactive properties are mainly used as immunostimulants. These includes probiotics, prebiotics and various plant by-products[446–449]. Very little have been done regarding the implementation of molecules with "osteoprotective" properties. Recent works showed how the supplementation of prebiotics promote the biomineralization and calcification of the skeleton in zebrafish[450,451].

In this context, among the most promising novel sources of nutrients and nutraceuticals for aquaculture there are marine microalgae. Being characterized by a well-established and technologically advanced production industry, microalgae biomass is considered a sustainable alternative source of ingredients for fish feeds[452]. In addition, microalgae have demonstrated to be a source of high quality nutrients for fish, including proteins[452], lipids[453], pigments[454], and have also been implemented as immunostimulants[455]. Most recently, microalgae extracts have been described to possess anti-bacterial properties in the model species *Danio rerio*[456].

Surprisingly, and despite the recently acquired importance of marine algae as a source of osteoactive compounds in drug discovery, very little effort has been dedicated to the search for compounds with osteoprotective properties for aquaculture uses.

In recent works, whole extracts from green macroalgae (*Codium fragile*, *Cladophora rupestris*) and red macroalgae (*Plocamium cartilagineum* and *Ceramium secundatum*) were able to stimulate mineralogenic activity in gilthead seabream-derived bone cell lines and showed osteogenic activity tested with *in vivo* zebrafish models[320,457].

The potential of microalgae biomass and microalgae-derived compounds as nutraceutical for aquaculture is just starting to emerge and is expected to become more and more relevant with the advancement of cultivation technologies.

1.3. PROJECT ALIGNMENT IN THE FRAMEWORK OF PHARMACEUTICAL RESEARCH

1.3.1. Screening approaches to drug discovery

When looking for bioactive compounds in the field of pharmaceutical research, two main "schools" have historically been dominating the process of drug discovery, these are defined as Forward Pharmacology (also known as "classical pharmacology") and Reverse Pharmacology. Forward Pharmacology adopts for the screening of potential drugs, an approach called phenotypic drug discovery (PDD). With this approach, compounds are tested in complex biological *in vivo* and *in vitro* models and the physiological response are monitored without, first, making any assumptions regarding the molecular mechanisms of action underlying the biological activity.

The potential of a compound to be used as drug will depend on its capacity of inducing or recovering from a desirable/pathological phenotype. Only later the research will focus on the understanding of the mechanism involved[458–460]. On the contrary, in Reverse Pharmacology is used an approach called target-based drug discovery (TDD) that relies on bioassays in which an isolated molecular target (such as an enzyme) is exposed to big libraries of compounds looking for the ones able to bind, activate or inhibit specific functions and pathways. This highly simplified tests are designed to give a direct insight on the predicted mechanisms of action of the compound, but they need to be further confirmed with *in vitro* and *in vivo* models. The main advantage of this method is that it allows to apply molecular knowledge to investigate a mechanism that has been hypothesized.

Phenotypic screening used to be historically the major approach in drug development but with the advances in genetic and molecular biology of the last decades, the target-based approach has become more popular thanks to the development of high-throughput screening (HTS) assays that take advantages of computational pharmacology, analytical chemistry and metabolomics which made available libraries of new biomolecule to test. Moreover, the knowledge of entire genomes sequenced allowed to gather an enormous set of information regarding genes involved and pathways.

Nowadays, robotics and artificial intelligence allow companies to conduct millions of highly standardized and automated assays in a short time[459]. Despite those advances, the pharmaceutical industry is facing a slow-down in the development of new medications, especially for the very appreciated "first-in-class" drugs, the ones acting through a newly discovered mechanism of action. Recently, phenotypic screening is making a return to drug discovery due to the idea, supported by recent studies[460,461], that the target-based approach can limit the discovery of novel compounds. Moreover, target-based screening is typically based on assays that are not able to fully reproduce the mechanisms involved in diseases and most of the hits generated with this approach are then rejected when the compound is tested with *in vivo* systems[459,462,463].

Phenotypic screening on the other side, when conducted on whole-animal models, gives an idea of the overall physiological effect of a molecule and is more reliable to predict toxicity. It can discover drugs which act on previously unknown or on different targets simultaneously[464]. This may explain why despite the target-based approach is more common, phenotypic approaches are responsible for the discovery of most of the first-in-class drugs recently discovered[460]. Furthermore, some recent advantages in the fields of genomics, transcriptomics and chemical proteomics allow a more successful identification of the mechanisms following the phenotypical screening provide a support to the phenotypic approach.

1.3.2. The role of academia and public institutions in drug development

Drug development is a time-consuming process by which newly discovered molecules are characterized and marketed as medications. The path leading to the approval of a new drug is complex, starting generally with the first *screening* of compounds that exhibit an interesting bioactivity. Only about 1 compound out of 10.000 selected during the screening phase, after nearly 10-15 years and an average investment of 2.6 billons USD, get approved and commercialized[465]. In this regard, the role of public-sector research institutions (PRIs) such as universities and non-profit organizations, traditionally marginal in the process of drug discovery, has nowadays dramatically changed[466]. While before the mid-70s, the contribution of PRIs to drug discovery was mainly the upstream research necessary to identify molecular targets for future drugs, after the dawn of the biotechnology era and the tools it brought, public institutions have acquired an increasingly important role in the applied phase of drug discovery, which is where small-molecules and bioactive compounds are discovered and patented[466]. A previous study[466] reported that, from 1990 to 2007, PRIs have contributed to the discovery of

9-21% of all the drugs approved during the same period. Also, recently published studies conducted in US on registration of FDA approved drugs, demonstrated that in the development of about a quarter of all the product patented is either directly involved a public research lab (19%) or a spin-off company (6%) generated from a public research institution[467]. For almost all the drugs considered, the contribution of the public institution was related to the initial discovery of the drugs, synthesis or key intellectual property leading to a patentable invention[467].

1.4. PROJECT AIM AND OBJECTIVES

Our aim in the scope of this research project was to screen extracts obtained from selected marine groups as sources of osteoactive compounds, and explore their potential applications for both the biomedical sector, as sources of future drugs to treat human bone erosive pathologies, and for the aquaculture industry, as nutraceuticals to be incorporated into fish feeds with the aim of improving the skeletal health of animals.

The specific objectives of this PhD project were:

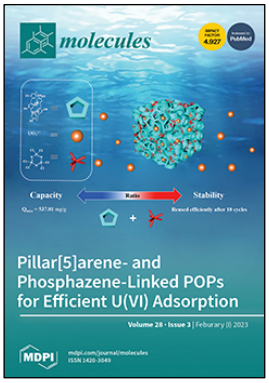
1. Screen extracts obtained from a variety of promising marine groups including holothurians, tunicates, and microalgae, by using the tools currently available for the screening of osteoactive compounds, including bone cell lines and *in vivo* screening systems based on zebrafish.
2. Perform the fractionation and chemical characterization of one promising extract, selected on the basis of good bioactivity and low toxicity, by the use of analytical tools, with the aim of identifying compounds as a potential candidate for being the one responsible for the positive effects on bone.
3. Provide a validation of the potential use of selected marine extracts for:
    a. Short-term applications in the aquaculture feed industry, by testing them as nutritional supplements for the commercially reared species gilthead seabream (*Sparus aurata*).
    b. On the longer-term, as treatment for human bone metabolic diseases, by testing them in fish biomedical models (zebrafish and medaka, and mammalian bone-derived lines.
4. Gain insights into the molecular mechanism of action for the selected extracts by the implementation of zebrafish (*Danio rerio*) transgenic reporter lines and though a transcriptomic (RNAseq) approach.

CHAPTER 2.

SCREENING OF NOVEL MARINE EXTRACTS WITH OSTEOGENIC BIOACTIVITY

Part of this chapter is or will be published in:

Carletti A, Cardoso C, Lobo-Arteaga J, Sales S, Juliao D, Ferreira I, Chainho P, Dionísio M A, Gaudêncio M J, Afonso C, Lourenço H, Cancela M L, Bandarra N M & Gavaia P J (2022). "Antioxidant and anti-inflammatory extracts from sea cucumbers and tunicates induce a pro-osteogenic effect in zebrafish larvae". *Frontiers in Nutrition* 9, 888360.



J.T. Rosa, A. Carletti, T. Varela, I. Vitorino, O. M. Lage, G. Lopes, V. Gonçalves, T. Santos, H. Galvão, M.L. Cancela, P. Gavaia, V. Laizé. "Phenotypic-based zebrafish screening pipeline for the identification of bone anabolic compounds". In preparation for submission to *Molecules* MDPI (by the first half of 2023).

CHAPTER OVERVIEW

Marine biodiversity represents a promising source of natural bioactive compounds with potential for the development of drugs to be used for the treatment of bone erosive disorders. As discussed in **Chapter 1**, several groups of marine organisms hold great potential in this sense. Among these, marine invertebrates are the most represented group when it comes to providing natural compounds with pharmacological potential. On the other side, marine microorganism such as microalgae, bacteria, and cyanobacteria are considered the most underexplored source of natural compounds, as an increasing number of studies are highlighting their bioactive potential. As such, in this second chapter we have focused on the search for marine compounds with osteogenic bioactivity by screening a set of extracts and fractions obtained from several species belonging to these aforementioned groups. With this aim, we have tested these extracts using the teleost zebrafish (*Danio rerio*), a well-established model for the study of skeleton pathologies due to the highly conserved disease mechanisms and its technical advantages over traditional rodent models. Therefore, zebrafish is a model of choice for large *in vivo* phenotypic screening assays aiming at identifying compounds with osteogenic potential.

In **Chapter 2.1**, published in *Frontiers in nutrition* (see details in chapter), we explored the bioactive potential of 14 extracts obtained from various species of marine invertebrates belonging to the groups of holothurians and tunicates, as sources of antioxidant, anti-inflammatory, and osteogenic compounds. We identified three ethanolic extracts characterized by a strong *in vivo* osteogenic activity, which positively correlated with their anti-inflammatory potential.

In **Chapter 2.2**, which is part of an article in preparation for submission to *Molecules*, we have conducted a vast screening activity on 133 extracts and fractions prepared from various marine microorganisms including cyanobacteria, actinobacteria, planctomycetes, and microalgae, and evaluated their capacity to increase bone growth by using the same zebrafish-based assay. As a result, we identified 6 ethanolic extracts with potent pro-osteogenic activity.

The identified extracts and fractions represent a promising starting point for the identification of compounds with future applications in pharmaceutical research for the treatment of human bone metabolic pathologies.

Author's note: The paper presented in Chapter 2.2 is the result of a collective work were different authors contributed to the screening of different extracts. In detail, I performed the screening of all the extracts from the microalgae species: Skeletonema costatum (SKLT), Nannochloropsis oculata (NANNO), and Tetraselmis striata CTP4 (CTP4).

2.1. INTRODUCTION

Noncommunicable diseases, also known as chronic diseases, are the utmost burden of the global health systems being by far the major cause of morbidity and deaths[468]. Metabolic bone disorders characterized by reduced bone mineral density (BMD), i.e. osteoporosis and osteopenia, are by far the most common diseases related to bone, with a global incidence of 40% in population over the age of 50[469,470]. When it comes to the etiology of osteoporosis, the full picture of the underlying mechanisms remains unclear, but a growing body of evidence has stressed the importance of inflammation and oxidative stress. The most common type of osteoporosis, type 1 or postmenopausal osteoporosis, is considered to have pathophysiological roots in the dysregulation of inflammatory processes prompted by the decrease of estrogen levels[36,471,472].

Current therapeutic approaches for the treatment of osteoporosis and other bone erosive pathologies rely on antiresorptive drugs that target osteoclasts, or on bone anabolic drugs, which act by pharmacologically increasing osteoblast-assisted mineral deposition. Despite being successfully implemented in osteoporotic patients, these therapies are still characterized by long-term associated side effects and do not lend themselves to the treatment of lifelong chronic conditions, like in the case of osteoporosis[48].

In this context, natural compounds with antioxidant, anti-inflammatory, and pro-osteogenic properties may represent alternative tools for the prevention and treatment of osteoporosis and other bone-related diseases[473]. Polyphenols are among the better known and naturally occurring compounds with antioxidant and anti-inflammatory properties, and have raised interest due to their therapeutic potential for the treatment of chronic disorders[474].

In the last decades, the marine environment has emerged as an interesting opportunity to discover new bioactives, and the wide metabolic diversity characterizing marine organisms has drawn the attention of the pharmaceutical industry[209]. Different groups of marine organisms hold great bioactive potential, including invertebrates such as sea cucumbers[475,476] and tunicates[480,48], and microorganisms like microalgae, bacteria and cyanobacteria[477].

The teleost fish zebrafish (*Danio rerio*) is well-established for its contribution to the study of skeletal pathologies due to the highly conserved disease characteristics and molecular mechanisms and its technical advantages over rodents, that makes it a model of choice for large *in vivo* phenotypic screening assays aiming at identifying compounds with osteogenic potential. Thus, in the present chapter, we have implemented the zebrafish operculum assay, a

quick and cost-effective tool that was previously developed for the screening of natural compounds and drugs with osteogenic potential[478].

In the first section of this chapter, we present the results of the screening for bioactivities of extracts produced from 3 species of sea cucumbers and 4 species of ascidians We extracted the water- and ethanol-soluble phases and explored their antioxidant potential, content of polyphenols, anti-inflammatory, and osteogenic activities.

Marine microorganisms such as microalgae, bacteria, and cyanobacteria are other taxonomic groups attracting a great deal of interest of the pharmaceutical industry, due to their potential for the isolation of bioactive compounds[215–219]. Thus, in the second part of this chapter we took full advantage of the short time needed to achieve reliable experimental results using the operculum assay[478], and set up a medium-scale screening project for evaluating the osteogenic activity for a total of 134 extracts/fractions obtained from 13 species of marine microalgae, 12 strains of bacteria, and 8 of cyanobacteria, with the scope of identifying the active extracts and make a selection of these for using in the next steps of this PhD project.

2.1. SCREENING OF EXTRACTS FROM MARINE INVERTEBRATES

THIS SECTION IS PART OF: Carletti A, Cardoso C, Lobo-Arteaga J, Sales S, Juliao D, Ferreira I, Chainho P, Dionísio M A, Gaudêncio M J, Afonso C, Lourenço H, Cancela M L, Bandarra N M & Gavaia P J (2022). "Antioxidant and anti-inflammatory extracts from sea cucumbers and tunicates induce a pro-osteogenic effect in zebrafish larvae". *Frontiers in Nutrition* 9, 888360.

2.1.1. Animal collection and Identification

2.1.1.1. Sea cucumber

Fresh samples of sea cucumber were captured by scuba diving in the coastal zone between Sesimbra and Sado Estuary (Setúbal, Portugal; 38°25'23.50"N; 9°0'45.06"W), from January to July 2019. A total of 208 animals were captured, *Holothuria (Roweothuria) arguinensis* (*n* = 62), *H. (Panningothuria) forskali* (*n* = 64) and *H. (Holothuria) mammata* (*n* = 82). After dissection and removal of internal organs and celomic fluid, specimens were cleaned under running water, minced, and stored at -80 °C until analysis.

2.1.1.2. Tunicates

Samples of 4 different tunicates species - Styela plicata, Aplidium sp., Botrylloides diegensis, and Ciona robusta - were collected by hand as epibionts of mussels cultured in the mussel aquaculture rafts of the Albufeira lagoon. The Albufeira lagoon is a semi-enclosed lagoon located 20 km south of Lisbon on a mesotidal area with a NE-SW orientation to the coast. The mussel rafts are located in the main water bodies of Lagoa Grande with an average depth of 4 to 5 m, with a maximum depth of 13 m.

2.1.1.3. Phylogenetic Identification

Specimens were identified to the lowest possible taxonomic level through integrative taxonomy, combining morphological and genetic approaches, when necessary. For the morphological approach, a stereomicroscope and identification keys were used. For the genetic approach, a small piece of tissue of each organism was used to extract total DNA using the E.Z.N.A Mollusc DNA Kit (Omega Bio-Tek, Norcross, US), following the manufacturer's instructions. An iCycler (Bio-Rad, Hercules, US) thermal cycler was used to amplify the fragment of the mitochondrial gene cytochrome c oxidase subunit I gene (COI-5P), using a

PCR mix (Invitrogen™, Waltham, US). Each PCR reaction contained 2.5 µL 10× Taq polymerase buffer, 0.75 µL of 50 mM MgCl2, 0.5 µL of 10 mM dNTP mixture, 0.1 µL of 5U/µL of Taq DNA polymerase, 1.5 µL (10 µm) of each primer (LoboF1 5'-KBTCHACAAAYCAYAARGAYATHGG-3' and LoboR1 5'-TAAACYTCWGGRTGWCCRAARAAYCA-3')[479], 1.25 µL of 1% W-1, 4 µL of DNA template and sterile Milli-Q water to make up a total volume of 25 µL. The conditions of the PCR thermal cycling were as follows: 1) 5 minutes at 94 °C; 2) 5 cycles: 30 seconds at 94 °C, 1 minute and 30 seconds at 45 °C, 1 minute at 72 °C; 3) 45 cycles: 30 seconds at 94 °C, 1 minute and 30 seconds at 54 °C, 1 minute at 72 °C; 4) 5 minutes at 72 °C. The amplified PCR products were purified using magnetic beads and subsequently sequenced bidirectionally with the BigDye Terminator 3 kit on an ABI 3730XL DNA analyzer (Applied BiosystemsTM, Waltham, US) by StabVida. Trace files obtained were carefully analyzed and sequences were aligned using MEGA version X. GenBank BLASTn search[480] and BOLD Identification System tool (BOLD-IDS)[481], were used for matching sequences. The specimen and sequence data obtained in this study is compiled in the Barcode of Life Data Systems project titled "SCUTU - Properties of extracts from sea cucumbers and tunicates". GenBank accession numbers are presented in Supplementary Table 1.

2.1.2. Ethanolic and Aqueous extraction

The samples of sea cucumbers and tunicates were freeze-dried in a Heto PowerDry PL3000 Freeze Dryer (Thermo Fisher Scientific, Waltham, US) for 72 hours. Freeze-dried biomass was grounded into a fine powder with a Retsch GM200 ball mill (Retsch GmbH, Haan, Germany). Ethanolic (HE) and aqueous (AQ) extracts were prepared through liquid extraction. Biomass powder was weighed and transferred into 50 mL Falcon tubes covered with aluminum foil, to prevent photo-oxidative processes, and solubilized in distilled water and 96% ethanol-water mixture respectively. A biomass-solvent ratio of 30 mL of solvent per 1 g of biomass was used. Solutions were homogenized with a UNIDRIVE X1000D Homogenising System (CAT, Deerfield, US) at 30,000 rpm for 1 min and then left on an orbital shaker (VWR International, Radnor, US) overnight (15h) to extract phenolic compounds. The next day, extracts were centrifuged at 1,000 × g for 5 minutes to allow the insoluble material to precipitate and the supernatant was transferred into new 50 mL falcon tubes. Centrifugation was repeated 3 times. HE and AQ extracts were aliquoted and a half was immediately used for polyphenol determination and antioxidant activity, while the other half was evaporated and completely

dried before testing anti-inflammatory activity and osteogenic activity. Fractions were then evaporated using a rotatory evaporator RV 10 digital (IKA-Werke GmbH & Co, Staufen, Germany), setting a temperature of 40 °C for ethanol extracts and 50 °C for aqueous extracts, and then stored at -80 °C. Before exposure to the fish, extracts were resuspended in their vehicle, ethanol (Merck, Darmstadt, Germany) and Milli-Q water (pH 7.4), respectively.

2.1.3. Total Polyphenols Content

Polyphenols content was determined in the AQ and HE extracts by colorimetry determination with phosphomolybdic-phosphotungstic acid with a protocol adapted from[482]. The assay is based on the reactivity of the Folin-Ciocalteu reagent, which consists of a yellow acidic solution containing complex polymeric ions formed from phosphomolybdic and phosphotungstic acids. In an alkaline solution, this reagent oxidizes phenolic compounds and is reduced producing a complex molybdenum-tungsten blue pigment, which can be spectrophotometrically detected by reading the absorbance at λ= 750 nm. The total polyphenols content is calculated relatively to the reactivity of gallic acid (3,4,5-trihydroxy benzoic acid), a known phenolic compound, under the same conditions. Folin-Ciocalteu reagent has been purchased from Merck and diluted 2 × (1:1) in Milli-Q water before utilization. For a volume of 100 µL of HE or AQ extract, 600 µL of Milli-Q water and 150 µL of Folin-Ciocalteu reagent (1:1 diluted) were added to test tubes. The mixture was then vortexed and incubated in the dark for 5 min at room temperature. Subsequently, 750 µL of 2% sodium carbonate (Na_2CO_3) prepared in water were added to each test tube. Mixtures were then vortexed and incubated in the dark for 90 min at room temperature. Each reaction was performed in triplicate for each extract. Absorbance was then detected at 750 nm using a UV-Vis spectrophotometer Helios Alpha-model (Unicam, Cambridge, UK). The calibration curve was calculated by using standard solutions of gallic acid (Merck) —0.025, 0.05, 0.1, 0.2, 0.3 mg/mL— obtained by serial dilutions from a stock solution (1 mg/mL) prepared in ethanol for both AQ and HE extracts. Total polyphenols content was assessed in triplicates and calculated from the gallic acid calibration curve as milligrams of gallic acid-equivalent (GAE) polyphenols per gram of dry biomass (mg GAE/100 g dw).

2.1.4. Antioxidant activity

Antioxidant potential was assessed for the HE and AQ extracts from sea cucumbers and tunicates. Free radical scavenging capacity was assessed with two assays based on a single electron transfer reaction (FRAP and $ABTS^{+}$ methods) and one assay based on a hydrogen atom transfer reaction (DPPH method), which are considered complementary methods for the evaluation of antioxidant capacity[483,484]. Antioxidant activities were analyzed in triplicate for all the tests performed.

2.1.4.1. FRAP assay

The ferric reducing antioxidant power (FRAP) was evaluated for HE and AQ extracts with a protocol based on the method adapted by[485]. The test is based on a redox reaction occurring between the substrate (electron donor) and Fe^{3+} ions (electron acceptor), producing $Fe2^{+}$ ions. This reduction is monitored spectrophotometrically by the change in the color of the solution of Fe^{3+} with TPTZ (2,4,6-tris(2-pyridyl)-s-triazine), which turns blue and absorbs electromagnetic radiation at λ= 595 nm. The ferric reducing antioxidant power of the extracts is calculated as related to the absorbance of a Fe^{2+} standard solution of iron sulphate ($FeSO_4$), tested in parallel. The FRAP reagent was prepared by mixing acetate buffer (0.3 mol/L), TPTZ (10 mmol/L) (Alfa-Aesar, Cityward Hill, US), and $FeCl_3 \cdot 6H_2O$ (20 mmol/L) (Merck) in the ratio 10:1:1. For a volume of 100 µL of HE or AQ extract, 3.0 mL of the FRAP reagent was added to test tubes and the mixture was then incubated in the dark for 30 min at 37 °C in a water bath. The absorbance of the samples was read in comparison to the blank (prepared by adding the reagent to 96% ethanol and water for AQ and HE extracts respectively) at a wavelength of 595 nm, using a UV-Vis spectrophotometer Helios Alpha-model (Unicam). The calibration curve was prepared using standard solutions of $FeSO_4$ (0.25, 0.5, 1, 1.5, 2 mmol/L) obtained by serial dilution from a stock solution (2 mmol/L). All measurements were conducted in triplicate and the results were expressed as micromoles of equivalents of iron sulphate per g of dry biomass (µmol eq $FeSO_4$/g dw).

2.1.4.2. $ABTS^{+}$ assay

The antiradical capacity of HE and AQ extracts was evaluated by following their effect on the stable free cation radical ABTS+• [2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)], following the protocol used by[486]. The assay is based on the scavenging capacity of antioxidants against ABTS+• radical cation, a blue/green chromophore, which has multiple

absorbance maxima at 645 nm, 734 nm, and 815 nm. ABTS+• is firstly produced by the reaction between ABTS and potassium persulfate (K2S2O8). The addition of antioxidants reduces it to ABTS resulting in the decolorization of the solution which is monitored via reading the reduction of the absorbance at λ= 734 nm. The scavenging capacity of the $ABTS^{+}$• radical cation is determined as a function of concentration and time and calculated relative to the reactivity of Trolox (6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid) as antioxidant standard, under the same conditions. ABTS, [2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)] diammonium salt, and potassium persulfate ($K_2S_2O_8$) were obtained from Merck. $ABTS^{+}$• solution (7 mM) was prepared by dissolving 10 mg of ABTS into a potassium persulfate solution (2.45 mM) prepared in Milli-Q water and incubating the mixture in the dark for 16 h at room temperature. Because ABTS and potassium persulfate reacts stoichiometrically at a ratio of 1:0.5, this results in incomplete oxidation of the ABTS and production of $ABTS^{+}$•. After the incubation, the $ABTS^{+}$• solution was diluted by adding 5 mM phosphate-buffered saline (PBS), pH 7.4 until obtaining a final absorbance of 0.7 ± 0.02 at 734 nm. For a volume of 20 µL of HE or AQ extract, 2.0 mL of $ABTS^{+}$• solution was added to test tubes. The mixture was then vortexed and incubated in the dark for 6 min at 37 °C in a water bath. Absorbance was detected again at 734 nm using a UV-Vis spectrophotometer Helios Alpha-model (Unicam). The calibration curve was calculated by using standard solutions of Trolox (Merck) (0, 100, 250, 500, 1000, and 2000 µM) obtained by serial dilution from a stock Trolox solution (3 mM) prepared in 96% ethanol for AQ and HE. $ABTS^{+}$• radical scavenging capacity was calculated as micromoles of equivalents of Trolox per 100 g of dry biomass (µmol eq TROLOX/100 g dw).

2.1.4.3. DPPH assay

Radical scavenging activity of AQ and HE extracts against the stable radical DPPH• (2,2-diphenyl-2-picrylhydrazyl hydrate) was determined spectrophotometrically by following a protocol adapted from[487]. When DPPH• reacts with an antioxidant compound able to donate a hydrogen proton, it is reduced to DPPH-H, and the color change which results can be detected by measuring the reduction of absorbance at λ= 517 nm. The scavenging capacity against the stable DPPH• radical is determined relative to the reactivity of ascorbic acid as an antioxidant standard, under the same conditions. DPPH solution (0.15 mM) was prepared by dissolving 11.8 mg of DPPH reagent (2,2-diphenyl-1-picrylhydrazyl, 95%, (Alfa-Aesar) into 200 ml of methanol in a volumetric flask. For a volume of 1 mL of ethanolic or aqueous extract, 2.0 mL of the DPPH solution was added to test tubes. Solutions were vortexed and incubated for 30

minutes in the dark at room temperature. Reduction in the absorbance at 527 nm was detected with a UV-Vis spectrophotometer Helios Alpha-model (Unicam). The absorption of a blank sample containing the same amount of DPPH• solution and ethanol 96% or water was measured for HE and AQ extracts, respectively. The experiment was carried out in triplicates. A calibration curve with ascorbic acid (Merck) was drawn by reading absorbance (517 nm) of standard concentrations of ascorbic acid (5, 10, 15, and 20 mg/L) obtained by serial dilution from the higher concentration and a calibration curve equation was calculated for both HE and AQ extracts. Radical scavenging capacity was calculated as milligrams of equivalents of ascorbic acid per 100 g of dry biomass (mg eq AA/100 g dw).

2.1.5. Anti-inflammatory activity

Anti-inflammatory activity was evaluated by the cyclooxygenase-2 (COX-2) inhibitory assay, an established biomarker of inflammation processes and target of common non-steroidal anti-inflammatory drugs (NSAID)[488]. Extracts were subjected to heat treatment (80 ºC during 1 h) and centrifuged (3,000 × g at 4 °C for 10 min). The supernatant was collected and the solvent was evaporated using a vacuum rotary evaporator with the water bath temperature at 65 °C. The residue was directly dissolved in 100% dimethyl sulfoxide (DMSO, Merck) to prepare a stock solution with a concentration of 10 $mg \cdot mL^{-1}$. Extracts were tested at 1 $mg \cdot mL^{-1}$ using a commercial COX-2 inhibitory screening assay kit, Cayman test kit-560131 (Cayman Chemical Company, Ann Arbor, US). A volume of 10 µL of extract or DMSO (blank) was used. Results were expressed as a percentage of inhibition of COX-2. Anti-inflammatory activity was analyzed in quadruplicates.

2.1.6. Acute toxicity and osteogenic activity

Adult zebrafish (AB wild-type strain) were crossed by using an in-house breeding program. Fertilized eggs were transferred into plastic 1 L tanks with static water conditions and maintained until hatching, at 3 days post-fertilization (dpf), with the following conditions: temperature 28 ± 0.1 °C, pH 7.5 ± 0.1, conductivity 700 ± 50 µS, NH_3 and NO_2 lower than 0.1 mg/L, NO_3 lower than 50 mg/L and a photoperiod of 14:10 h light-dark. Fish water was prepared by adding a salt mixture (Instant Ocean, Blacksburg, US) and sodium bicarbonate to reverse osmosis treated water in order to maintain stable pH and conductivity. The osteogenic activity was evaluated for HE and AQ extracts as described by[478] and the process is

schematized in Figure 2.1. All extracts were initially solubilized in either ethanol or water at the higher concentration possible, i.e. 200 µg/mL. Briefly, at 3 dpf, hatched embryos (n = 15) were transferred to 6 well-plates and treated each extract and their vehicle as the control group (ethanol 0.1% for HE and water for AQ extracts). Calcitriol (1α,25-dihydroxy vitamin D_3, Sigma-Aldrich, St. Louis, US), was used as a positive control at a concentration of 10 pg/mL in ethanol. Extracts were tested in consecutive experiments at different concentrations and developmental toxicity was evaluated to calculate the maximum non-lethal dose (MNLD) over a 72h period. Toxicity was recorded daily by monitoring the mortality induced in each condition. Larvae were considered not viable when one of the following observations occurred: all or part of larval body degraded, severe growth retardation, a developmental anomaly with severity non-consistent with larval survival. Whenever the extracts resulted in toxicity, the concentration was reduced to 100 µg/mL and the experiment was repeated. At 6 dpf, larvae were sacrificed with a lethal dose of MS-222 (0.6 mM, pH 7.0, Sigma-Aldrich), stained for 20 min at room temperature with 0.03% alizarin red S (AR-S) prepared in Milli-Q water (pH 7.4), and washed twice with Milli-Q water for 5 min. Euthanized larvae were placed in a lateral position on top of a 2.5 % agarose gel plate and imaged using a Leica MZ10F fluorescence stereomicroscope (Leica, Wetzlar, Germany) equipped with a green fluorescence filter (λex= 546/10 nm), a barrier filter (λem =590 nm), and a DFC7000T camera (Leica). Images were acquired using the following parameters: exposure time 300 milliseconds, gamma 1.00, image format 1920×1440 pixels, binning 1×1. Fluorescence images were analyzed using ImageJ software version 2.0.0-rc-69/1.52p. For morphometric analysis, the images were processed by using the toolbox available within the "ZFBONE" macro toolset for Fiji[489]. The area of the operculum and the area of the head were digitally measured with the support of an Intous M drawing tablet (Wacom, Ōtone, Japan). The ratio of the operculum area over the head area was calculated, since the area of the head is the most accurate morphological parameter to normalize inter-specimen size variations[478].

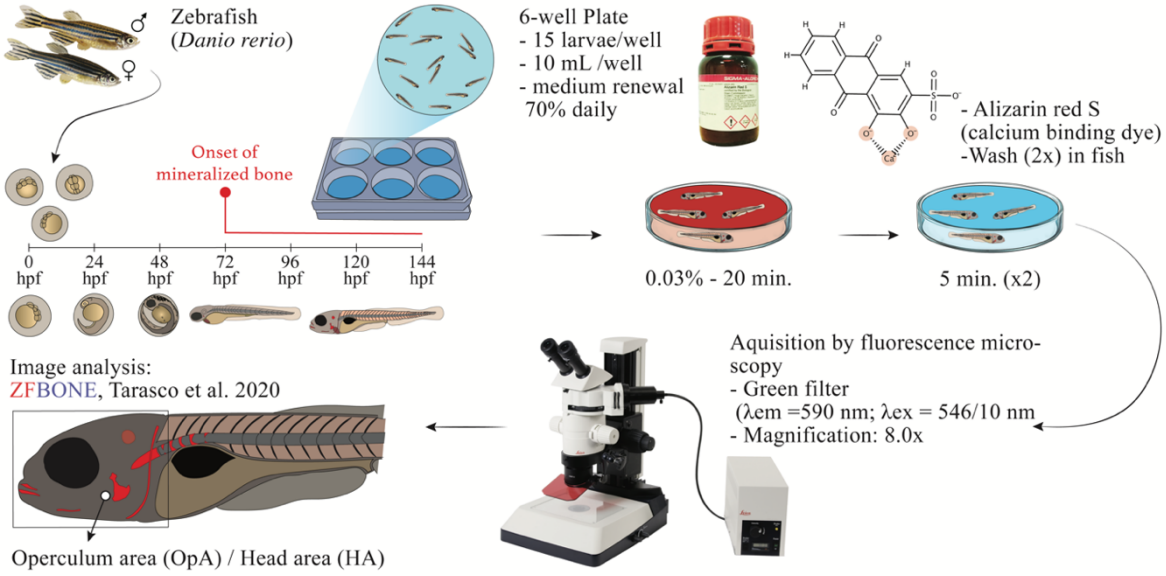


Figure 2.1. Scheme of the protocol used for the evaluation of the osteogenic activity.

2.1.7. Statistical analysis

For all the experiments, normality was tested with a D'Agostino-Pearson omnibus normality test or with an Anderson-Darling test ($p < 0.05$). Homoscedasticity was tested through the Brown-Forsythe test ($p < 0.05$). When the distribution of the data of all the experimental groups resulted normal and homogeneous, statistical differences between the control and the extracts were tested with a one-way ANOVA followed by Dunnett's multiple comparison test ($p < 0.05$). If the distribution of the data resulted non-normal or non-homogeneous, statistical differences were tested with a non-parametric test followed by Dunn's multiple comparison test ($p < 0.05$). Statistical analyses were performed using Prism version 8.00 (GraphPad Software, Inc. La Jolla, USA).

2.1.8. Results

2.1.8.1. Total Polyphenols Content

Tunicate extracts displayed a high content of polyphenolic compounds, with higher values for the HE fractions. The extracts with the highest content were the ones obtained by HE fractions of *Botrylloides diegensis*, 669 ± 11 mg GAE/100 g dw, *Aplidium* sp, 365 ± 21 mg GAE/100 g dw and *Ciona robusta*, 206 ± 14 mg GAE/100 g dw (Table 2.1). Polyphenol contents from the

extracts of holothurians were generally lower than ascidians, never exceeding 100 mg GAE/100 g dw, and were higher in the AQ extraction compared to the HE (Table 2.1).

Table 2.1. Polyphenol content in AQ and HE extracts of the tunicates and sea cucumbers. Values are presented as average ± S.D. nd – not detected. Different lowercase letters within a column correspond to statistical differences ($p < 0.05$) between different species and same extract type (ethanolic or aqueous, respectively). Different uppercase letters within a row correspond to statistical differences ($p < 0.05$) between aqueous and ethanolic extracts.

Species	**Polyphenol Content (mg GAE/100 g dw)**	
	Extract	
	Aqueous ($n = 3$)	**Ethanolic ($n = 3$)**
Styela plicata	31 ± 6^{aA}	40 ± 7^{bA}
Aplidium sp.	260 ± 13^{dA}	$\mathbf{365 \pm 21^{dB}}$
Botrylloides diegensis	428 ± 10^{eA}	$\mathbf{669 \pm 11^{eB}}$
Ciona robusta	103 ± 8^{cA}	$\mathbf{206 \pm 14^{cB}}$
H. (Roweothuria) arguinensis	84 ± 4^{bcA}	9 ± 5^{abB}
*H. (*Holothuria) *mammata*	79 ± 1^{bA}	21 ± 17^{abB}
H. (Panningothuria) forskali	48 ± 2^{aA}	nd^{aB}

2.1.8.2. Antioxidant activity

The antioxidant activity was measured by the use of three alternative methodologies (ABTS, FRAP, and DPPH) in the extracts from tunicates and sea cucumbers (Table 2.2). Three species of tunicates - *Aplidium* sp, *Botrylloides diegensis*, and *Ciona robusta* - displayed the highest antioxidant activities with consistent results across the different methodologies used. When measured by DPPH and FRAP assays, the HE extracts displayed higher antioxidant activity. However, when tested with ABTS assay, the AQ extract also displayed high antioxidant activity, surpassing the ones of the HE extracts. Moderate antioxidant activity was reported also for the AQ extracts from *H. arguinensis* and *H. mammata* when assessed by ABTS assay, but always lower compared to tunicates.

Table 2.2 (next page). Antioxidant activity as measured by ABTS, FRAP, and DPPH in AQ and HE extracts of tunicates and sea cucumbers. Values are presented as average ± S.D. nd – not detected. For all groups, $n = 3$. Different lowercase letters within a column correspond to statistical differences ($p < 0.05$) between different species and same extract type. For each antioxidant methodology, different uppercase letters within a row correspond to statistical differences ($p < 0.05$) between aqueous and ethanolic extracts.

Species	ABTS (µmol eq TROLOX/100 g dw)		FRAP (µmol eq $FeSO_4$/g dw)		DPPH (mg eq AA/100 g dw)	
	Extract		Extract		Extract	
	Aqueous (n = 3)	Ethanolic (n = 3)	Aqueous (n = 3)	Ethanolic (n = 3)	Aqueous (n = 3)	Ethanolic (n = 3)
Styela plicata	1,126 ± 81^{aA}	552 ± 60^{bB}	2.9 ± 0.5^{aA}	5.5 ± 0.1^{bB}	6.9 ±	nd^{aB}
Aplidium sp.	**6,206 ±**	**5,020 ± 20^{dB}**	**34.3 ±**	**34.2 ± 0.1^{dA}**	nd^{aA}	**102.0 ±**
Botrylloides	**6,735 ± 11^{gA}**	**5,886 ± 83^{eB}**	**54.6 ±**	**56.5 ± 0.0^{eB}**	16.7 ±	**179.0 ±**
Ciona robusta	**4,271 ±**	**3,027 ± 56^{cB}**	**19.7 ±**	**15.6 ± 0.6^{cB}**	**151.7 ±**	**162.7 ±**
H. arguinensis	2,234 ±	166 ± 35^{aB}	7.0 ± 0.2^{bA}	2.0 ± 0.1^{aB}	nd^{aA}	13.5 ±
H. mammata	3,089 ±	33 ± 57^{aB}	6.2 ± 0.0^{bA}	1.5 ± 0.2^{aB}	31.8 ±	5.7 ± 9.8^{aB}
H. forskali	1,883 ± 81^{bA}	nd^{aB}	8.9 ± 0.5^{cA}	2.1 ± 0.6^{aB}	106.1 ±	nd^{aB}

2.1.8.3. Anti-inflammatory activity

The anti-inflammatory activity was measured by the percentage of inhibition of COX-2 (Table 2.3). HE extracts generally showed higher anti-inflammatory activity than the AQ extracts. HE fractions from sea cucumbers exhibited relatively low values of COX-2 inhibition ranging from 16.4 to 41.8 %, while the AQ extracts did not present any detectable activity. AQ extracts from tunicates presented a moderate anti-inflammatory activity, ranging from 8.9 ± 3.7 % in *Botrylloides diegensis* to 33.2 ± 5.2 % for *Ciona robusta*. However, the HE fractions from the four species of tunicates showed high anti-inflammatory activity, three of which in the 70.2-76.7 % interval, whit the highest activity measured for the extracts from *Ciona robusta*, showing 92.2 ± 8.5 % inhibition of COX-2 activity.

Table 2.3. Anti-inflammatory activity in AQ and HE extracts of tunicates and sea cucumbers. Values are presented as average ± S.D. nd – not detected. Different lowercase letters within a column correspond to statistical differences ($p < 0.05$) between different species within same type of extract. Different uppercase letters within a row correspond to statistical differences ($p < 0.05$) between AQ and HE extracts.

Species	Anti-Inflammatory Activity (% Inhibition COX-2)	
	Extract	
	Aqueous (n = 4)	Ethanolic (n = 4)
Styela plicata	19.6 ± 5.8^{cA}	**76.7 ± 3.4^{cB}**
Aplidium sp.	27.8 ± 2.5^{dA}	**70.2 ± 6.3^{cB}**
Botrylloides diegensis	8.9 ± 3.7^{bA}	**75.9 ± 3.0^{cB}**
Ciona robusta	33.2 ± 5.2^{dA}	**92.2 ± 8.5^{dB}**
H. (Roweothuria) arguinensis	nd^{aA}	40.0 ± 7.6^{bB}
H. (Holothuria) mammata	nd^{aA}	41.8 ± 4.9^{bB}
H. (Panningothuria) forskali	nd^{aA}	16.4 ± 9.7^{aB}

2.1.8.4. Developmental toxicity and establishment of maximum non-lethal dose

Acute developmental toxicity was evaluated in zebrafish larvae and the Maximum Non-Lethal Dose (MNLD) of each extract was determined during the operculum assay and results are summarized in Table 2.4. None of the extracts resulted in toxicity at the higher concentration tested (200 µg/mL), except for the HE extracts of *H. forskali*, which induced mortality. Because of this observation, all ethanolic extract were also tested at 100 µg/mL. Control groups – Calcitriol (10 pg/mL), water, and ethanol 0.1 % (v/v) did not cause mortality in any of the assays performed.

Table 2.4. Acute developmental toxicity of the AQ and HE extracts from sea cucumbers and tunicates assessed in zebrafish larvae. Two to 3 concentrations have been tested for each extract. Final survivorship after 72 hours of exposure (S_{72h}) was calculated. Depending on the results of the experiment, the concentrations were either increased to a maximum of 200 µg/ml or decreased following a half-logarithmic dilution (3.16, 10, 31.6, 100 µg/mL). Maximum non-lethal dose (*, **bold**); concentration not tested (-).

Species	***Extract***	***Concentration (µg/ml)***	***S_{72h}***	***Concentration (µg/ml)***	***S_{72h}***	***Concentration (µg/ml)***	***S_{72h}***
S. plicata	AQ	**200 ***	15/15	-	-	-	-
S. plicata	HE	**200 ***	15/15	100	15/15	-	-
Aplidium sp.	AQ	**200 ***	15/15	-	-	-	-
Aplidium sp.	HE	**200***	15/15	100	15/15	-	-
C. robusta	AQ	**200 ***	15/15	-	-	-	-
C. robusta	HE	**200 ***	15/15	100	15/15	-	-
B. diegensis	AQ	**200 ***	15/15	-	-	-	-
B. diegensis	HE	**200 ***	15/15	100	15/15	-	-
H. forskali	AQ	200	15/15	-	-	-	-
H. forskali	HE	200	11/15	**100 ***	15/15	-	-
H. arguinensis	AQ	**200 ***	15/15	-	-	-	-
H. arguinensis	HE	**200 ***	15/15	100	15/15	-	-
H. mammata	AQ	**200 ***	15/15	-	-	-	-
H. mammata	HE	**200 ***	15/15	100	15/15	-	-

2.1.8.5. Osteogenic activity

To determine the pro-osteogenic potential of the extracts studied, the effect of HE and AQ extracts on bone formation during zebrafish development was assessed using the operculum assay[478]. None of the extracts significantly affected the area of the head, indicating that there was not a significant variation in growth among treatments (Supplementary Figure 1). In particular, the HE extracts from three tunicates species (*Aplidium* sp., *Botrylloides diegensis*, and *Ciona robusta*) at 200 µg/mL were characterized by improved osteogenic activity, inducing an increase in the mineralized area of the opercular bone by 41.7 ± 16.6 %, 31.1 ± 13.8 %, and ± 20.0 12.7%, respectively (Figure 2.2). Statistical differences between the two

concentrations tested for the HE extracts were reported for *Aplidium* sp. ($p = 0.0186$) and *Ciona robusta* ($p < 0.0001$) indicating that a dose-dependent effect was present, but not for *Botrylloides diegensis* ($p = 0.0975$).

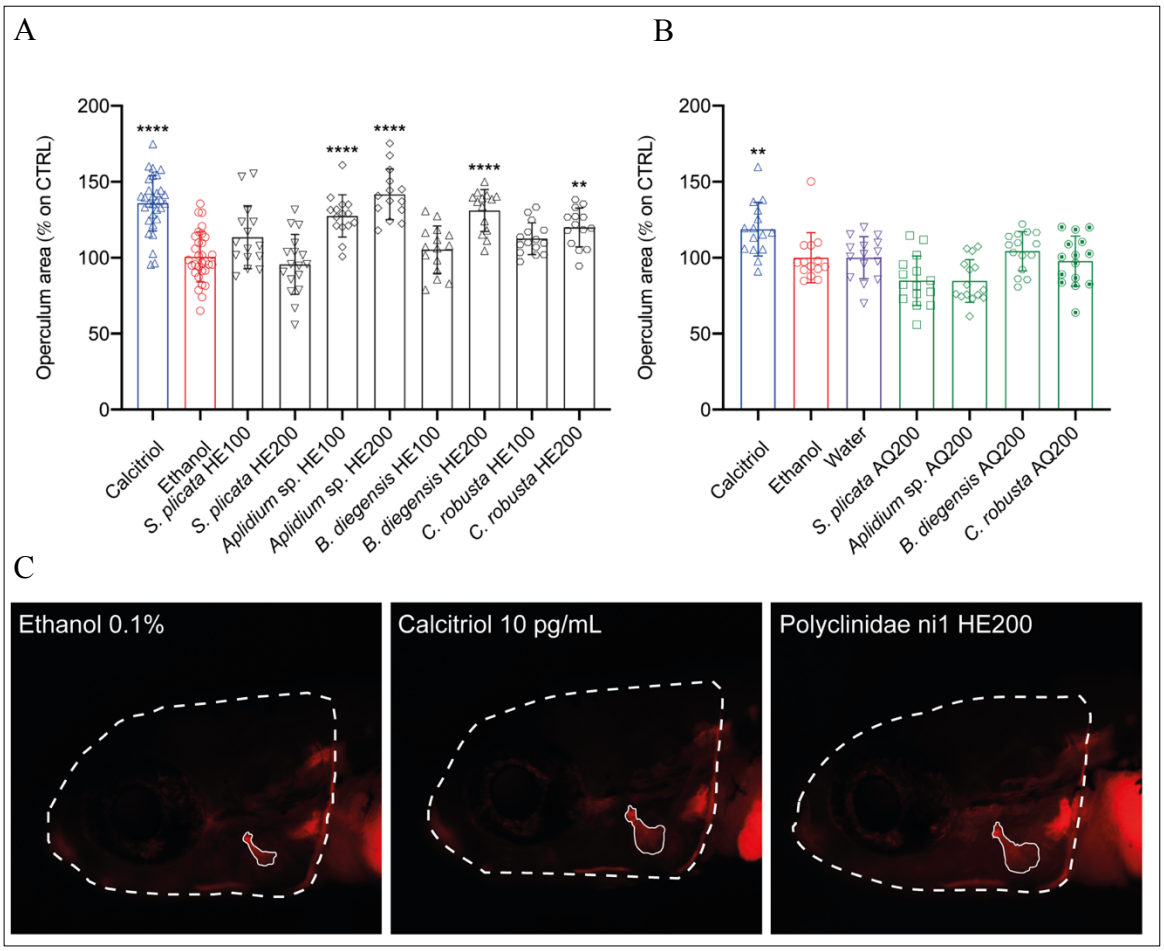


Figure 2.2. Osteogenic activity of aqueous (**A**) and ethanolic (**B**) extracts from tunicates in zebrafish larvae. Results are displayed as corrected operculum area (operculum area/head ratio) and expressed as percentage of increase over the control. Representative image (**C**) of a fish treated with the negative control (ethanol), the positive control (Calcitriol 10 pg/mL) and the most powerful osteogenic extracts from tunicates (*Aplidium* sp. HE200 – HE at 200 µg/mL). Statistical differences among the means were tested through One-way ANOVA followed by Dunnett's multiple comparison test ($p < 0.05$) or, whenever normality and homoscedasticity were not met, through a non-parametric test followed by Dunn's multiple comparison test ($p < 0.05$). *p* values are indicated as follow: 0.0332 (*), 0.0021. (**), 0.0002 (***), < 0.0001 (****). HE - ethanolic extracts, AQ – aqueous extracts, 100 – 100 µg/mL, 200 – 200 µg/mL.

In contrast, the AQ extracts did not display any evident osteogenic activity at the concentrations tested. Interestingly, also the HE extracts from two species of sea cucumbers, *H. arguinensis*

and *H. mammata*, induced a potent pro-osteogenic effect by increasing the area of the opercular bone in 33.0 ± 19.98 %, and 38.8 ± 22.8 %, respectively (Figure 2.3). For HE extracts from holothurians, when differences between 200 vs 100 µg/mL were tested through Student's *t* test ($p < 0.05$), there were no statistical differences for *H. arguinensis* ($p = 0.1942$), while these were present for *H. mammata* ($p = 0.0014$).

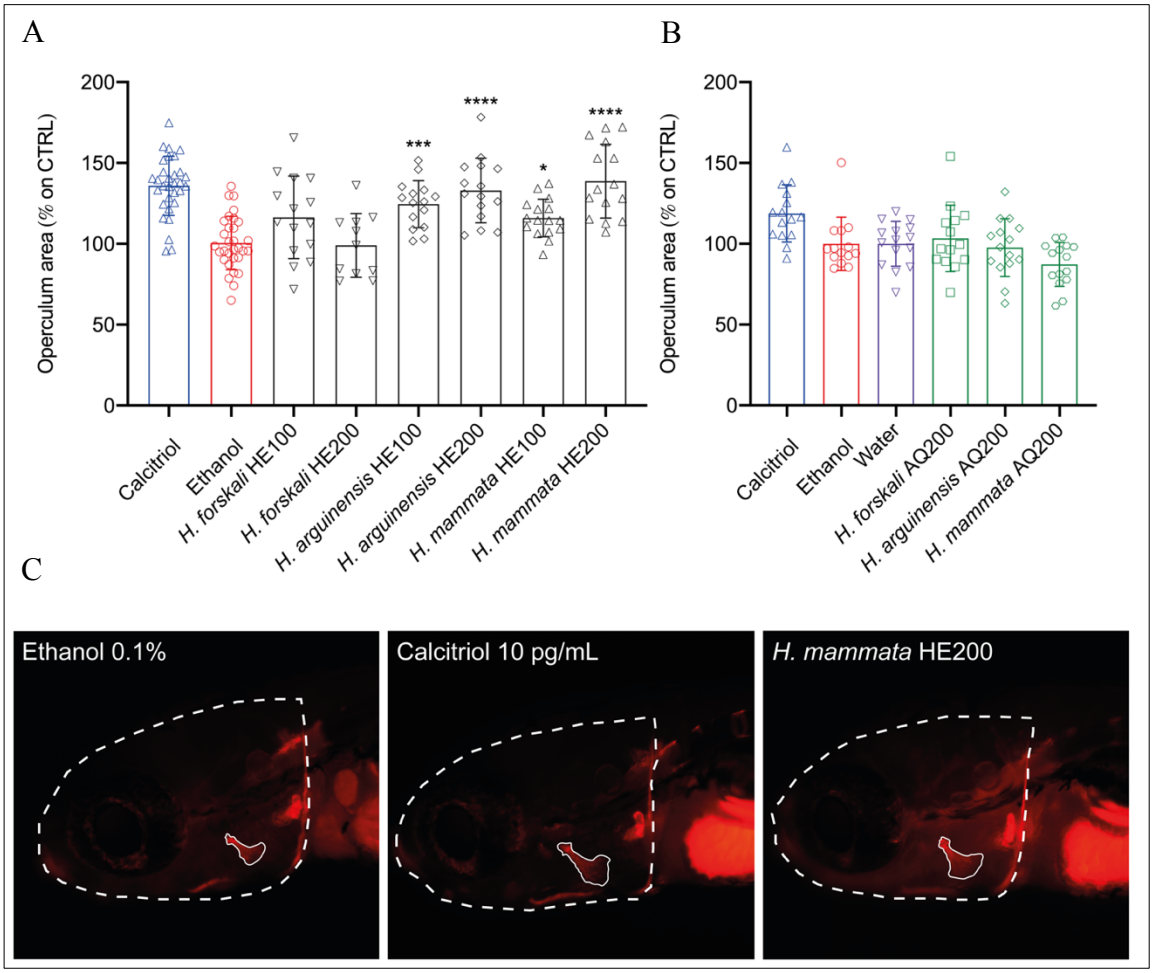


Figure 2.3. Osteogenic activity of aqueous, AQ (**A**) and ethanolic, HE (**B**) extracts from three species of sea cucumbers in zebrafish larvae. Results are displayed as corrected operculum area (operculum area/head ratio) expressed as percentage of increase over the control. Representative image (**C**) of a fish treated with the negative control (ethanol), the positive control (Calcitriol 10 pg/mL) and the most powerful osteogenic extracts among holothurians (*H. mammata* HE200 – ethanolic extract at the concentration of 200 µg/mL). Statistical differences among the means were tested through One-way ANOVA followed by Dunnett's multiple comparison test ($p < 0.05$) or, whenever normality and homoscedasticity weren't met, through a non-parametric test followed by Dunn's multiple comparison test ($p < 0.05$). *p* values are indicated as follow: 0.0332 (*), 0.0021. (**), 0.0002 (***), < 0.0001 (****). HE - ethanolic extracts, AQ – aqueous extracts, 100 – 100 µg/mL, 200 – 200 µg/mL.

2.2. SCREENING OF EXTRACTS FROM MARINE MICROORGANIMS

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2.2.1. Preparation of microalgae extracts

All microalgae species were cultured by the Marine Biotechnology Laboratory, Centre of Marine Sciences (CCMAR, Portugal). Extracts were produced from 13 species of marine microalgae: *Isochrysis* sp. (ISO), *Phaeodactulum fusiformis* (PAT), *Porphyridium* sp. (POC), *Cylindrotheca fusiformis* (PT05), *Tetraselmis striata* (PT08), unidentified species 1 (PT14), *Tetraselmis rubens* (IMP3), unidentified species 2 (SAG05), unidentified species 3 (PTG14), unidentified species 4 (PT-LS), *Skeletonema costatum* (SKLT), *Nannochloropsis oculata* (NANNO), and *Tetraselmis striata* CTP4 strain (CTP4). Ethanol, ethyl acetate and aqueous extracts were produced from freeze-dried algal biomass with a solvent extraction protocol, showed in Figure 2.4. A total number of 39 extracts were so produced. Algal biomass was transferred into a 500-mL Erlenmeyer covered with aluminum foil to prevent photo-oxidative degradation, and solubilized in ethanol, ethyl acetate or distilled water respectively. The biomass-solvent ratio was of 1g each 40 mL of solvent. Solutions were left on a magnetic stirrer overnight (12-15h) to extract soluble compounds. The next day, extracts were transferred to 50 ml plastic tubes and centrifuged at 2500 rpm for 5 minutes to allow the insoluble material to precipitate. Centrifugation was repeated 3 times.

Extracts were then vacuum filtered to an Büchner flask by passing through a sequence of filters with progressively smaller mesh (0.70 µm, 0.45 µm and 0.2 µm filters). Paper filters (LLG GmbH, Am Hambuch, Germany) were used for the first step of filtration, while hydrophilic Nylon-66 membrane filters (Labbox Labware S.L., Barcelona, Spain) were used during the remaining steps. The extracts were then evaporated by using a rotatory evaporator RV 10 digital (IKA-Werke GmbH & Co, Staufen, Germany), setting a temperature of 40 °C for ethanol extracts and 50 °C for aqueous extracts, and then stored at -80 °C. When the extracts were almost completely dry, they were transferred to 2 ml amber HPLC vials (amber glass w/t PTFE caps, Supelco), completely evaporated under a flow of N_2 and then stored at -80 °C until use. Before fish exposure, ethanolic, ethyl acetate and water extracts were resuspended in

ethanol (Merck, Darmstadt, Germany), DMSO (Sigma-Aldrich, St. Louis, USA), and Milli-Q water (pH 7.4), respectively.

2.2.2. Aquatic bacteria extract

Twelve marine bacterial strains from the culture collection of the Faculty of Science, University of Porto were used, namely: *Alienimonas chondri* (LzC2), *Rhodopirellula lusitana* (CcC6), *Rhodopirellula rubra* (LF2), *Rhodopirellula baltica* (MsF2), *Bremerella* sp. (FF15), all belonging to the Phylum: Planctomycetes; *Aestuariibius insulae* (LzU14), *Proteus mirabilis* (118-13), from the Phylum: Proteobacteria; *Micrococcus luteus* (B02-26), *Gordonia* sp. (B02-2.22), *Dermacoccus* sp. (91-17), from the Phylum: Actinobacteria; and *Enterococcus faecalis* (118-3), Phylum: Firmicutes. One freshwater strain, *Aquisphaera giovannonii* (OJF2, Phylum: Planctomycetes), was also included in the study. Marine planctomycetal strains LzC2, LF2, CcC6, MsF2 and FF15 and freshwater strain OJF2 were fermented in 250 mL of M600 broth[490] or PYGV broth[491], respectively, for 14 days at 25°C under constant shaking (120 rpm). Cultures were then freeze-dried and extracted with acetone for 1h (CcC6, MsF2, FF15, and OJF2) or independently and consecutively for 1 hour with ethyl acetate, acetone and methanol (LzC2 and LF2).

The extracts were dried in a rotary vacuum evaporator until solid residues were achieved. Dichloromethane was then used to transfer each extract to a glass vial for final evaporation and dry weight calculated. Strains LzU14, 118-13, B02-26, B02-2.22, 91-17 and 118-3 were fermented in 100 mL of marine broth for 5 days at 25°C under constant shaking (120 rpm). 100 mL of acetone was then added to the cultures and incubated for 1h to assure cellular lysis. The acetone was evaporated in a rotary vacuum evaporator and the aqueous phase collected. 10g of the resin Amberlite™ XAD 16N (Supelco, Sigma) was added to each vial and incubated for 2 hours, for capture of metabolites. The Amberlite resin was then collected by filtration and subjected to double salt cleaning with 100 mL Millipore water. Finally, 50 mL of acetone were added to the resin for extraction of metabolites, for 1h. The Amberlite resin was discarded and the acetone was collected and dried in a rotary vacuum evaporator. Dichloromethane was then used to transfer each extract to a glass vial for a final evaporation and dry weight calculated. A total of 12 extracts were produced as a result. Before exposure to the fish, extracts were resuspended in DMSO (Sigma-Aldrich)

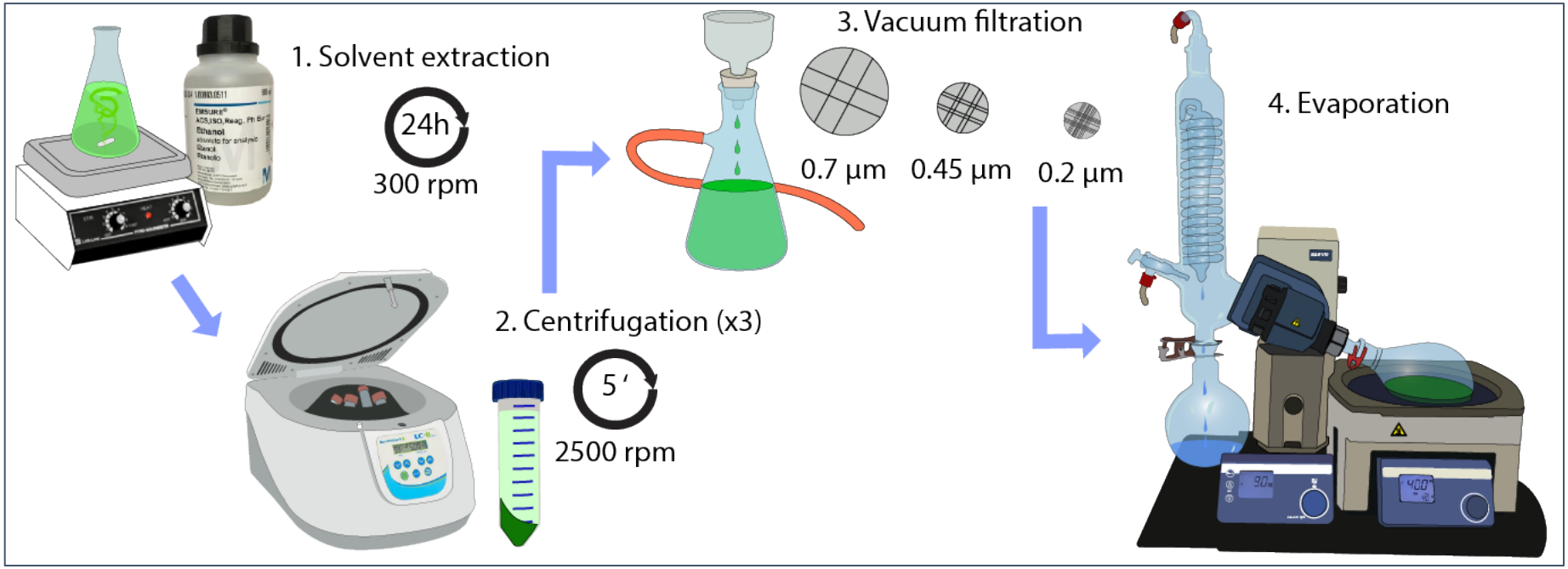


Figure 2.4. Extraction protocol used for the production of ethanol, ethyl acetate and aqueous extracts from 13 species of marine microalgae.

2.2.3. Cyanobacteria extraction and fractionation

Eight cyanobacteria strains isolated from the Portuguese coast and maintained in the LEGE Culture Collection (LEGE-CC), of the Interdisciplinary Centre of Marine and Environmental Research (CIIMAR, Portugal) were selected for bioactivity screening assays, namely: Unidentified colonial (UC) LEGEYY1 (E13012), *Nodosilinea* sp. LEGE06001 (E13019), UC LEGEYY2 (E14023), UC LEGEYY3 (E14027), *Synechococcales* LEGE10388 (E14034), UC LEGEYY4 (E14067), *Tychonema* sp. LEGE07215 (E15077), and UC LEGEYY5 (E15082). Strains were cultured in Z8 medium or Z8 supplemented with marine tropical salt (25 g/L), at 25ºC, with a photoperiod of 14 h light/10 h dark and at a light intensity of 10–30 μmol photons·m^{-2}·s^{-1}. The strains were grown in 20-L flasks, with constant aeration. At the exponential phase, cells were harvested through centrifugation, and subsequently frozen and freeze-dried. The dry biomass was extracted by repeated percolation with a warm mixture of CH_2Cl_2/MeOH (2:1, v/v). Crude extracts were fractionated by normal-phase Silica gel 60, 0.015–0.040 mm (Merck) VLC (vacuum liquid chromatography) with an increasing polarity grade, from 90% n-hexane to 100% ethyl acetate and 100% methanol, giving a total of 83 fraction were produced (from nine to eleven fractions each species, depending on the strain). Before exposure to the fish, extracts were resuspended in DMSO (Sigma-Aldrich).

2.2.4. Zebrafish husbandry

Broodstock of adult zebrafish (AB wild-type strain; ZFIN ID: ZDB-GENO-960809-7) were maintained at the zebrafish facility of the Centre of Marine Sciences (CCMAR, Portugal) in a ZebTEC recirculating system (Tecniplast, Italy) under the following conditions: temperature 28 ± 0.1ºC, pH 7.5 ±0.1, conductivity 700 ± 50 μS and a 14|10 h light/dark photoperiod. Conductivity and pH were stabilized using Instant Ocean salt mixture (Aquarium Systems, Sarrebourg, France) and sodium bicarbonate (Sigma-Aldrich), respectively, dissolved in reverse osmosis-treated water. Nitrogen compounds were monitored weekly and maintained within the following limits: lower than 0.1 mg/L for ammonia and nitrites, and lower than 50 mg/L for nitrates. Sexually mature zebrafish were crossed following an in-house breeding program and fertilized eggs were transferred into a 1-L container with static water conditions with the same parameters described above, and methylene blue (0.0002% w/v) was added to prevent fungal growth.

2.2.5. In vivo operculum screening assay

In order to evaluate the osteogenic potential of the extracts/fractions on bone growth, zebrafish larvae at 3 days post-fertilization (dpf) were exposed to the extracts/fractions at different concentrations in a 12-well plate (10 fish/well in 4 mL of fish water) and placed in the dark (to avoid photo-degradation of the compounds) at 28.5ºC. Positive control was10 pg/mL of calcitriol (1α,25-dihydroxyvitamin D_3, Sigma-Aldrich), and 0.1% DMSO or 0.1% ethanol (vehicle for extracts/fractions and calcitriol, respectively) were used as negative controls. Treatments were renewed (70% of the total volume) daily until the end of the treatment. At 6 dpf, and following the protocol previously described in **Chapter 2.1**, larvae were sacrificed with a lethal dose of MS-222 (0.6 mM, pH 7.0, Sigma-Aldrich), stained for 20 min at room temperature with 0.03% alizarin red S (AR-S) prepared in Milli-Q water (pH 7.4), and washed twice with Milli-Q water for 5 min. Stained larvae were then imaged in a fluorescence stereomicroscope (Leica)and analyzed morphometrically using ImageJ software version 2.0.0-rc-69/1.52p and the toolbox available within the "ZFBONE" macro toolset for Fiji[489].

2.2.6. Results

A total of 134 extracts/fractions, of which 39 from marine microalgae (Figure 2.5), 12 from aquatic bacteria, and 83 from marine cyanobacteria (Figure 2.6) were screened for their capacity to affect the ossification of the opercular bone in zebrafish larvae. Overall, 34 were able to significantly increase the mineralized area of the operculum:19 extracts from microalgae, 4 from aquatic bacteria, and 11 fractions from cyanobacteria. Of these extracts, 7 were mildly osteogenic, inducing an increase in the size of the operculum by 1-20% compared to their vehicle, and 22 were moderately osteogenic, inducing an increase in the size of the operculum by 20-40%. Five extracts and fractions demonstrated to be strongly pro-osteogenic, by increasing the size of the operculum in 40-60%. These were respectively, the ethanolic extracts from the microalgae *Skeletonema costatum* (+59%), *Tetraselmis rubens* (+46%), *Nannochloropsis oculata* (+42%), *Tetraselmis striata* CTP4 (+40%), and the F fraction of the extract from the cyanobacteria *Nodosilinea* sp. (Table 2.5). The aqueous extract from *Nannochloropsis oculata*, and 3 fractions from marine cyanobacteria (E13012-G, E14027-F, and E15077-C) were characterized by having anti-osteogenic activity.

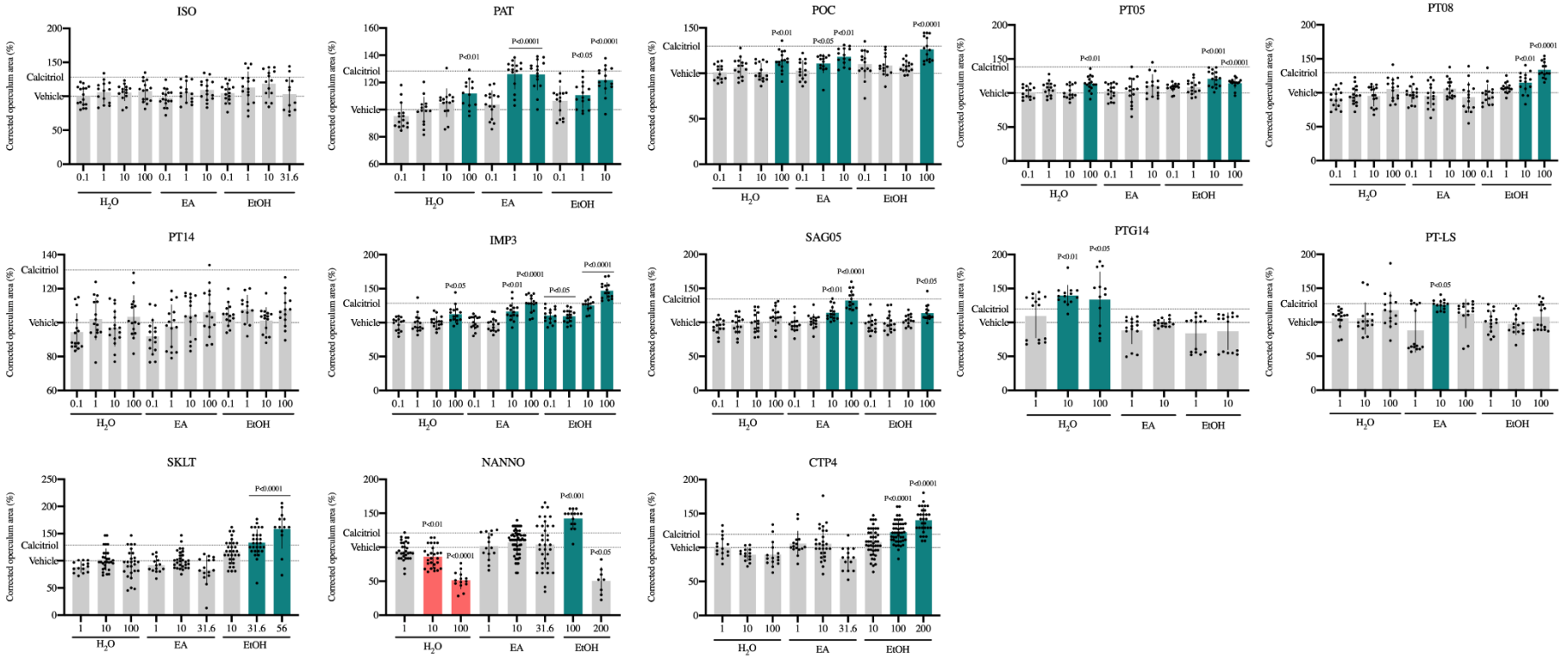


Figure 2.5. Osteogenic activity of microalgae-derived extracts. Results are displayed as corrected operculum area and expressed as percentage of variation relative to the negative control (vehicle used to solubilize the extract). Statistical differences were tested through one-way ANOVA followed by Dunnett's multiple comparison test ($p < 0.05$) or, whenever normality and homoscedasticity weren't met, through a non-parametric test followed by Dunn's multiple comparison test ($p < 0.05$). Statistically different p values are indicated on top of each column.

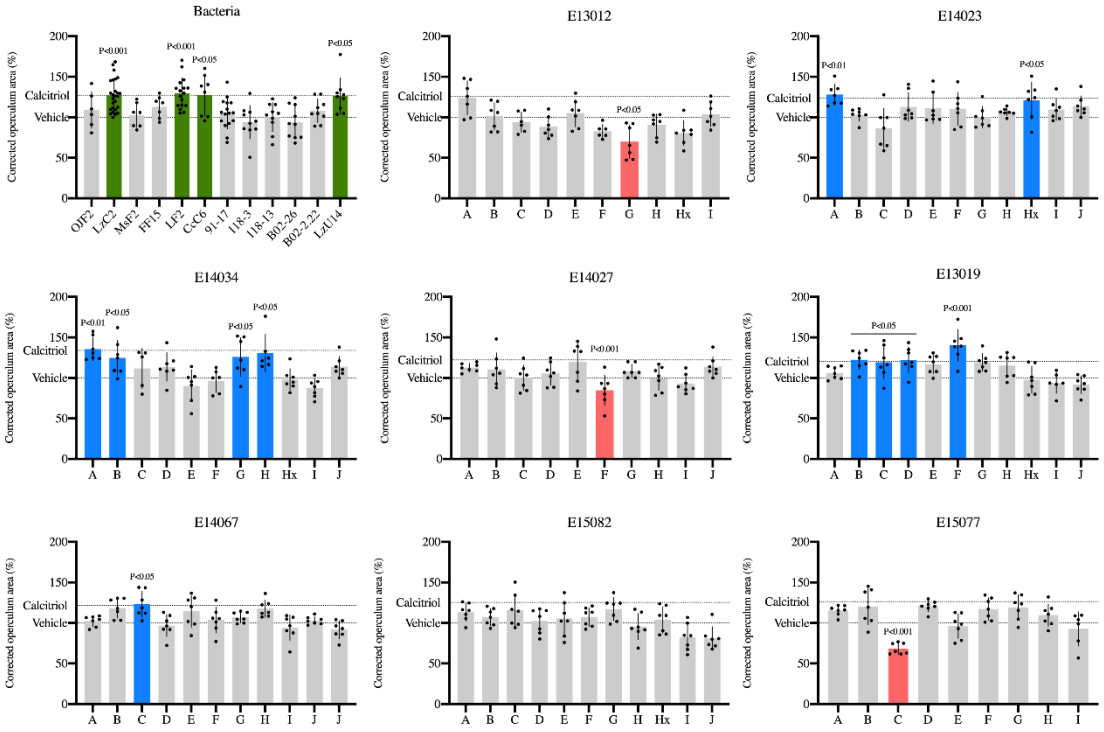


Figure 2.6. Osteogenic activity of extracts from aquatic bacteria and fractions obtained from cyanobacterial extracts. Results are displayed as corrected operculum area and expressed as percentage of variation relative to the control. Statistical differences among the means were tested through one-way ANOVA followed by Dunnett's multiple comparison test ($p < 0.05$) or, whenever normality and homoscedasticity weren't met, through a non-parametric test followed by Dunn's multiple comparison test ($p < 0.05$). Statistically different p values are indicated on top of each column.

Table 2.5. List of all the extracts/fractions screened with the operculum assay. EA, ethyl acetate; EtOH, ethanol; nd, not statistical different. Red text, increase in. operculum area > +40% in respect to the vehicle.

Group	Specie	Extraction method	Fraction	Increase in operculum area (%)
Microalgae	ISO, *Isochrysis* sp.	H_2O	-	nd
		EA		nd
		EtOH		nd
	PAT, *Phaeodactulum fusiformis*	H_2O	-	+11.9
		EA		+26.1
		EtOH		+21.7
	POC, *Porphyridium* sp.	H_2O	-	+13.9
		EA		+18.3
		EtOH		+26.5
	PT05, *Cylindrotheca fusiformis*	H_2O	-	+13.9
		EA		nd
		EtOH		+20.9
	PT08, *Tetraselmis striata*	H_2O	-	nd
		EA		nd
		EtOH		+33.9

	PT14	H_2O	-	nd
		EA		nd
		EtOH		nd
	IMP3, *Tetraselmis rubens*	H_2O	-	+12.5
		EA		+27.8
		EtOH		**+46.3**
	SAG05	H_2O	-	nd
		EA		+32.1
		EtOH		+13.6
	PTG14	H_2O	-	+39.6
		EA		nd
		EtOH		nd
	PT-LS	H_2O	-	nd
		EA		+25.5
		EtOH		nd
	SKLT, *Skeletonema costatum*	H_2O	-	nd
		EA		nd
		EtOH		**+58.9**
	NANNO, *Nannochloropsis oculata*	H_2O	-	-48.6
		EA		nd
		EtOH		**+42.3**
	CTP4, *Tetraselmis striata* CTP4 strain	H_2O	-	nd
		EA		nd
		EtOH		**+40.2**
Planctomycetes	*Alienimonas chondri*	ethyl acetate, acetone and methanol	LzC2	+27.0
	Rhodopirellula lusitana	Acetone	CcC6	+27.4
	Rhodopirellula rubra	ethyl acetate, acetone and methanol	LF2	+29.6
	Bremerella sp.	Acetone	FF15	nd
	Aquisphaera giovannonii	Acetone	OJF2	nd
Proteobacteria	*Aestuariibius insulae*	Acetone-resin-Acetone	LzU14	+26.5
	Proteus mirabilis	Acetone-resin-Acetone	118-13	nd
Actinobacteria	*Micrococcus luteus*	Acetone-resin-Acetone	B02-26	nd
	Gordonia sp.	Acetone-resin-Acetone	B02-2.22	nd
	Dermacoccus sp.	Acetone-resin-Acetone	91-17	nd
Firmicutes	*Enterococcus faecalis*	Acetone-resin-Acetone	118-3	nd
Cyanobacteria	UC LEGEYY1	E13012	A	nd
			B	nd
			C	nd
			D	nd
			E	nd
			F	nd
			G	-30.1
			H	nd
			Hx	nd
			I	nd
	UC LEGEYY2	E14023	A	+28.0
			B	nd
			C	nd
			D	nd
			E	nd
			F	nd
			G	nd
			H	nd
			Hx	+20.7
			I	nd
			J	nd
	Synechococcales LEGE10388	E14034	A	+35.5
			B	+24.7
			C	nd
			D	nd
			E	nd
			F	nd

			G	+25.9
			H	+30.8
			Hx	nd
			I	nd
	UC LEGEYY3	E14027	A	nd
			B	nd
			C	nd
			D	nd
			E	nd
			F	-15.3
			G	nd
			H	nd
			I	nd
	Nodosilinea sp. LEGE06001	E13019	A	nd
			B	+22.3
			C	+19.3
			D	+22.2
			E	nd
			F	**+40.2**
			G	nd
			H	nd
			Hx	nd
			I	nd
			J	nd
	UC LEGEYY4	E14067	A	nd
			B	nd
			C	+23.1
			D	nd
			E	nd
			F	nd
			G	nd
			H	nd
			I	nd
			J	nd
	UC LEGEYY5	E15082	A	nd
			B	nd
			C	nd
			D	nd
			E	nd
			F	nd
			G	nd
			H	nd
			Hx	nd
			I	nd
			J	nd
	Tychonema sp. LEGE07215	E15077	A	nd
			B	nd
			C	-31.8
			D	nd
			E	nd
			F	nd
			G	nd
			H	nd
			I	nd

2.3. DISCUSSION AND CONCLUSIONS

Given the compelling need for novel therapies for the treatment of bone disorders associated with mineral loss, and considering the rising interest of the pharmaceutical industry towards marine bioactive compounds, in the present chapter we explored the bioactive potential of various promising groups of marine organisms – i.e. sea cucumbers, tunicates, microalgae, bacteria, and cyanobacteria. Two separate screening projects were performed.

The first focused on aqueous (AQ) and ethanolic (HE) extracts from 3 species of sea cucumbers and 4 species of tunicates, two somewhat underexplored groups of marine invertebrates. An increasing body of evidences suggest that oxidative stress and inflammation are related to the etiology of osteoporosis[36]. Thus, screened these extracts for their antioxidant and anti-inflammatory activities, and their content of polyphenols, by applying several *in vitro* assays.

Concerning the sea cucumbers, previous reports described the presence of compounds with antioxidant activity, including polyphenols, in extracts of sea cucumbers[492]. A significant presence of phenolic substances was observed in species closely related to the ones studied in the present work including *H. atra*[492], *H. scabra*[493], and *H. arguinensis,* one species analyzed in the present work[494]. In the study by Roggatz et al[494], a cold-water extract of *H. arguinensis* was found to have a polyphenol content of 14.2 mg GAE/100 g dw, while no polyphenols were detected in the ethanolic extract.

Accordingly, also in our study a higher content of polyphenols was found in the aqueous extracts compared with the ethanolic. However, the extracts from sea cucumber showed lower content of polyphenolic compounds, when compared with the ones from tunicates. A lower antioxidant and anti-inflammatory potential were also reported for these species. Results from ABTS assay highlighted a moderate antioxidant potential for the AQ extracts of the three holothurians studied, but this observation was not consistent with the antioxidant capacity when tested through FRAP assay, which is based on a similar mechanism used to determine the presence of potential electron donors[485]. On the other side, the DPPH assay, which is used for the determination of compounds with proton donor potential[487], did not reveal a high antioxidant activity when compared with the other species studied. The presence of polyphenolic compounds and a moderate antioxidant potential reported for the AQ extracts from holothurians may indicate that these species of sea cucumbers deserve further attention as natural sources of polyphenolic compounds.

Tunicates yielded the most interesting results in terms of all bioactivities analyzed. In the literature, very few studies investigated the presence of antioxidant compounds in tunicates. A

radical scavenging potential for hot water extracts of *Styela clava* measured by ABTS assay was previously reported[495]. Consistently, we observed antioxidant activity on both AQ and HE extracts for the closely related species *Styela plicata*, measured by ABTS, DPPH, and FRAP assays. However, the species showing higher antioxidant potential were the three tunicates (*Aplidium* sp., *Botrylloides diegensis*, and *Ciona robusta*). These species presented the highest content of polyphenols, for both AQ and HE extractions, but with the HE extracts yielding higher contents.

The same three species were also characterized by the higher antioxidant activities, as measured by different methodologies. A positive correlation was found between polyphenol content and antioxidant potential measured with all the three methodologies used, namely, through the DPPH ($R^2 = 0.29$, $p = 0.0311$), ABTS ($R^2 = 0.70$, $p < 0.0001$), and FRAP ($R^2 = 0.32$, $p = 0.0212$), supporting the hypothesis that the antioxidant potential observed may be related to their high content in polyphenolic compounds.

HE fractions from all the four species of tunicates also presented the highest anti-inflammatory potential among all the extracts. No previous studies investigated the presence of polyphenolic compounds with anti-inflammatory activity in tunicates, but other compounds with anti-inflammatory properties were already described. For instance, it was previously reported the presence of a dermatan sulfate, similar in structure to the mammalian heparin, isolated from *Styela plicata* that was shown to display anti-inflammatory activity[496]. Moreover, two new tricyclic thiazine-containing quinolinequinone alkaloids, ascidiathiazones A and B, that were isolated from *Aplidium* sp. collected from the coast of New Zealand, were reported to inhibit the production of superoxide by human neutrophils[497].

Following the promising results obtained with antioxidant and anti-inflammatory activities, we tested the extracts for their osteogenic potential. For the AQ extracts from holothurians, the antioxidant activity reported was not translated into an appreciable effect in terms of induction on bone formation and mineralization *in vivo*. Conversely, HE extracts from two holothurians – *H. mammata* and *H. arguinensis* induced a pro-osteogenic effect in zebrafish larvae. However, these extracts were characterized by a low polyphenolic content, antioxidant activities and anti-inflammatory activities, compared with the tunicates, suggesting that non-phenolic compounds in holothurians may be involved in the effect observed. Nevertheless, holothurians have been described to be able to synthesize compounds with pro-osteoblastogenic potential, as observed by increased viability and activity (ALP) in a human osteoblastic cell line[353] and in rat bone marrow mesenchymal stem cells[354]. These observations

highlight the need for further investigating these two species as potential sources of osteogenic bioactives.

Concerning the tunicates, while AQ extracts did not induce osteogenic effects, the HE extracts from *Aplidium* sp., *Botrylloides diegensis*, and *Ciona robusta* were capable of inducing an increase in mineralized area of the opercular bone in zebrafish larvae. This increase was in a similar extent to the positive control (Calcitriol), and in the case of *Aplidium* sp. at 200 µg/mL (*Aplidium* sp. HE200), even surpassing the positive control value. These observations indicate that pro-osteogenic compounds were isolated through ethanolic extraction from the three tunicates species.

Overall, the *in vivo* pro-osteogenic effect combined with the high content of polyphenols, antioxidant and anti-inflammatory activities of the tunicates here studied led us to formulate the hypothesis that the pro-osteogenic activity induced by HE extracts can be due to polyphenolic compounds. Previous reports showed that polyphenols can stimulate osteoblast function by directly interacting with different molecular pathways involved in osteoblast differentiation[498,499], by attenuating detrimental effects from pro-inflammatory signals[500] and by exerting a protective effect against reactive oxygen species on osteoblastic cells[501]. Interestingly, a positive correlation was found between the pro-osteogenic and the anti-inflammatory bioactivities for the extracts from tunicates ($R^2 = 0.59$, $p = 0.0266$). This may indicate that the presence of an anti-inflammatory activity can be directly involved in the pro-osteogenic effects reported for the extracts.

Due to their richness in polyphenolic compounds, antioxidant and anti-inflammatory activities associated with their high pro-osteogenic potential, we identified potential candidates for the discovery of compounds with anti-osteoporotic potential in three species of tunicates *Aplidium* sp., *Botrylloides diegensis*, and *Ciona robusta*.

In the second screening project, we have conducted the screening of 134 extracts and fractions obtained from a total of 33 marine microbial species including microalgae, bacteria and cyanobacteria.

Marine microorganisms are considered an untapped source of bioactive compounds and previous reviews on the topic have reported that they provided between 22 and 34% of all marine bioactive compounds discovered so far[214,215]. Among those, microalgae, bacteria, and cyanobacteria are gaining momentum as highly promising sources of natural compounds with applications as pharmaceuticals and nutraceuticals[216–219]. Focusing on compounds with osteoactive potential, in the timeframe comprised between 1999 and 2022, marine microalgal species were the origin of 6 newly identified osteoactive compounds, while from bacteria and

cyanobacteria species 14 new osteoactives were isolated. As we highlighted in our review in **Chapter 1**, most of these compounds act through an antiresorptive mechanism.

Overall, with the two screening projects, we have evaluated a total of 148 extracts from 40 species of marine organisms. Out of these, six extracts, all obtained by ethanolic extraction, induced an increase in the size of the operculum in zebrafish larvae by more than 40% relative to the negative control (the vehicle used to solubilize the extract) – i.e. four from microalgal species - *Skeletonema costatum*, *Tetraselmis rubens*, *Nannochloropsis oculata*, and *Tetraselmis striata* CTP4; one from a cyanobacterium - *Nodosilinea* sp.; and one from a tunicate species - *Aplidium* sp. (Figure 2.7).

Regarding the interpretation of the read-out from the methodology used for the screening project, the operculum assay, it is important to notice that at the stage of zebrafish development used for analysis, neither active osteoclasts nor bone resorption occurs[478,502]. Therefore, the effect here observed for the extracts is due to pro-osteogenic rather than anti-resorptive mechanisms. As a result, two microalgal extracts were chosen as the focus of the next steps of this PhD project, the ethanolic extract from *Skeletonema costatum*, for being the most potent osteogenic inductor (+59% in operculum area), and the ethanolic extract from *Tetraselmis striata* CTP4, due to its potent osteogenic activity (+40% increase of the operculum), associated to the absence of toxic effects at all the concentration tested.

These findings provided evidence for the presence of pro-osteogenic compounds in some of the extracts tested, opening the opportunity for further isolation and characterization of compounds with potential for the treatment of bone erosive disorders in the species studied. Future research must be directed at the isolation of such compounds and at the dissection of the molecular mechanism of action involved in these osteogenic activities. As such, in the next chapter (**Chapter 3**) we will attempt the chemical characterization of the ethanolic extract from the microalga *Tetraselmis striata* CPT4, aiming at isolating putative compounds responsible for the osteogenic effects here observed, while in **Chapters 4**, **5**, and **6**, potential applications of these extracts in the fields of aquaculture and biomedicine will be explored.

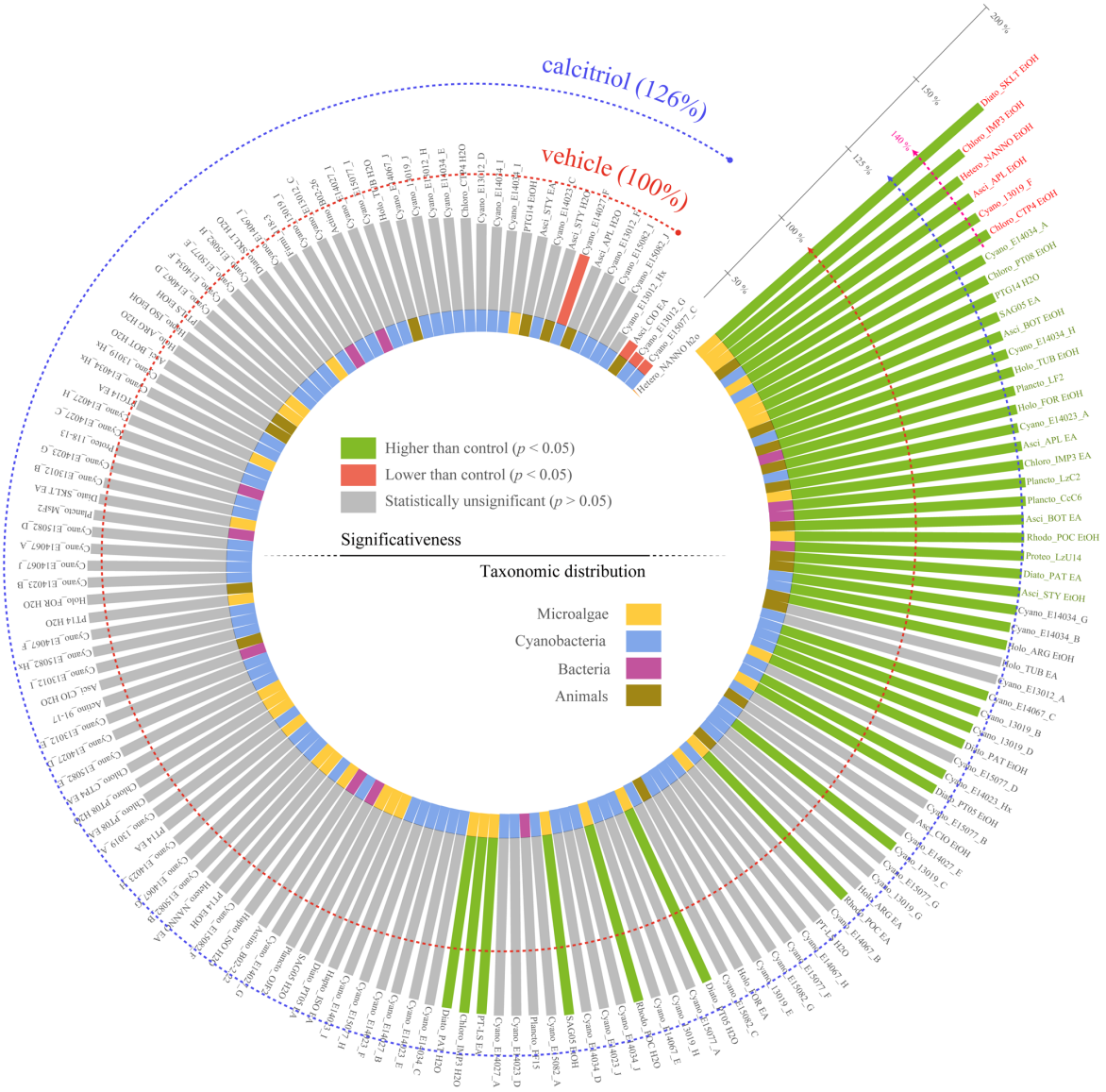
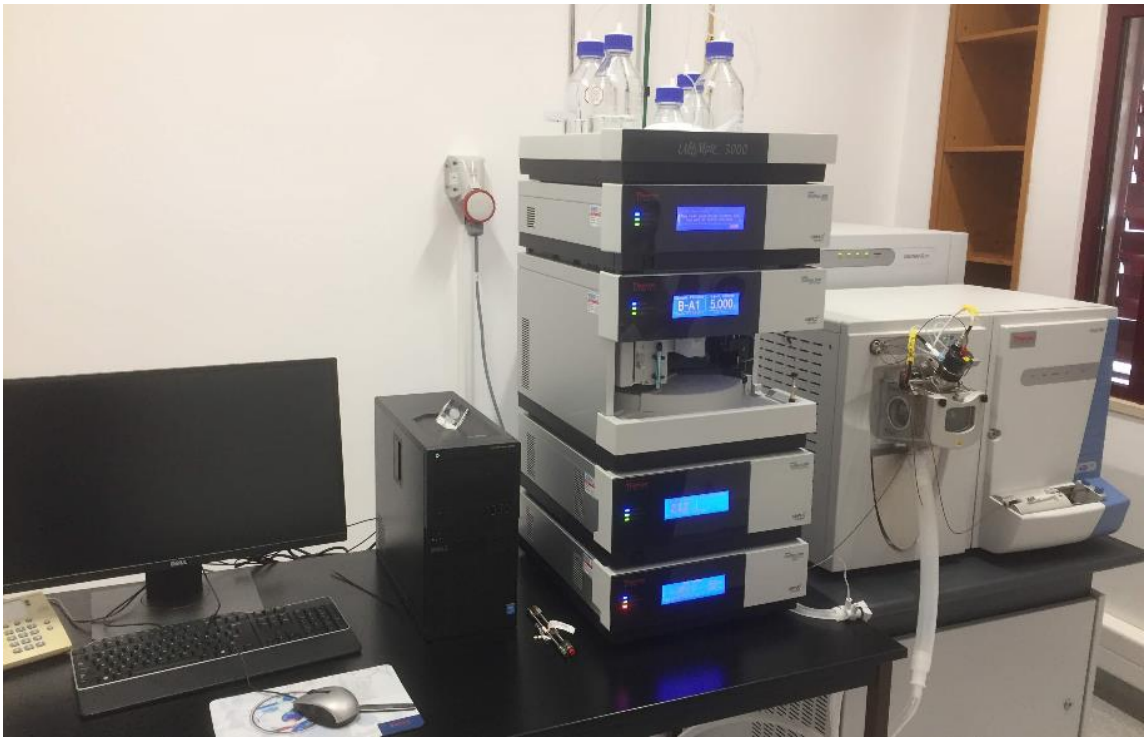


Figure 2.7. Osteogenic activity of extracts obtained from marine invertebrates, microalgae, bacteria, and cyanobacteria. Results are displayed as operculum area and expressed as percentage of variation over each extracts vehicle (negative control). Red dashed line indicates y = 100%, corresponding to the average value of the negative control group (vehicle); Blue dashed line indicates y = 126%, corresponding to the average of the positive control group (calcitriol at the concentration of 10 pg/mL); Pink dashed line indicates y = 140%. Statistically different values ($p < 0.05$) are indicated in green (osteogenic molecules) or red (anti-osteogenic molecules) in the top part of each column.

CHAPTER 3.

CHEMICAL CHARACTERIZATION OF A SELECTED MICROALGAE EXTRACT



UHPLC-HR-MS^n apparatus used for the chemical characterization of the microalgal extracts, at the analytical chemistry platform of the Centre for marine sciences (CCMAR), University of Algarve. Picture by Alessio Carletti.

3.1. Introduction

Following the screening of microalgae extracts (described in **Chapter 2.2**), four of these extracts were selected as the most promising ones. The ethanolic extracts from *Tetraselmis rubens*, *Skeletonema costatum*, *Nannochloropsis oculata*, and *Tetraselmis striata* CTP4 strain, were chosen due to their pro-osteogenic effect observed during zebrafish bone development. The ethanolic extract from *Tetraselmis striata* CTP4 (just CTP4, hereon) was selected as best candidate to perform the chemical characterization, following two criteria: (a) The absence of toxicity at all the concentrations tested in zebrafish larvae (up to 200 µg/mL); (b) The consistent commercial availability by the producer and industrial partner (Necton SA, Belamandil-Olhão, Portugal) of large amounts of microalgae biomass used to prepare the extract. Consequently, the CTP4 extract was processed through a bioassay-guided identification pipeline with the aim of identifying a set of compounds as potential candidates for being the ones responsible for the positive osteogenic effect reported in zebrafish larvae.

3.2. A bioassay-guided identification pipeline

CTP4 ethanolic extract chemical profiling was performed, in collaboration with colleagues from the Marine Biotechnology Group (MarBiotech) and the Analytical and Structural Chemistry Platform of the Centre for Marine Sciences (CCMAR). The identification of the compounds has been performed following a bioassay-guided identification pipeline as shown in Figure 3.1.

Briefly, the extract was fractionated by High Performance Liquid Chromatography (HPLC) through the use of a semi-preparative column, resulting in several fractions. These purified fractions were subsequently tested again for osteogenic activity through the zebrafish operculum assay. Fractions that retained the biological effect were further analyzed through Ultra High Performances Liquid Chromatography followed by Tandem Mass Spectrometry (UHPLC-HR-MS^n), for identifying compounds that are highly represented in the fractions. Compounds commercially available were, whenever possible, purchased and tested again with the zebrafish operculum assay to confirm or reject the hit.

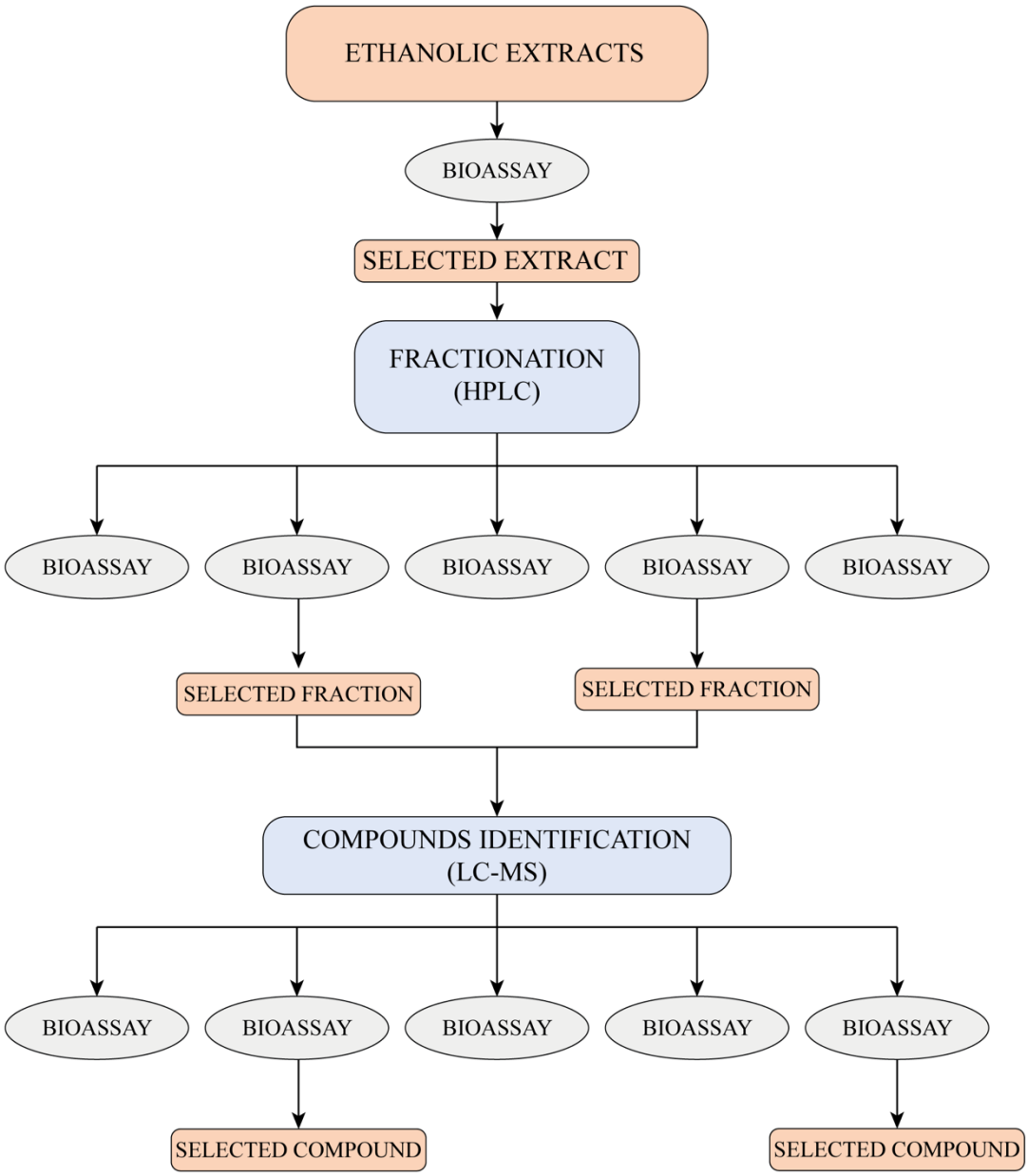


Figure 3.1. Bioassay-guided identification pipeline used for the identification of osteogenic compounds from the CTP4 extract.

3.3. Fractionation of CTP4 extract by High Performance Liquid Chromatography

Fractionation of the CTP4 extracts was performed by isocratic gradient HPLC, with the aim of isolating the compounds responsible for the osteogenic and mineralogenic effect in purified fractions. The instrumentation used was a preparative HPLC system (Knauer Wissenschaftliche Geräte GmbH, Berlin, Germany) equipped with a UV detector able to screen

absorbance in 4 channels (C1: 254 nm, C2: 280 nm, C3: 350 nm, C4: 444nm). The extract was run through an HPLC preparative column with load capacity from 1 to 25 mg (Phenomenex®, Torrance, USA) and eluted with a set of solvents spanning a wide range of polarity (hydrophobicity). Milli-Q water, Acetonitrile (C_2H_3N, Fisher Scientific, Waltham, USA) and Ethyl acetate 99.9% ($C_4H_8O_2$, Fisher Scientific) were filtered through a 0.2 µm PFOE filter and degasified by the use of a 2510EMTH ultrasonic cleaner (Bransonic®, Emerson, St. Louis, USA) before use. The extract was resuspended in absolute ethanol to obtain a concentration of 50 mg/ml, in order to not exceed the loading capacity of the column (25 mg, 250 µL injection loop), and filtered through a 0.2 µm PFOE filter. The solvent program used for the elution is presented in Table 3.1. Before injecting the sample, the solvent lines were purged and the column was cleaned by running the starting solvent mix (90%H_2O – 10 % Acetonitrile) slowly increasing the flow rate from 0.1 ml/min to 4 ml/min and then allowed to flow with these condition for 30 minutes, in order to stabilize the internal pressure of the column. When the pressure stabilized at 190-220 x 0.1 MPa, 250 µL of extract were injected into the column automatically starting the elution program.

In an exploratory run, the UV spectrometer detected numerous peaks, as shown by the chromatogram in Figure 3.2, highlighting that the extract is characterized by a high chemical complexity. As such, the extract was separated into 15 fractions, by sampling the eluted compounds every 5 minutes along the 70 minutes program. It was expected to find polar compounds in the first fraction eluted by the initial solvent mix (90%H_2O – 10 % Acetonitrile), while more and more non-polar compounds were expected in the last fractions, to be eluted by hydrophobic solvents such as the ethyl acetate. The run was repeated 12 times, and eluted fractions were accumulated in 40 ml amber vials. The 15 fractions obtained were placed under a mild flow of N_2 for 1 week (12 h/day), until complete evaporation of the solvents and stored at -30 °C in amber HPLC vials (Supelco®, Sigma) until use.

Table 3.1. Solvents elution program for the preparative HPLC used to fractionate CTP4 ethanolic extract.

Step	Time (min)	H_2O (%)	Acetonitrile C_2H_3N (%)	Ethyl acetate C_4H_8O (%)	Flow rate (ml/min)
1	initial	90	10	0	4.000
2	2.00	90	10	0	4.000
3	37.00	0	100	0	4.000

4	42.00	0	100	0	4.000
5	45.00	0	100	0	4.000
6	55.00	0	40	60	4.000
7	57.00	0	40	60	4.000
8	72.00	0	100	0	4.000
9	74.00	0	100	0	4.000
10	76.00	90	10	0	4.000

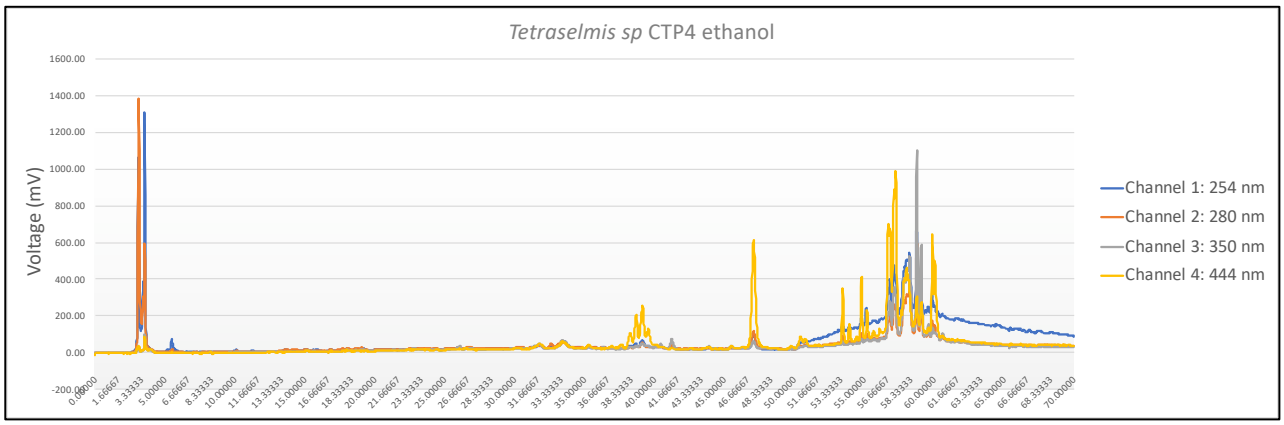


Figure 3.2. HPLC chromatogram of the CTP4 extract monitored by UV absorption at 254 nm, 280 nm, 350 nm and 444 nm.

3.4. Screening of the purified fractions

Fractions produced by HPLC were screened with the operculum assay, to assess if any fraction had retained the biological effect observed in the whole extract. All the fractions were resuspended in absolute ethanol and sonicated with a 2510EMTH ultrasonic cleaner, to achieve complete solubilization of the fraction. Each fraction was resuspended in a variable volume of ethanol, in order to obtain the higher concentration possible to be tested in zebrafish larvae. Due to the differences in the relative content of the compounds present in the extract, yields of the 15 fractions were highly variable. All the 15 fractions were screened with the operculum assay by using the methodology described in **Chapter 2.2**. Only the higher possible concentration was tested for each condition (Table 3.2), together with their respective solvent (0.1% v/v ethanol) as negative control, and calcitriol (Sigma-Aldrich) at 10 pg/mL as positive control. Mortality occurred for two of the fractions: F8 and F9, by 40 and 80%, respectively.

In order to test if these fractions could have a valuable biological activity, they were diluted 1:10 and tested again.

Table 3.2. Concentrations tested by the operculum assay for the 15 fractions obtained from the CTP4 ethanol extract by HPLC. When mortality was observed, indicated as: * (% mortality) in the table, the fractions were tested again at a lower concentration (1:10).

Fraction	***Concentrations tested (µg/mL)***
1	200
2	20
3	50
4	20
5	20
6	20
7	40
8	100* (40%), 10 (0%)
9	50* (80%), 5 (0%)
10	100
11	80
12	100
13	50
14	20
15	10

The fraction F7 (40 µg/mL) was able to increase the area of the head in 6 dpf zebrafish embryos (Figure 3.3A), although the standard length was unchanged (Figure 3.3B). Surprisingly, 9 fractions were able to significantly increase opercular growth (Figure 3.4A). The fractions that induced a higher increase of the operculum area were F5 (+56.2%), F7 (+60.6 %), F12 (+67 %), F13 (+62 %), and F11 (+121.7 %). None of the 2 fractions tested at lower concentration (1:10) were able to affect the size of the operculum (results not shown).

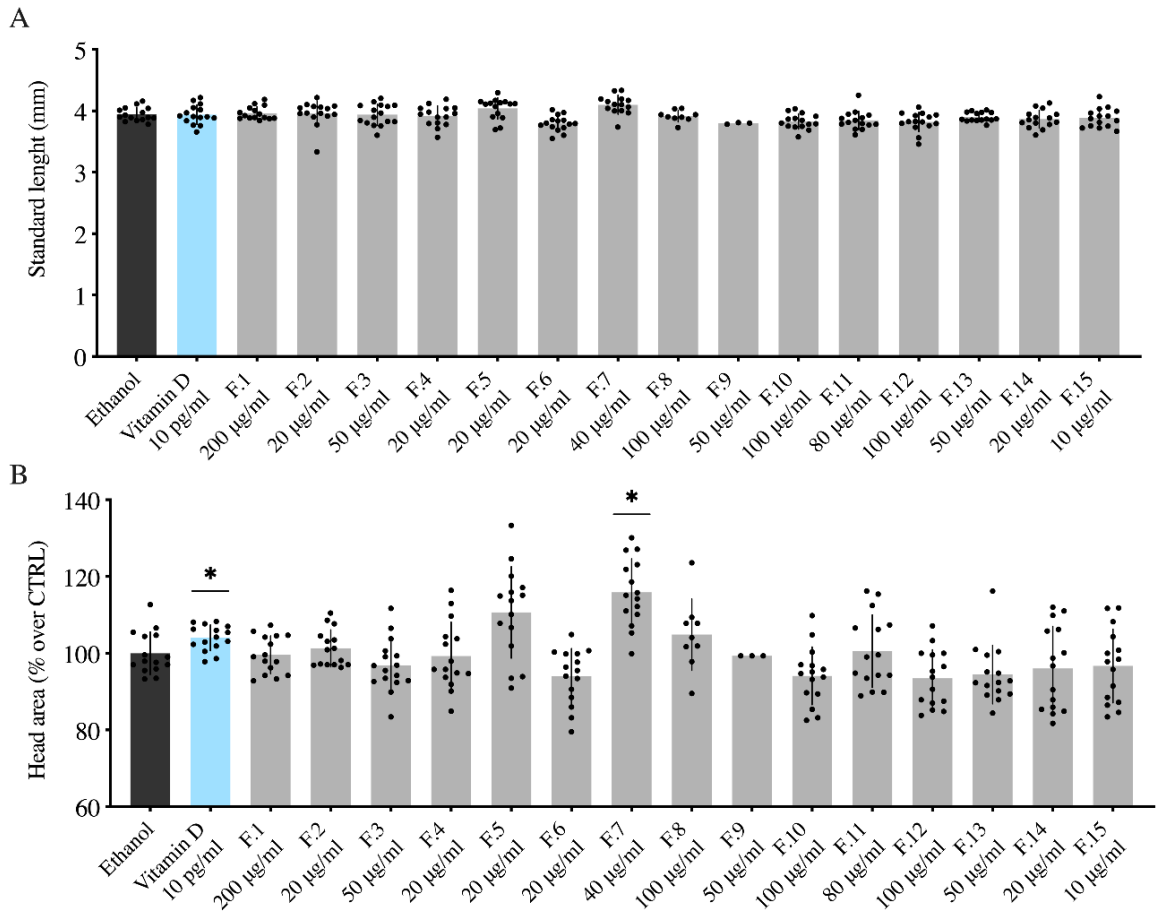


Figure 3.3. Effect of the 15 fractions (F1-F15) from CTP4 extract on the standard length (A), and on the head area (B) of 6 dpf zebrafish larvae. Statistical differences were tested through a one-way ANOVA. *p* values are indicated as follow: 0.0332 (*).

Various fractions displayed an increased the number of mineralized centra at 6 dpf compared with the negative control (Figure 3.4B). In details, for fractions F10 and F11, an effect on the mineralization patterning of the notochord was observed. In particular, 53 % and 67 % of F10 and F11 fish, displayed an early fusion of the 3rd, 4th, and 5th vertebral bodies, respectively (Figure 3.5). This effect is remarkably similar to what was reported by previous works to be caused by treatment with high concentrations of retinoic acid, and its capacity to inhibit the intervertebral disk formation[503,504]. Accordingly, fractions F10 and F11 contained compounds with elution times between 45-60 minutes, which were placed in an area of the chromatogram where liposoluble compounds, including retinoids, are expected to be eluted. Among those, some carotenoids like zeaxanthin, produced by *Tetraselmis striata* and other microalgae, have been reported to be able to bind the retinoic acid receptor and activate downstream signaling pathways[505,506].

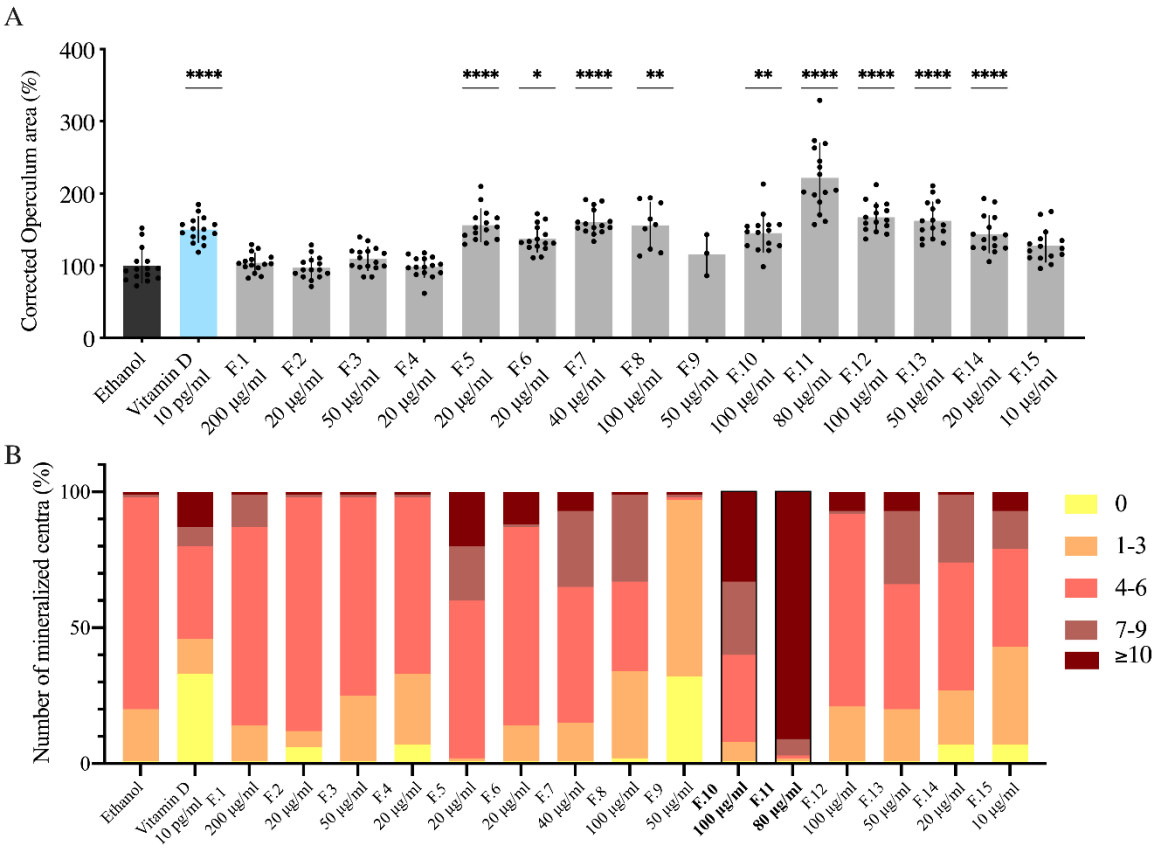


Figure 3.4. Osteogenic effect of the 15 fractions (F1-F15) from CTP4 extract on the operculum area (**A**), and on the number of mineralized vertebral centra (**B**) in 6 dpf zebrafish larvae. In (**B**) **Bold text** and column borders - fractions where fish had fusion of vertebral bodies. In (**A**) Statistical differences were tested through a one-way ANOVA. *p* values are indicated as follow: 0.0332 (*), 0.0021. (**), < 0.0001 (****).

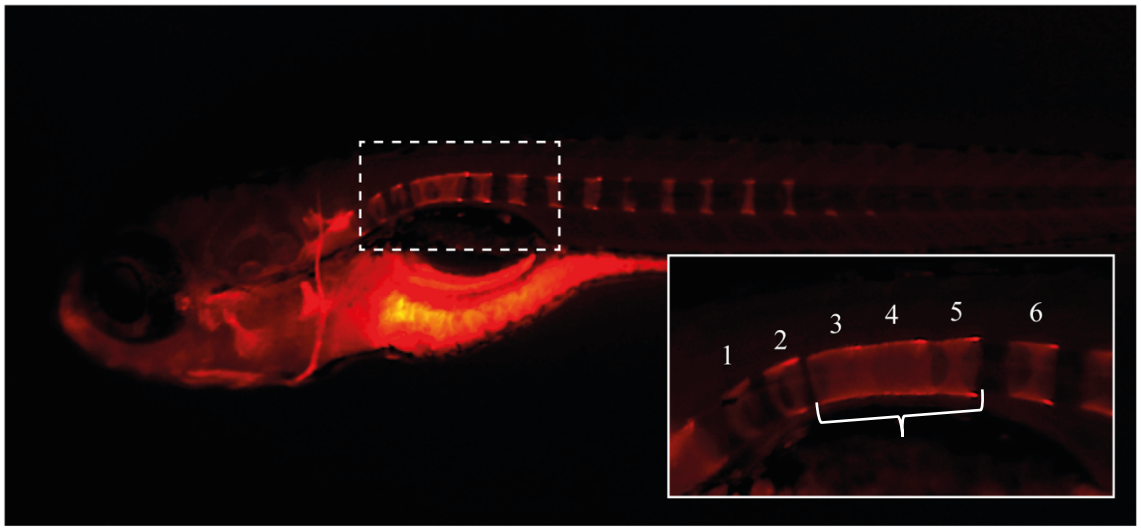


Figure 3.5. Representative images of the vertebral fusion induced by F11 in 6 dpf zebrafish larvae. Fused vertebral bodies 3, 4, and 5 are indicated in the fluorescent picture of an AR-stained larva.

These results indicate that the fractionation allowed the separation of some of the compounds responsible for the effect in the original extract. The fractions F5 and F7 were selected for further analysis as the most promising, as they were showing high osteogenic effect at low concentrations (20 and 40 µg/mL for F5 and F7, respectively) and were not associated with any detectable adverse effects.

3.5. Chemical characterization of candidate fractions though UHPLC-HR-MS^n

Fractions F5 and F7 were selected and firstly run through UHPLC-HR-MS^n against their vehicle (ethanol 100%) with the aim of exploring their chemical complexity.

Chromatographic separation was performed on an *Ultimate* 3000 UHPLC (Thermo Fisher Scientific, Waltham, USA). The column used was a Thermo Scientific Accucore RP-18 (2.1 × 100 mm, 2.6 µm). The mobile phase composition was prepared with water and acetonitrile, both containing 0.1 % of formic acid. The gradient started with 100 % of water for 2 min, increased linearly to 30 % of acetonitrile in 13 min, to 100 % of acetonitrile in 16 min, and was maintained for 4 min, then returned to 100% of water in 1 min and was maintained for 4 min. The flow rate was 0.3 mL/min and the injection volume 5 µL.

Mass analyses were performed on an Orbitrap Elite (Thermo Scientific) mass spectrometer with a Heated EletroSpray Ionization source (HESI-II). Acquisition was performed under positive and negative polarities. High Resolution Tandem Mass Spectrometry (HR-MS^n) data was acquired using the following ionization parameters: spray voltages, 3.7 kV (positive polarity) and 4.0 kV (negative polarity); sheath gas, 40 arbitrary units; auxiliary gas, 10 arbitrary units; heater temperature, 300 °C; capillary temperature, 350 °C; S-Lenses RF level, 64.9 %. Scan range was 100-1000 m/z. Fragmentation spectra were obtained by running the system in data dependent mode using dynamic exclusion. Liquid Chromatography-Mass Spectrometry (LC-MS) profiles were analyzed using Compound Discoverer 3.1 which search compounds hits within two databases: mzVaults (Software Version 2.1, Thermo Scientific), and with the mzCloud™ online spectral library[507].

A first data mining, conducted by considering all the hits within the compound libraries, reported that more than 8000 compounds were present in the two fractions, thus suggesting a

very complex chemical composition. As such, a second run was performed with the aim of identifying candidate osteogenic compounds. This time, a comparative strategy was used in order to reduce the list of possible candidate compounds. The fractions F5 and F7 were run against a couple of arbitrary reference fractions represented by the fractions immediately nearby on the sequence of the fractionation by HPLC (F4 and F6 against F5, and F6 and F8 against F7, respectively). The reference fractions were chosen as they showed a lower or no osteogenic bioactivity compared with F5 and F7, suggesting that compounds responsible for the biological activity observed were likely to be found at higher relative concentration within the fractions F5 and F7. The alignment of the mass spectra with the databases yielded 7778 compound hits. In order to reduce the number of candidate compounds, a threshold of 70% similarity match in the mass spectra profile between the hits and the unknown compounds studied was chosen. This allowed reducing the number of candidate compounds to 133. Furthermore, a second threshold filter was applied, this time selecting only the compounds with a higher relative concentration in the fractions F5 and F7 (with a factor of 10) than in the reference fractions. This operation allowed to reduce the number of candidate compounds to 15 (Table 3.3).

Table 3.3. Compounds selected as possible candidates for having osteogenic activity in the fractions F5 and F7 of CTP4 ethanolic extracts.

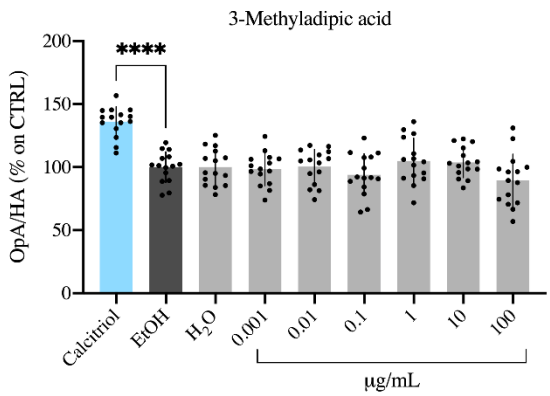
N°	Compounds name	mzVault/mzCloud best match (%)
1	3-Methyladipic acid	99
2	5-(6-hydroxy-6-methyloctyl)-2,5-dihydrofuran-2-one	80
3	(1S,6R,11aR,13R,14aS)-1,13-dihydroxy-6-methyl 1H,4H,6H,7H,8H,9H,11aH,12H,13H,14H,14aH-cyclopenta[f]oxacyclotridecan-4-one	90
4	10-Nitrolinoleate	73
5	1-Linoleoyl glycerol (1-Monolinolein)	98
6	Glyceryl monooleate (Monoolein)	99
7	2-Arachidonoyl glycerol	91
8	(9E)-9-Tetradecenamide	71
9	[5-hydroxy-3-(hydroxymethyl)-2-oxo-6-propan-2-ylcyclohex-3-en-1-yl] 3-methylbutanoate	71
10	(E)-6-hydroxyoctadec-4-enoic acid	82

11	(10E,12E)-9-hydroxyoctadeca-10,12-dienoic acid	89
12	hexadecanedioic acid	89
13	(10Z,13Z)-15,16-dihydroxyoctadeca-10,13-dienoic acid	97
14	17-(2,6-dihydroxy-6-methyl-3-oxoheptan-2-yl)-2,3,16-trihydroxy-4,4,9,13,14-pentamethyl-1,2,3,7,8,10,12,15,16,17-decahydrocyclopenta[a]phenanthren-11-one	73
15	10-ethyl-7,9-dimethyl-4,8-dioxospiro[4.5]dec-9-ene-1-carboxylic acid	78

Seven commercially available compounds were purchased (Table 3.4) and tested at different concentrations using the operculum assay, to evaluate their osteogenic bioactivity and confirm the hits from the LC-MS analysis. Overall, none of the molecules tested was able to induce a substantial osteogenic effect (Figure 3.6).

Table 3.4. Compounds purchased for testing for osteogenic activity.

Name (IUPAC)	Common name	Molecular Weight	mzVault match (%)	mzCloud match (%)	Peak aera						PubChem Reference
					Ethanol	F4^{R}	F5*	F6^{R}	F7*	F8^{R}	
3-methylhexanedioic acid	3-Methyladipic acid	160.0733	82.0	98.7	6.2E+04	7.7E+05	3.4E+05	7.2E+05	4.7E+06	2.4E+06	https://pubchem.ncbi.nlm.nih.gov/compound/12292
(9E,12Z)-10-nitrooctadeca-9,12-dienoic acid	10-Nitrolinoleate	307.2132	72.6	-	4.1E+04	1.7E+05	3.4E+05	1.2E+06	2.4E+06	1.8E+05	https://pubchem.ncbi.nlm.nih.gov/compound/5282259
2,3-dihydroxypropyl (9Z,12Z)-octadeca-9,12-dienoate	1-Linoleoyl glycerol	354.2753	98.1	74.1	4.0E+04	1.6E+05	7.1E+06	6.0E+05	2.0E+07	5.2E+05	https://pubchem.ncbi.nlm.nih.gov/compound/5283469
2,3-dihydroxypropyl (Z)-octadec-9-enoate	Glyceryl monooleate	356.2908	99.2	95.9	3.5E+04	4.0E+04	3.1E+05	2.2E+06	1.5E+07	1.1E+05	https://pubchem.ncbi.nlm.nih.gov/compound/5283468
1,3-dihydroxypropan-2-yl (5Z,8Z,11Z,14Z)-icosa-5,8,11,14-tetraenoate	2-Arachidonoyl glycerol (2-AG)	378.2752	91.2	-	1.9E+04	2.3E+04	3.4E+04	1.8E+05	2.7E+06	3.3E+04	https://pubchem.ncbi.nlm.nih.gov/compound/5282280
Hexadecanedioic acid	Thapsic acid	308.1975	88.6	-	5.2E+04	2.7E+06	1.2E+07	5.1E+06	4.7E+05	5.6E+05	https://pubchem.ncbi.nlm.nih.gov/compound/10459
(1S,6R,11aR,13R,14aS)-1,13-dihydroxy-6-methyl-1H,4H,6H,7H,8H,9H,11aH,12H,13H,14H,14aH-cyclopenta[f]oxacyclotridecan-4-one	Brefeldin A	280.1665	90.4	-	1.2E+05	2.8E+05	4.0E+05	1.3E+05	3.0E+07	1.2E+06	https://pubchem.ncbi.nlm.nih.gov/compound/5287620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OpA/HA (% on CTRL)
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150
100
50
0
****
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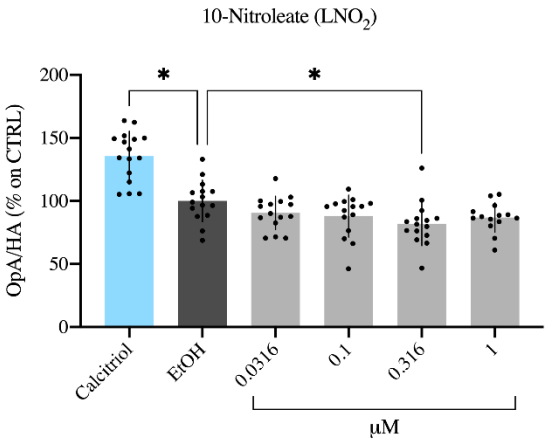
0.01
0.1
1
10
100
µg/mL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OpA/HA (% on CTRL)
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50
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*
*
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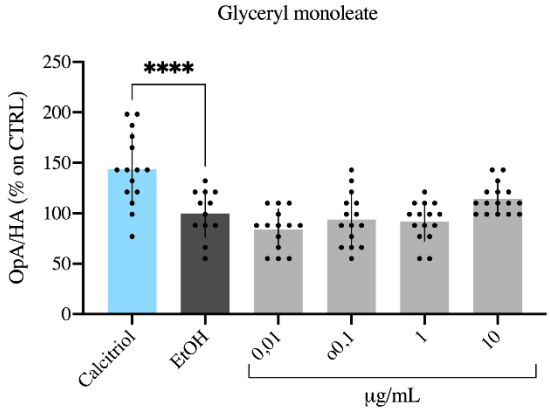
0.316
1
µM


Glyceryl monoleate
OpA/HA (% on CTRL)
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200
150
100
50
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Calcitriol
EtOH
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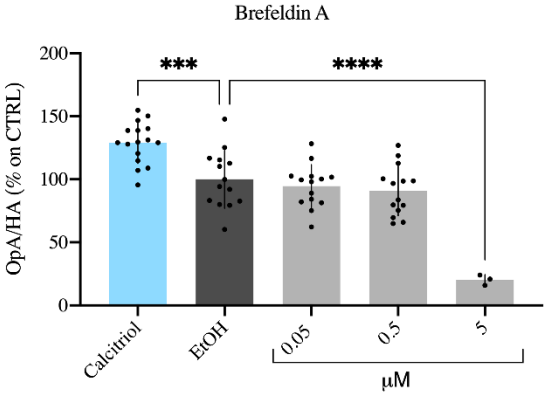
0,1
1
10
µg/mL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OpA/HA (% on CTRL)
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50
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***
****
Calcitriol
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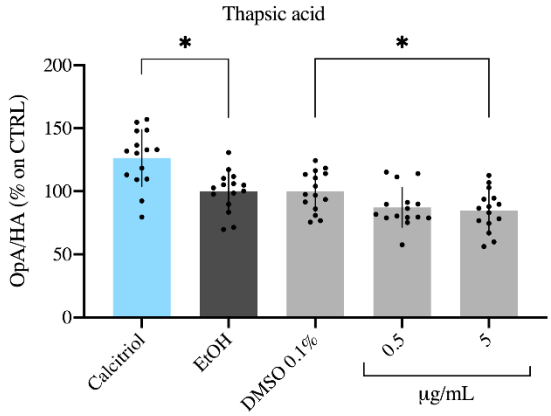
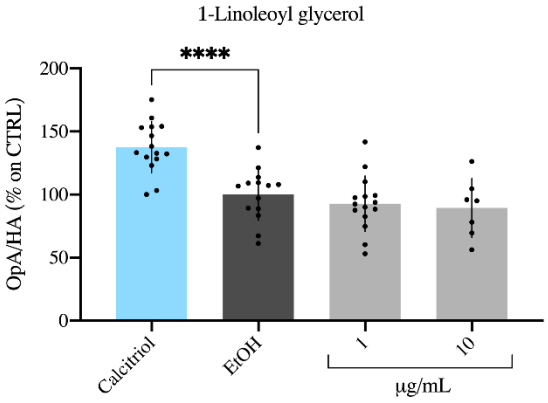
0.5
5
µM


Thapsic acid
OpA/HA (% on CTRL)
200
150
100
50
0
*
*
Calcitriol
EtOH
DMSO 0.1%
0.5
5
µg/mL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OpA/HA (% on CTRL)
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150
100
50
0
****
Calcitriol
EtOH
1
10
µg/mL

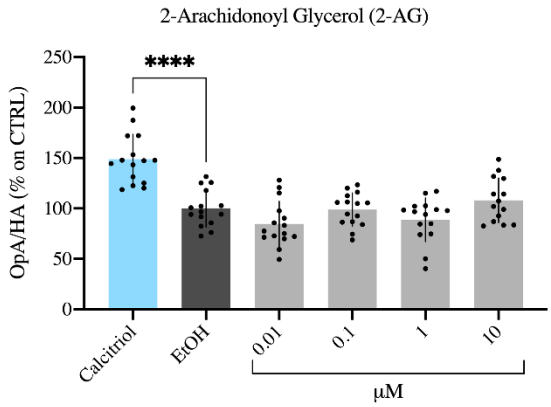

2-Arachidonoyl Glycerol (2-AG)
OpA/HA (% on CTRL)
250
200
150
100
50
0
****
Calcitriol
EtOH
0.01
0.1
1
10
µM

Figure 3.6. Screening of 7 candidate compounds using the zebrafish operculum assay. Normality was tested for each individual group through D'Agostino-Pearson omnibus normality test. Statistical differences among the means were tested through one-way ANOVA and Dunnett's multiple comparison test ($p < 0.05$) whenever distribution resulted normal. Otherwise, differences were tested with a non-parametric Kruskal-Wallis test followed by Dunn's multiple comparison test ($p < 0.05$). p values are indicated as follows: 0.0332 (*), 0.0002 (***), < 0.0001 (****).

3.7. Discussion and Conclusions

In the present work, we have performed the chemical characterization of CTP4 extract by applying a bioassay-guided identification pipeline where the extract was fractionated, and the resulting fractions were tested with the same assay used for detecting the bioactivity of the crude extract.

Bioactivity-based pipelines are commonly used for the identification of bioactive compounds from natural substrates that bear the intrinsic challenge of unambiguously identify which compound is responsible for the biological effect observed during bioassays[508–510]. In the present study, the chromatographic fractionation of CTP4 extract yielded 15 fractions, 8 of which showed a higher osteogenic effect and for some of these fractions the effect was achieved at lower concentrations when compared with the crude extract.

The observation of different fractions retaining a biological activity, despite containing compounds with different physical-chemical properties, suggests that the bioactivity of the crude extract might be attributed to the synergy of different compounds. Fractions F5 and F7 were chosen for being the fractions with the higher osteogenic effect, lacking at the same time negative effects on the skeleton of zebrafish larvae. Fractions F10 and F11, although strongly pro-osteogenic, were characterized by the occurrence of vertebral fusions in the zebrafish larvae and were therefore, discarded. Fractions 12, 13, and 14 were discarded for being the ones eluted by the most non-polar solvent mixtures, which contains high concentration of ethyl acetate, and therefore are expected to contain a wide range of already well known pro-osteogenic compounds produced by marine microalgae, including liposoluble vitamins (A, D, E, K) and strongly non-polar fatty acids[511,512].

Nonetheless, conducting a more in-depth characterization of the chemical composition of the fractions 10-14 in the future should be a priority, in order to explore whether unknown osteogenic compounds might be found. In this regard, the application of liquid chromatography

coupled with mass spectrometry (LC-MS) is considered an optimal strategy for compounds dereplication in drug discovery, with the aim of excluding the already known compounds from the list of possible candidates for the bioactivity observed[513–516].

Fractions F5 and F7 were processed through LC-MS analysis, which resulted into more than 8000 molecule hits between the two fractions. This observation highlighted an extremely complex chemical composition which represent a limit for compound identification. In order to reduce the number of candidate compounds for biological testing and hit confirmation, we selected compounds based on their relative abundance within the fractions F5 and F7, in respect to neighboring reference fractions chosen among the less active ones (F4, F6, and F8).

As a result, fifteen compounds were identified as possible candidates based on their high relative abundancy in F5 and F7. Among these, 8 were fatty acid-derived compounds and 1 a terpenoid. Some of these compounds were previously reported for having biological activity. In detail, 1-linoleoyl glycerol (1-monolynolein) and glyceryl monooleate (compounds 5 and 6 in Table 3.3, respectively), are plant-derived natural products related to linoleic acid and oleic acid. Both 1-linoleoyl glycerol and glyceryl monooleate are found in several plants and bacterial species (see PUBCHEM CIDs 5283469, and 5283468) and were described to have antiviral activity[517]. In addition, an herbal extract containing 1-monolinolein as main ingredient was shown to possess anti-osteoclastogenic activity and anti-osteoporotic activity in ovariectomized rats, although 1-monolinolein alone resulted uneffecitve[518]. 10-Nitrolinoleic acid (LNO_2, compound 4 in Table 3.3, PUBCHEM CID 5282259), is a nitrified fatty acid found in humans as derivative of linoleic acid and a component of the nitric oxide (NO) signaling cascade. It acts as a potent endogenous anti-inflammatory mediator through the activation of endothelial heme oxygenase 1 (HMOX1) expression[519,520]. LNO_2 was also found to have a role in Neutrophils activation [521], Lymphocytes adhesion[522], and was described to be an endogenous ligand of the peroxisome proliferator-activated receptor γ (PPARγ)[523,524].

2-Arachidonoylglycerol (2-AG, compound 7 in Table 3.3, PUBCHEM CID 5282280), is a human endocannabinoid derived from arachidonic acid, and an endogenous agonist of the cannabinoid receptors (CNR1 and CNR2)[525–527]. It has a role in synaptic transmission[528], immune response and inflammation[529]. The endocannabinoid system was previously described to regulate bone metabolism. In more detail, osteoblasts and osteoclasts produce 2-AG and express the cannabinoid type 2 receptor (CNR2). In previous studies, CNR2-deficient mice (*Cnr2*$^{-/-}$) showed an accelerated age-related bone loss, and SNPs in the *CNR2* gene in osteoporotic women are associated with lower bone mineral density[530]. In addition, the cannabinoid type 1 receptor (CNR1), is expressed in sympathetic nerves in proximity to

osteoblastic cells, and its activation by 2-AG signaling, expressed as a response of bone to mechanical damage, stimulates bone anabolic processes[531]. 2-AG was also described to have a dual effect on human osteoblasts, by increasing early- but decreasing late differentiation markers[532].

Of the fifteen compounds selected following the mass spectrometric analysis, seven compounds were commercially available and were therefore purchased and screened for osteogenic potential with the operculum assay. However, none of them resulted positive for osteogenic activity in zebrafish larvae.

In this regard, several sources of variation might be at the base of the occurrence of false hits during compound selections. These include: The variability of the experimental conditions and equipment used among different laboratories contributing to the mass spectrometry databases[533], the highly complex composition of the fractions, and the presence of structural isomers, which are not discerned by normal mass analysis. These factors might have concurred into the misidentification of the compounds selected, and it is not to exclude that the candidate compounds purchased might indeed be only similar in chemical nature to the ones that are found in the extract.

To explore this possibility in the future, a potential strategy would be to run the extract and the standard compounds parallelly, and then compare the resulting mass spectra. However, the negative results obtained might also not be related to the misidentification of compounds, but rather to the fact that the compounds responsible for the positive effect observed in the extracts have very high bioactivity or act at very low concentrations, therefore being poorly represented in terms of relative abundance. If this is the case, our strategy used for identifying the best candidate compounds would not be suitable for the identification of poorly represented compounds.

In addition, the approach of looking for hits within libraries of mass spectra databases intrinsically exclude the possibility of identifying completely novel compounds. As such, the optimal strategy which may be applied in the future to identify such compounds consist on further sub-fractionating the extract up to isolating pure compounds. These sub-fractions may allow for purification of unknown compounds that could be studied by the application of other analytical techniques such as Nuclear Magnetic Resonance (NMR) Spectroscopy. In the present work, it was not possible to proceed with further fractionation, as this task would require the investment of time and resources which are not suitable for a single PhD project. However, the results here obtained provide a proof of concept of the opportunities offered by

Tetraselmis striata CTP4, not only as source of valuable nutrients, but also of bioactive compounds with potential pharmaceutical and nutraceutical applications.

CHAPTER 4.

MICROALGAE EXTRACTS PROMOTE MINERALOGENESIS DURING ZEBRAFISH BONE DEVELOPMENT

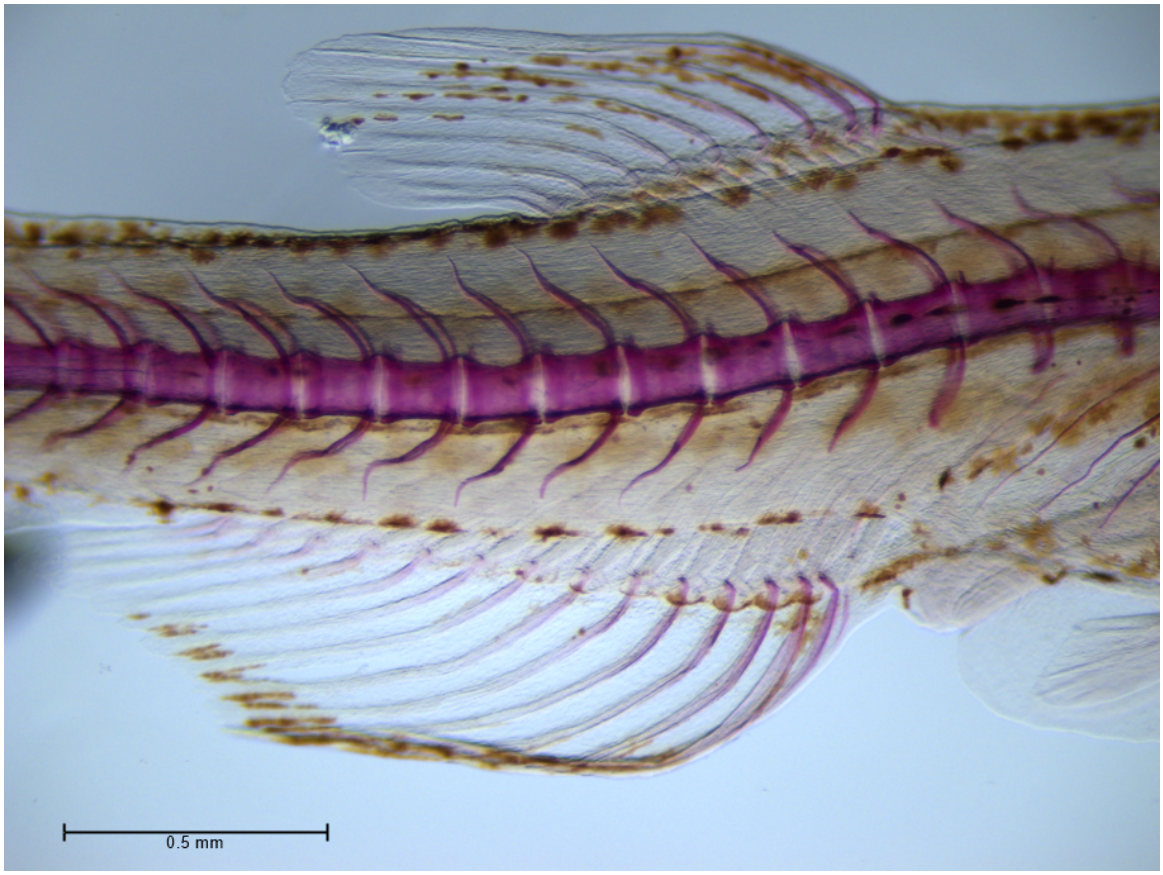
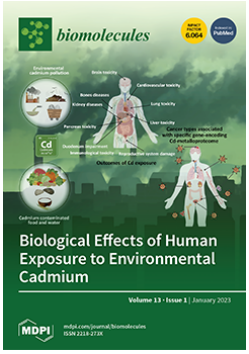


Image depicting a zebrafish (Danio rerio) juvenile with ossified structures highlighted by alizarin red staining. Picture by Alessio Carletti.



This section will be part of: A. Carletti, J.T. Rosa, K. Pes, I. Borges, L. Barreira, T. Santos, J. Varela, H. Pereira, M.L. Cancela, P. Gavaia, V. Laizé. "The osteogenic and mineralogenic potential of the microalgae *Skeletonema costatum* and *Tetraselmis striata* CTP4 in fish models". Currently submitted to *Biomolecules*.

Abstract

Technological advances on microalgal culture methods have allowed microalgae to become a relevant asset in the field of drug discovery as a source of bioactive molecules. Bone erosive pathologies such as osteoporosis are a global health burden that requires the provision of new drugs, to expand the therapeutic options available and bypass the common side effects associated with the use of the current therapeutic approaches. After recent evidence that microalgae may produce osteoactive compounds, we used zebrafish (*Danio rerio*) *in vivo* assays and gilthead seabream (*Sparus aurata*) *in vitro* cell systems to assess osteogenic and mineralogenic molecules in ethanolic extracts prepared from two commercial microalgae strains, *Skeletonema costatum* and *Tetraselmis striata* CTP4. Both extracts increased extracellular matrix mineralization in fish bone-derived cell lines and promoted osteoblastic differentiation in zebrafish larvae. Long-term dietary exposure to both extracts increased bone mineralization in zebrafish, upregulated genes involved in bone formation (*sp7*, *col1a1a*, *oc1*, and *oc2*), bone remodeling (*acp5a*), and antioxidant defenses (*cat*, *sod1*). Extracts also improved the skeletal status of zebrafish juveniles by reducing the incidence of skeletal anomalies. Our results demonstrate the presence of osteoactive molecules in *S. costatum* and *T. striata* CTP4 with the potential to improve bone formation and mineralization and widen the spectrum of therapeutic options to treat bone erosive disorders.

4.1. Introduction

Marine microalgae have gained momentum in applications related to the blue economy and sustainable growth[534], ranging from clean energy production[535], wastewater treatment[536], and animal feeds[452,537], to human nutrition and medicine[538,539]. They are optimal nutritional sources of essential nutrients such as vitamins, minerals, and polyunsaturated fatty acids[540], and are able to produce molecules with the potential to be used as nutraceuticals and pharmaceuticals. As such, they are expected to fuel the pharmaceutical industry with a plethora of bioactives with unique and innovative activities for human health[541,542]. The increasing interest of academia and industries in microalgae has stimulated the development of methods for the large-scale production of biomass from a wide range of strains, which is the starting material for the isolation of bioactive compounds[543]. In the latest years, genetic engineering tools have been successfully applied to microalgae and have allowed for the development of transgenic

lines optimized for the synthesis of specific compounds, shaping the idea of microalgae as full-fledge bioreactors for the production of biomolecules[327,328].

Osteoporosis, osteopenia, and Paget's disease of bone are human disorders characterized by erosive processes that result in bone structure deterioration and increased susceptibility to fracture[1,4,29,31]. These disorders represent an urgent pharmaceutical challenge because of the limited number and poor variety of treatments available for their treatment[3]. Current drugs are classified, based on their mechanism of action, as antiresorptive and osteoanabolic molecules. Antiresorptives such as bisphosphonates (e.g. alendronate, risedronate, ibandronate, and zoledronate) and antibody-based therapies (e.g., Denosumab) are among the most efficient and cost-effective treatments for patients suffering bone erosive pathologies, achieving a rapid reduction of fracture risk[146,147,158,544–546].

However, the intrinsic inability of the antiresorptive approach to compensate for the loss of bone occurred before the beginning of the treatment has raised concerns about the adequateness of this therapeutic strategy, and osteoanabolic agents are now considered the preferable therapeutics to tackle such diseases[546]. Unfortunately, the only osteoanabolic therapies currently approved for treating bone erosive pathologies - the parathyroid hormone (PTH) analogues, teriparatide and abaloparatide - have been correlated in animal studies to the occurrence of osteosarcoma[546], have shown reduced effect in patients previously treated with antiresorptive drugs[546] and lose efficiency over time, limiting their implementation for long term treatments[163,546].

In this scenario, molecules able to stimulate bone anabolic mechanism in a more optimal manner are sought and natural resources have been prospected for bioactives that could be the basis of a new generation of therapeutics for bone erosive pathologies[547–549]. Despite a growing interest for microalgae-derived bioactives, very few studies have explored microalgae as a source of osteogenic and mineralogenic compounds, possibly because of the perceived lack of suitable systems to assess these bioactivities. In this regard, fish are becoming increasingly relevant as animal models to study skeletal processes and can be used to create knowledge translatable to human medicine[399,550,551]. Small fish such as the zebrafish (*Danio rerio*) bears several technical advantages over mammalian models, such as lower maintenance costs, small size, short life cycle, high fecundity, easier genetic manipulation, and translucent embryonic stages [397,402,403]. Fish-based *in vitro* and *in vivo* systems to evaluate the bone-related bioactivity of compounds have been recently described[397,402,403] and successfully used to assess the presence of osteoactive compounds found in marine invertebrates[360], seaweeds[320], and halophytes plants[552].

In this work, extracts from two well-established and commercially available species of marine microalgae – *Skeletonema costatum* and *Tetraselmis striata* CTP4 – were prepared through ethanolic maceration and evaluated for the presence of osteogenic and mineralogenic compounds using fish systems.

4.2. Materials and Methods

4.2.1. Ethical statement

Procedures involving animals were performed following the EU and Portuguese legislation for animal experimentation and welfare (Directives 86/609/CEE and 2010/63/EU; Portaria 1005/92, 466/95 and 1131/97; Decreto-Lei 113/2013). Animal handling and experimentation were performed by qualified operators accredited by the Portuguese Direção-Geral de Alimentação e Veterinária under the authorization no. 012769/2021. All efforts were made to minimize pain, distress, and discomfort. Experiments were terminated (fish were returned to normal conditions or euthanized) whenever adverse effects were observed.

4.2.2. Preparation of ethanolic extracts

Freeze-dried biomass of *Skeletonema costatum* (Necton S.A., Olhão, Portugal) and *Tetraselmis striata* CTP4 (ALLMICROALGAE - Natural Products S.A., Pataias, Portugal) was macerated with 96% ethanol (Merck, Darmstadt, Germany) at a ratio of 1 g of biomass for 40 mL of solvent, under gentle agitation at 24 °C for 18 h. Macerates were centrifuged for 5 min at 1,000 x g and supernatants were collected. Pellets were washed twice with 96% ethanol and supernatants were pooled and then vacuum filtered sequentially through 0.45 µm and 0.22 µm nylon membranes (Labbox Labware S.L. Barcelona, Spain). Filtrates were kept at -20 °C until concentrated using a rotatory evaporator RV 10 digital (IKA-Werke GmbH & Co, Staufen im Breisgau, Germany) at 40 °C. Extracts were evaporated until a dense paste was formed. Extraction yields were determined from 2 mL of each concentrated solution placed under a gentle flow of 99.8% nitrogen until complete evaporation. The yield of the extraction process was 37.9% for *S. costatum* and 19.6% for *T. striata* CTP4 (Figure 4.1A).

4.2.3. Cell culture and extracellular matrix mineralization

Gilthead seabream (*Sparus aurata*) osteochondroprogenitor VSa13 cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, USA), 1% penicillin–streptomycin, 1% L-glutamine, and 0.2% fungizone (all from GIBCO, ThermoFisher Scientific, Waltham, USA) at 33 °C in a 10% CO2-

humidified atmosphere[553,554]. Pre-confluent cell cultures were sub-cultured 1:4 twice a week using trypsin-EDTA solution (0.2% trypsin from GIBCO, 1.1 mM EDTA, pH 7.4). Mineralization assays were conducted in 48-well plates seeded at a density of 1.25 × 104 cells per well (1.56 × 104 cells /cm2) and incubated until cell confluency. Extracellular matrix (ECM) mineralization was induced by supplementing culture medium with L-ascorbic acid (50 μg/mL), β-glycerophosphate (10 mM) and calcium chloride (4 mM). Mineralizing cell cultures were treated with the ethanolic extracts from *Skeletonema costatum* or *Tetraselmis striata* CTP4 (thereafter referred to as SKLT and CTP4) at different concentrations, or with 0.1% ethanol (CTRL). Cells cultured in non-supplemented medium (i.e. without mineralogenic cocktail and extracts) were also used a control (Min^-). After 17 days of culture, mineral deposition was assessed through alizarin red S (AR-S, Sigma-Aldrich) staining and quantified by spectrophotometry as previously described[555].

4.2.4. Zebrafish maintenance

Zebrafish wild type (AB) and transgenic lines *Tg(Hsa.RUNX2-Mmu.Fos:EGFP)*^{zf259}, *Tg(Ola.Sp7:mCherry-Eco.NfsB)*^{pd46} and *Tg(Ola.osteocalcin:EGFP)*^{hu4008}, hereafter referred to as *Tg(runx2:EGFP)*, *Tg(sp7:mCherry)* and *Tg(oc:EGFP)*[556,557], were maintained in a ZebTEC water recirculating system (Tecniplast, Buguggiate, Italy) at the aquatic animal facilities of the Centre of Marine Sciences (CCMAR, Faro, Portugal). Eggs were obtained following an in-house breeding program using sexually mature adult zebrafish. Viable fertilized eggs were transferred into plastic 1 L tanks and maintained for 3 days in reverse osmosis-treated water supplemented with salts (Instant Ocean, Blacksburg, USA), sodium bicarbonate (Fisher Chemicals, Hampton, USA), and methylene blue (0.0002% w/v, Sigma-Aldrich). Water parameters were: temperature 28 ± 0.1 °C, pH 7.5 ± 0.1, conductivity 700 ± 50 μS, ammonia and nitrites lower than 0.1 mg/L, and nitrates lower than 50 mg/L. Photoperiod was set to 14:10 h light-dark.

4.2.5. Waterborne exposure of zebrafish larvae to microalgae extracts

At 3 days post-fertilization (dpf), hatched larvae were transferred to 6 well-plates at a density of 15 larvae/well, and exposed to ethanolic extracts, 0.1% ethanol (vehicle and negative control) or 10 pg/mL of calcitriol (1α,25-dihydroxy vitamin D_3, Sigma-Aldrich; positive control)[478]. Treatment (10 mL/well) was renewed – 70% of the total volume, i.e., 7 mL – daily until the end of the experiment. Mortality was monitored at the time of treatment renewal. At 6 dpf, larvae were sacrificed with a lethal dose of MS-222 (0.6 mM, pH 7.0, Sigma-Aldrich)

then stained for 20 min with 0.03% alizarin red S (AR-S) or 0.5% calcein prepared in Milli-Q water (pH 7.4; Millipore, Burlington, USA), and washed twice with Milli Q water for 5 min. Larvae were imaged immediately upon staining, and 30 larvae per treatment were pooled (10 larvae/pool, $n = 3$,) for RNA extraction.

4.2.6. Imaging and morphometric analysis

Euthanized larvae were placed in a lateral position on top of a 2.5% agarose gel plate and imaged using an MZ10F fluorescence stereomicroscope (Leica, Wetzlar, Germany) equipped with a DFC7000T color camera (Leica). A green fluorescence filter (λex = 546/10 nm) and a barrier filter (λem = 590 nm) were used to image AR-S stained wild-type larvae and *Tg(sp7:mCherry)* transgenic larvae, while a blue fluorescence filter (λex = 470/40 nm) and a barrier filter (λem = 515 nm) were used to image calcein stained wild-type larvae, and *Tg(runx2:EGFP)* and *Tg(oc:EGFP)* transgenic larvae. Images were acquired using the following parameters: exposure time of 300 ms (mCherry and AR-S) or 1 s (calcein and EGFP); gamma 1.00; resolution 1920×1440 pixels; binning 1×1. Fluorescence images were analyzed using ImageJ (version 1.52p). For morphometric analysis, images were processed using ZFBONE macro toolset for Fiji[489]. The area of the operculum (OpA), the area of the head (HA), the splanchnocranial area (SA), and the areas positive for reporter signals within the head ($sp7^+$, oc^+) and within the splanchnocranial area ($runx2^+$) were determined using an Intous M drawing tablet (Wacom, Kazo, Japan). To correct for inter-specimen size variations, OpA, $sp7^+$, and, oc^+ were normalized using HA[478], and $runx2^+$ was normalized using SA.

4.2.7. Preparation of feeds supplemented with ethanolic extracts

Ethanolic extracts SKLT and CTP4 were vacuum coated onto a zebrafish base diet (Sparos Lda, Olhão, Portugal) at 0.5% and 2.5% each. The five diets – CTRL (no extract), SKLT 0.5%, SKLT 2.5%, CTP4 0.5%, and CTP4 2.5% (Figure 4.1B) were analyzed for phosphorus (P), calcium (Ca), potassium (K), and magnesium (Mg) contents. For this, diets were completely dried at 120 °C for 24 h in a VENTI-Line drying oven (VWR International, Radnor, USA), weighted and solubilized in nitric acid (Sigma-Aldrich). Mineral content was determined by microwave plasma atomic emission spectroscopy (4200 MP-AES, Agilent Technologies, Santa Clara, USA) using P, Ca, K, and Mg standards.

4.2.8. Dietary exposure to ethanolic extracts

At 3 dpf, zebrafish AB larvae were randomly distributed into 2.5 L plastic tanks filled with water from the recirculating system filtered using 0.22 µm nylon membranes (Labbox) and maintained in static conditions at a density of 150 larvae/tank. Water parameters were: temperature 28 ± 0.1 °C, pH 7.5 ± 0.1, conductivity 700 ± 50 µS, ammonia and nitrites lower than 0.1 mg/L, and nitrates lower than 50 mg/L. Photoperiod was set at 14:10 h light-dark. Experimental procedures were conducted in triplicates for all conditions and the feeding regimen was adjusted with the growth of the fish (Figure 4.1C). From 5 to 7 dpf, larvae were fed exclusively with live rotifers (*Brachionus plicatilis*). From 8 to 19 dpf, larvae were maintained in a regimen of co-feeding with rotifers and the experimental diets, gradually decreasing the number of rotifers and increasing the amount of dry food. From 20 to 55 dpf, fish were fed three times per day solely with the experimental diets. Food size was <100 µm until 16 dpf and 100-200 µm until 55 dpf. At 20 dpf, approximately 150 fish were sampled to determine the total length (TL), while the remaining fish were transferred to 2.8 L glass tanks and maintained in recirculating water conditions until 55 dpf at a density of 100 larvae/tank. At the end of the trial, juveniles were given lethal anesthesia with MS-222 then randomly pooled for the assessment of dry weight (n = 12, 3 fish/pool), content of Ca and P (n = 6, 3 fish/pool), and gene expression by qPCR (n = 6, 3 fish/pool), as described below. The remaining fish ($n \geq 80$) were placed in a lateral position over a 2.5% agarose gel and photographed under a Leica MZ10F stereomicroscope equipped with a Leica DFC7000T color camera. Total length was assessed from bright-field images using ImageJ, then fish were fixed for 16 h in 4% paraformaldehyde prepared in phosphate-buffered saline (PBS, pH 7.4), then washed with PBS for 5 min and dehydrated in an increasing ethanol series. Subsequently, specimens were stained for 20 min with 0.05% AR-S in 1% KOH, then washed in 1% KOH for 48 h and preserved in glycerol. Incidence of skeletal anomalies and rate of skeleton mineralization were assessed in stained fish. To estimate the mineralization status of the skeleton, a color code was attributed to each skeletal element according to a mineralization index, where 0 is for elements with no mineralization, 1 for elements with a low level of mineralization, 2 for elements with intermediate levels of mineralization, and 3 for elements fully mineralized.

4.2.9. RNA extraction and qPCR analysis

Total RNA was extracted from pools of larvae at 6 dpf (n = 3, 10 larvae/pool) or juvenile fish at 55 dpf (n = 6, 3 fish/pool) using NZYol (NZYTech, Lisbon, Portugal) and quantified using a NanoDrop OneC spectrophotometer (ThermoFisher Scientific). RNA integrity was

confirmed using an Experion Automated Electrophoresis system (Bio-Rad, Hercules, USA). Total RNA (1 µg) was reverse-transcribed for 1 h at 37 °C using M-MLV reverse transcriptase, oligo-d(T) primer and RNAseOUT (all from ThermoFisher Scientific). Quantitative real-time PCR (qPCR) reactions were performed using qPCR NZYSpeedy Mastermix (2x) ROX Plus (NZYTech), 10 µM of gene-specific primers (Supplementary table 2) and 1:10 dilution of reverse-transcribed RNA, in a CFX Connect Real-Time PCR detection system (Bio-Rad). PCR amplification was as follows: an initial denaturation step of 2 min at 95 °C and 40 cycles of amplification (10 s at 95 °C and 20 s at 65 °C). Efficiency of amplification was above 95% for all primer sets. Primer specificity was confirmed through sequence analysis and qPCR specificity was assessed by melting curve analysis at the end of each PCR run. Levels of gene expression were calculated using the ΔΔCt method[558] and normalized using the average value of three housekeeping genes, *ef1a*, *actb1*, and *rps18*.

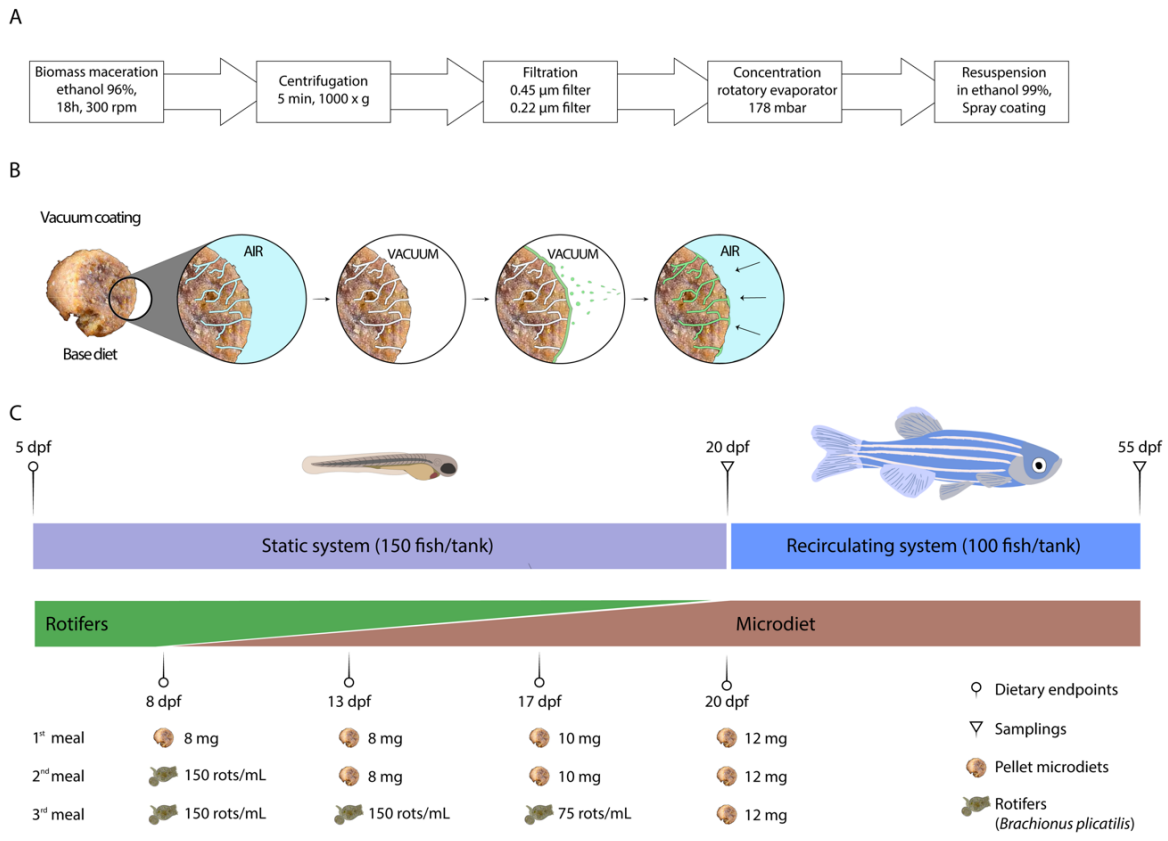


Figure 4.1. (**A**) Schematic representation of the production of microalgal extracts. (**B**). Vacuum coating of commercial diets with algal extracts. (**C**) Time course and feeding table of the nutritional trial. rots - rotifers.

4.2.10. Statistical analysis

For all the experiments, normality was tested with a D'Agostino-Pearson omnibus normality test or with an Anderson-Darling test ($p < 0.05$). Homoscedasticity was tested through the Brown-Forsythe test ($p < 0.05$). If data distribution was normal and homogeneous in all the experimental groups, differences were tested with an unpaired *t* test (gene expression, $p < 0.05$) or a one-way ANOVA followed by Dunnett's multiple comparison test (all other parameters, $p < 0.05$). If data distribution was not normal or not homogeneous, differences were tested with a Mann-Whitney test (gene expression, $p < 0.05$) or a non-parametric test followed by Dunn's multiple comparison test (all other parameters, $p < 0.05$). Statistical analyses were performed using Prism version 8.00 (GraphPad Software Inc., La Jolla, USA).

4.3. Results

4.3.1. Microalgae extracts induce matrix mineralization in fish bone cell lines

As a first approach to evaluate the osteogenic potential of the ethanolic extracts prepared from *S. costatum* (SKLT) and *T. striata* CTP4 (CTP4), mineralogenic cells VSa13 were exposed for 17 days to 3 concentrations of the two extracts. While SKLT increased ECM mineralization at the two highest concentrations (50 and 100 µg/mL; Figure 4.2A), CTP4 showed a strong mineralogenic activity at all the concentrations tested (25, 100, and 300 µg/mL; Figure 4.2B) and in a dose-dependent manner, indicating the presence of mineralogenic compounds in both extracts.

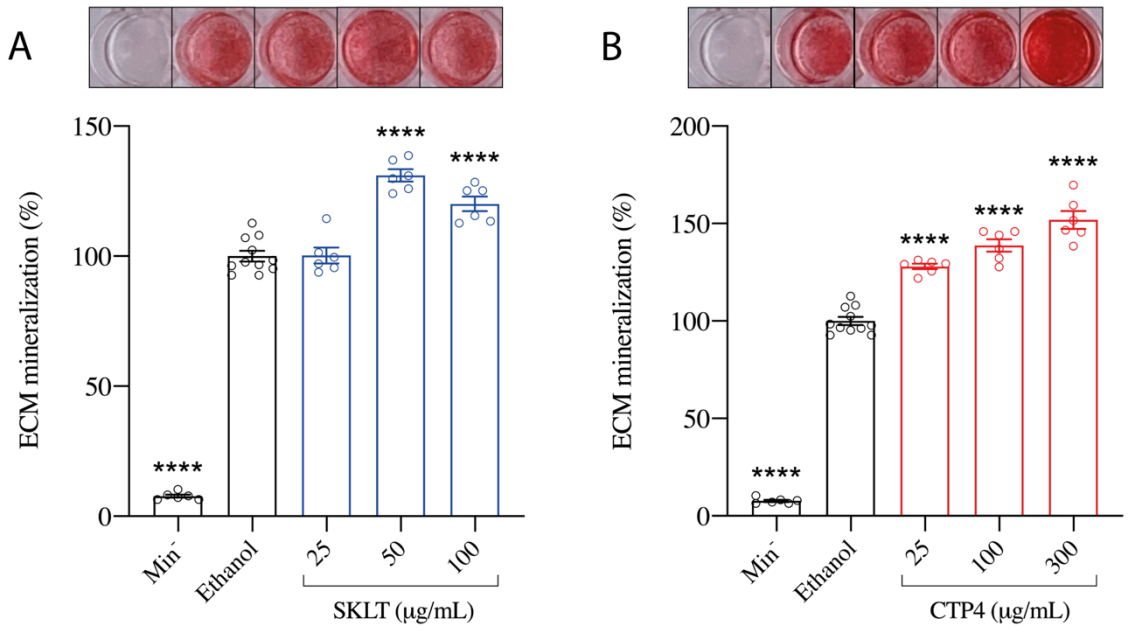


Figure 4.2. Mineralization of the extracellular matrix (ECM) of VSa13 cells exposed to ethanolic extracts prepared from (**A**) *Skeletonema costatum* (SKLT), and (**B**) *Tetraselmis striata* CTP4 (CTP4). Values are presented as mean ± standard deviation and as a percentage over the control group (Ethanol, $n = 6$). Picture panels above each graph display images of alizarin red-stained cell cultures. Normality was tested through Anderson-Darling test ($p < 0.05$). Asterisks indicate values significantly different according to one-way ANOVA followed by post-hoc Dunnett's or Kruskal-Wallis test. Each experimental group was tested against the control group (Ethanol). $p < 0.0001$ (****). Min^-, No mineralogenic cocktail control.

4.3.2. Microalgae extracts promote bone formation by stimulating osteoblastic differentiation in vivo

The osteogenic potential of both extracts was assessed in zebrafish via waterborne exposure of 3-dpf larvae to different concentrations of SKLT and CTP4 followed by morphometric analysis of the opercular bone (Figure 4.3A). Non-toxic concentrations – below 56 µg/mL for SKLT and 200 µg/mL for CTP4 – induced an increase of the mineralized area of the opercular bone in a dose-dependent manner (Figures 4.3B and 4.3C), indicating the presence of osteogenic compounds in both extracts. While CTP4 did not affect the head area at the concentrations tested, SKLT increased it at 31.6 µg/mL and 56 µg/mL, suggesting that other developmental processes (e.g. cranial development) might be affected (Supplementary figure 2). Interestingly, the highest concentrations of both extracts (56 µg/mL for SKLT and 200 µg/mL for CTP4) could increase the operculum area to levels higher than those observed for the positive control (Figures 4.3B and 4.3C).

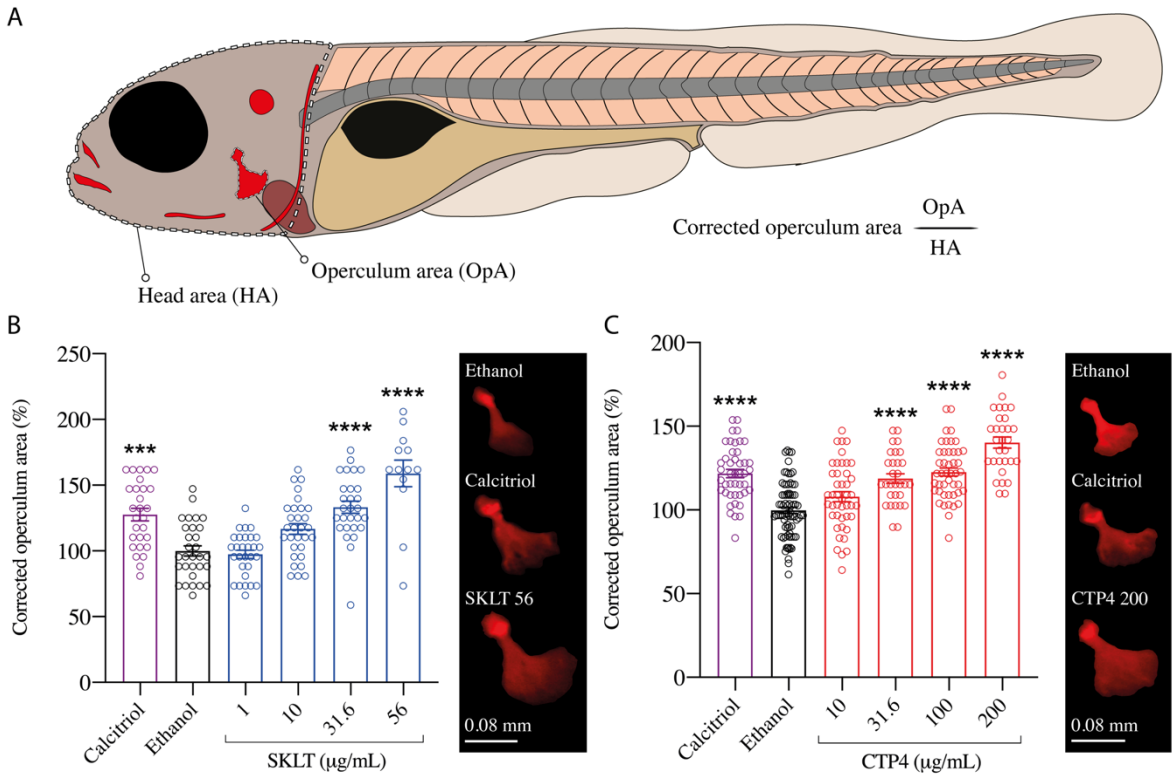


Figure 4.3. Mineralogenic effect on the operculum of 6-dpf zebrafish larvae exposed to *Skeletonema costatum* (SKLT) and *Tetraselmis striata* CTP4 (CTP4) extracts. (**A**) Composite image of a 6-dpf zebrafish larva, where operculum area (OpA) and head area (HA) are highlighted with a dashed line and where AR-S stained mineralized structures detected by fluorescence microscopy appear in red. (**B-C**) Effects of SKLT (**B**) and CTP4 (**C**) on the operculum area. Values are presented as mean ± standard error and as a percentage over the control (Ethanol) ($n > 15$). The picture panels on the right side of each graph present images of opercular bones from control fish and fish exposed to the higher concentration of each extract. Normality was tested though Anderson-Darling test ($p < 0.05$). Asterisks indicate values significantly different according to one-way ANOVA followed by post-hoc Dunnett's or Kruskal-Wallis test ($p < 0.05$). $p < 0.0002$ (***) and $p < 0.0001$ (****).

To investigate a possible role of osteoblasts in the pro-osteogenic capacity of microalgae extracts, cellular dynamics were monitored using transgenic reporter lines: *Tg(runx2:EGFP)* for osteoprogenitor cells, *Tg(sp7:mCherry)* for immature osteoblasts and *Tg(oc:EGFP)* for mature osteoblasts. A significant increase in the signal associated with osterix ($sp7^+$ cells) and osteocalcin (oc^+ cells), but not of *runt related transcription factor 2* ($runx2^+$ cells) was observed, suggesting that ethanolic extracts may increase operculum mineralized area through

a specific action on committed osteoblast differentiation, but not on early differentiation of mesenchymal precursors (Figures 4.4B, 4.4D and 4.4F). qPCR data confirmed that the expression of *runx2a* and *runx2b* was indeed unaffected in SKLT treated fish, while the expression of *sp7* was increased. Accordingly, expression of *col10a1a* (*collagen type X alpha 1a*), whose expression is directly controlled by *sp7*, was upregulated in SKLT treated fish. In contrast, expression of *sp7*, *oc1* and *oc2* was not affected in CTP4 treated fish (Figure 4.4G).

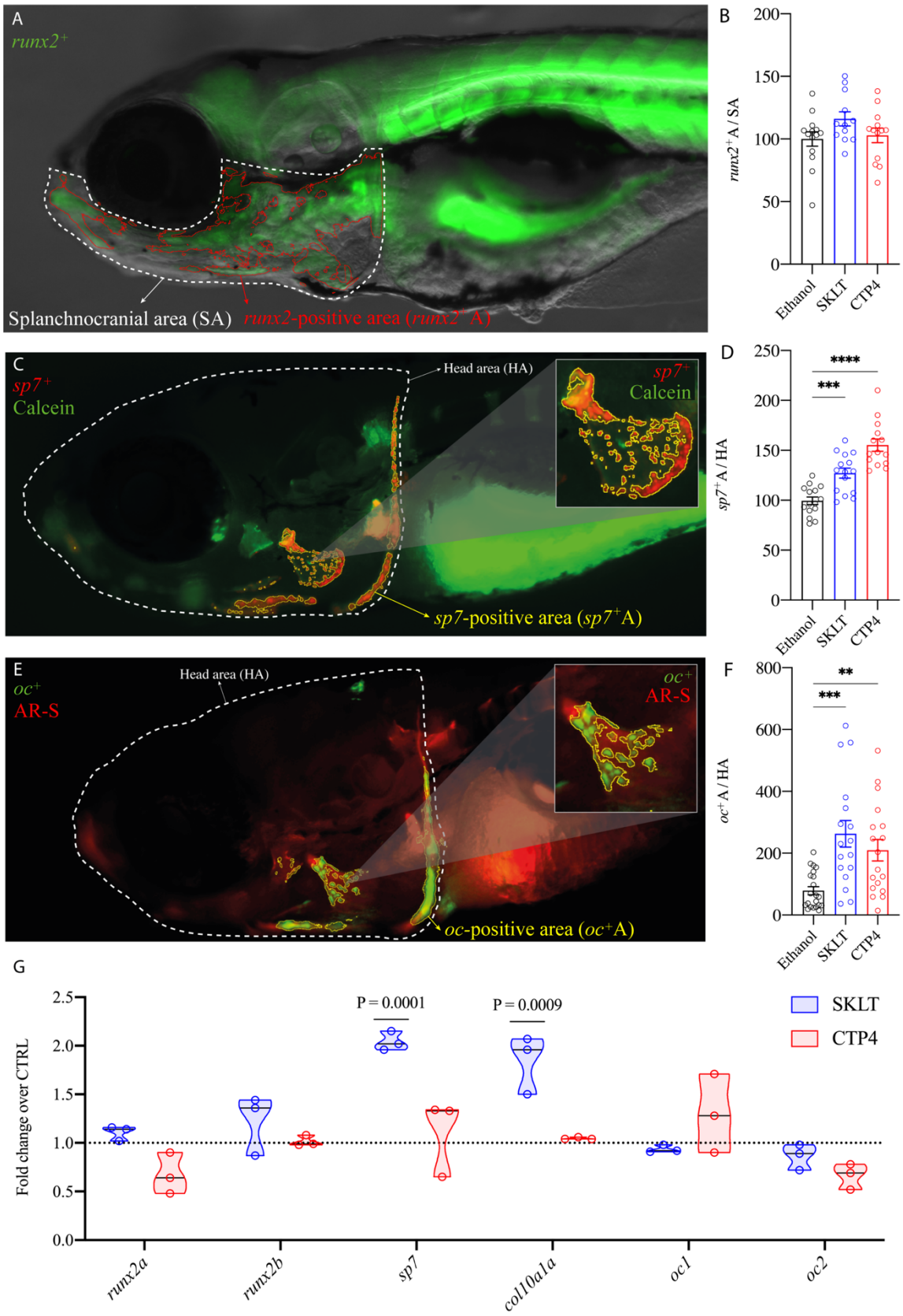


Figure 4.4. Expression of osteoblast differentiation markers in 6-dpf zebrafish larvae exposed to ethanolic extracts of *Skeletonema costatum* (SKLT) and *Tetraselmis striata* CTP4 (CTP4). Representative images of (**A**) *runx2*-positive osteoprogenitor cells, (**C**) *sp7*-positive immature osteoblasts, and (**E**) *oc1*-positive mature osteoblasts, and quantification of fluorescence signal

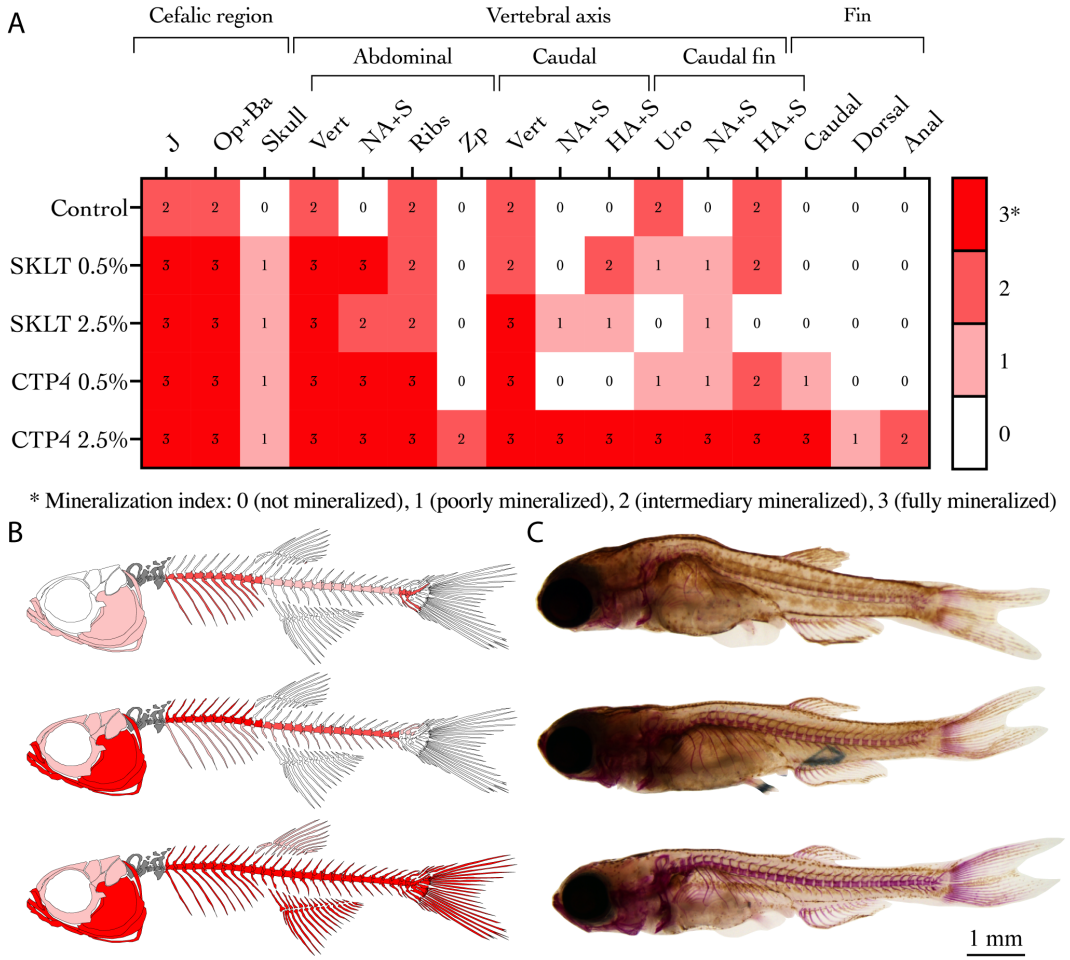
area for *runx2* (**B**), *sp7* (**D**), and *oc1* (**E**) positive cells. Normality was tested though Anderson-Darling test ($p < 0.05$). For fluorescence signals, differences were tested through one-way ANOVA or Kruskal-Wallis test. Asterisks indicate $p < 0.0021$ (**), $p < 0.0002$ (***) and $p < 0.0001$ (****). For gene expression, differences were tested through Student's *t* test and a $p < 0.05$ was considered significant.

4.3.3. Dietary exposure to ethanolic extracts promotes bone formation and mineralization, and decrease the incidence of skeletal anomalies

To gain further insights into the osteogenic and osteoblastogenic effects of the extracts, zebrafish were fed diets supplemented with CTP4 and SKLT from 8 to 55 dpf. The five experimental diets produced were homogeneous in terms of proximal composition (Supplementary Table 4) and content of P, Ca, K, and Mg (Supplementary Figure 3). Fish fed SKLT 0.5% and CTP4 2.5% were longer than control fish at 20 dpf (Supplementary Figure 4), but all fish had similar length and weight at 55 dpf. Our data suggests that none of the extracts significantly affected fish growth.

Although Ca and P contents did not significantly change in fish fed experimental diets (Figure 4.5D), a general increase of the mineralization index attributed to each skeletal structure was observed in those fish. In fact, levels of mineralization increased in all the skeletal regions and structures considered and, in a dose-dependent manner for both extracts, with the fish fed SKLT 2.5% and CTP4 2.5% displaying the highest mineralization indexes (Figure 4.5A-C). Accordingly, the expression of marker genes for osteoblast differentiation (*sp7*, *oc1*, *oc2*, *col1a1a*) was increased at 55 dpf in a dose-dependent manner and in fish fed the experimental diets (Figure 4.5E). In opposition to the data collected after the short exposure, *runx2b* expression was downregulated in fish fed at both supplementation levels in SKLT diets. Interestingly, fish fed SKLT 2.5%, CTP4 0.5% and CTP4 2.5% also displayed an increased expression of *acp5a*, a marker gene for active osteoclasts and bone resorption (Figure 4.5E), suggesting that extracts may also affect bone remodeling. The expression of marker genes for antioxidant function (*cat* and *sod1*) was also increased, suggesting a potentiation of the enzymatic removal of Reactive Oxygen Species (ROS) by the extracts (Supplementary Figure 5). The occurrence of skeletal anomalies was assessed in AR-S stained fish and all groups displayed a similar distribution pattern, with opercular bones, abdominal vertebrae, caudal fin complex, and unpaired fins being the most affected structures (Supplementary Figure 6). A more detailed analysis of the differences in incidence between the levels affecting the individual skeletal structures showed that fish fed experimental diets, in particular SKLT 2.5%

and CTP4 0.5%, had a lower incidence of anomalies compared to the control group (Figure 4.6). Interestingly, fish fed CTP4 2.5% had a lower incidence of caudal vertebrae anomalies and anomalies on all the five fins, but a higher incidence of anomalies affecting the skeletal elements associated with abdominal vertebrae.


A
Cefalic region
Vertebral axis
Fin
Abdominal
Caudal
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J
Op+Ba
Skull
Vert
NA+S
Ribs
Zp
Vert
NA+S
HA+S
Uro
NA+S
HA+S
Caudal
Dorsal
Anal
Control
SKLT 0.5%
SKLT 2.5%
CTP4 0.5%
CTP4 2.5%
3*
2
1
0
* Mineralization index: 0 (not mineralized), 1 (poorly mineralized), 2 (intermediary mineralized), 3 (fully mineralized)
B
C
1 mm
D
Ca (ppm/mg)
P (ppm/mg)
Ratio Ca/P
Control
SKLT 0.5%
SKLT 2.5%
CTP4 0.5%
CTP4 2.5%
E
SKLT 0.5%
SKLT 2.5%
CTP4 0.5%
CTP4 2.5%
Fold change over CTRL
bmp2b
runx2b
sp7
col1a1a
oc1
oc2
alpl
ctsk
acp5a

Figure 4.5. Mineralization status of zebrafish juveniles fed diets supplemented with the ethanolic extracts of *S. costatum* (SKLT) and *T. striata* CTP4 (CTP4). (**A**) Heatmap displaying the group modal values for the mineralization index assigned to each skeletal structure. (**B**) Schematic representation of the modal mineralization status of the fish fed CTRL, SKLT 2.5% or CTP4 2.5%. (**C**) Representative images of fish illustrated in C. (**D**) Content of P, Ca, and Ca/P ratio in juveniles fed experimental diets. (**E**) Expression of marker genes for osteoblastic differentiation, ECM mineralization, and osteoclast function. For gene expression, differences were tested through Student's *t* test. Asterisks indicate $p < 0.0332$ (*), $p < 0.0021$. (**), $p < 0.0002$ (***), and $p < 0.0001$ (****). J, jaws; Op+Ba, operculum and branchial arches; Vert, vertebral bodies; NA+S, neural arches and spines; HA+S, hemal arches and spines; Uro, caudal vertebrae bodies and urostyle.

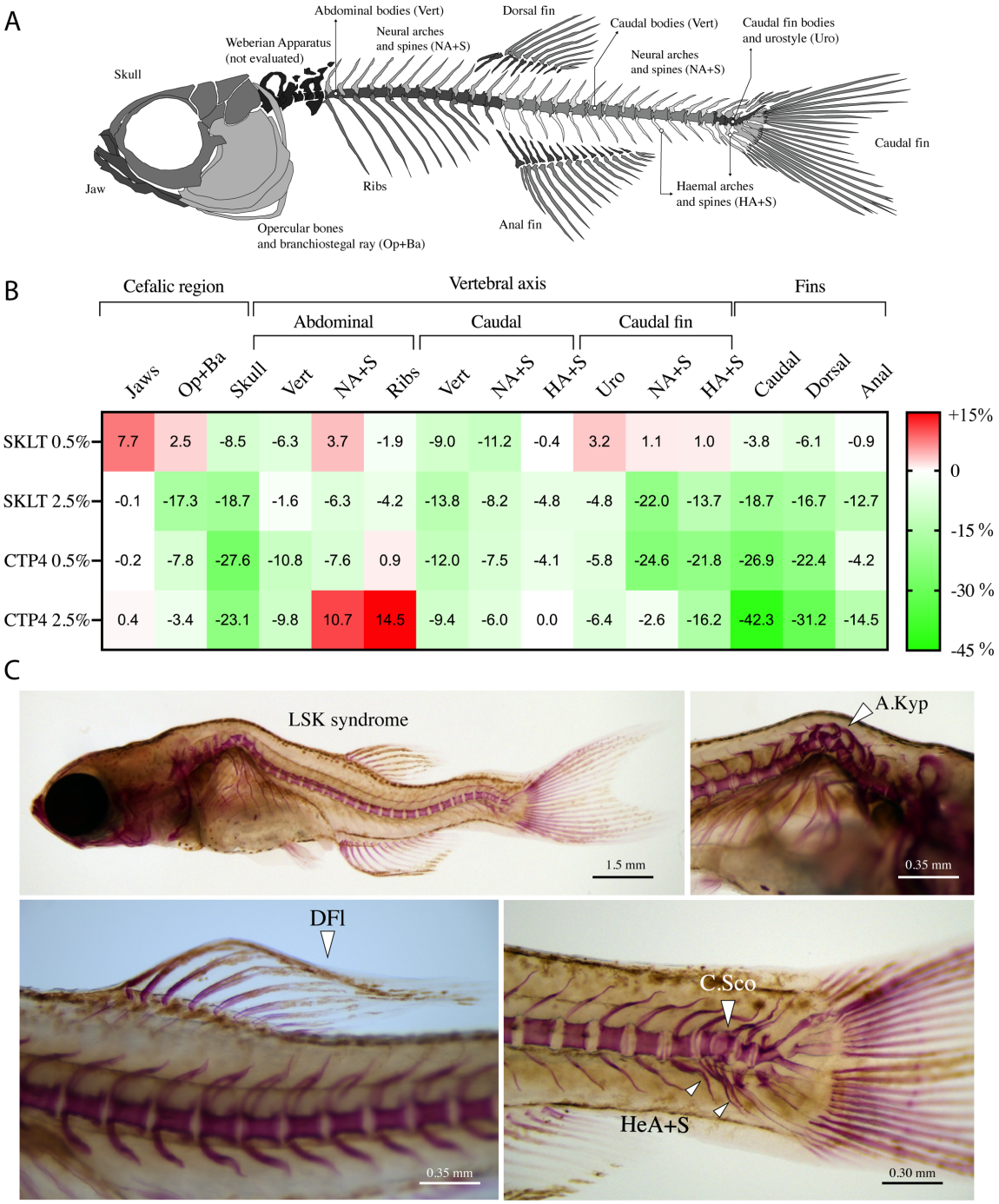


Figure 4.6. Incidence of skeletal anomalies in zebrafish juveniles fed diets supplemented SKLT and CTP4 extracts. (**A**) Scheme illustrating the skeletal elements considered in the analysis of skeletal anomalies. (**B**) Incidence of anomalies expressed as a percentage of increment/decrement of anomalies relative to the control group. (**C**) Representative images of commonly found skeletal anomalies in AR-S stained fish for all experimental groups. Op+Ba, operculum and branchial arches; Vert, vertebral bodies; NA+S, neural arches and spines; HA+S, hemal arches and spines; Uro, caudal vertebrae bodies and urostyle. LSK (lordosis-

scoliosis-kyphosis); DFI, deformed fin lepidotrichia; A.Kyp, abdominal kyphosis; C.Sco, caudal scoliosis; HeA+S, deformed hemal arches and spines.

4.4. Discussion

Microalgae species belonging to the genera *Skeletonema* spp. and *Tetraselmis* spp. are commonly cultured worldwide as a food source for aquatic animals due to favorable nutritional properties, e.g. high protein contents and diversified fatty acid profiles[559].

Here, we have shown that ethanolic extracts from *Skeletonema costatum* and *Tetraselmis striata* (CTP4 strain) contain compounds with strong pro-osteoblastogenic and pro-mineralogenic activities using *in vitro* and *in vivo* fish models. Both extracts demonstrated pro-mineralogenic properties, an effect that could be related to an increased rate of osteoblastic differentiation. This hypothesis is supported by *in vivo* data collected in zebrafish transgenic lines showing that both extracts selectively increased the populations of both immature (*sp7*$^{+}$) and mature (*oc*$^{+}$) osteoblasts, but not of osteoprogenitor cells (*runx2*$^{+}$*)*. This was further supported by expression data showing a reduced expression of *runx2b* in fish fed SKLT diets. Previous studies have shown that Runx2 regulates osteo-blast differentiation in a dualistic manner. It induces the osteoblastic commitment of mesenchymal stem cells at early stages of differentiation but must be downregulated at the final stages of osteoblast maturation because of its capacity to maintain osteoblasts in an immature state[560–563]]. We propose that the increase of *sp7* and *oc* expression in larvae exposed to microalgae extracts and the decrease of *runx2b* expression observed in juveniles fed the same extracts is indicative of an accelerated osteoblast maturation that could explain the improved mineralogenic performances observed in exposed fish.

Furthermore, both the larvae exposed to the extracts for a short period (3 days) and the juveniles fed the extracts for a long period (50 days) showed a clear increase in the mineralization of skeletal elements (operculum for larvae and overall skeleton for juveniles). It suggests that the stimulation of osteoblastic differentiation by the microalgae extracts may be then translated into an increased mineralization status. We have also observed that both CTP4 and SKLT diets elevated the expression of genes involved in ECM formation (*col10a1a*, *col1a1a*) and resorption (*acp5a*), suggesting that compounds present in both extracts may have the ability to modulate bone remodeling. This process is highly regulated and governed by molecular programs that mitigate bone anabolic processes by the stimulation of osteoclastic differentiation, which is controlled by both osteoblasts and osteocytes through the RANK-

RANKL-OPG signaling pathway[564]. The stimulation of resorptive processes is coherent with an overall increase of bone formation and osteoblastic maturation induced by the extracts. For example, human patients receiving treatment with PTH analogues display an increased osteoclastic differentiation, which is a secondary effect of the activation of anabolic mechanisms for a prolonged time[175].

Fish fed microalgae extracts were also characterized by a reduced incidence of skeletal anomalies in most skeletal elements, indicating an overall improvement of the skeleton health status. Still, an increase in skeletal anomalies associated to the accessory elements of abdominal vertebrae (ribs, neural arches and spines) was observed in fish fed CTP4 2.5%. It might not be a coincidence that this group was also the one presenting the stronger induction of mineralization. In this aspect, it has been shown in zebrafish that conditions that overstimulate bone mineralization, as it is the case for high levels of dietary phosphorus, may trigger an increased incidence of skeletal abnormalities[404]. In this work, phosphorus content was similar in all the experimental groups and cannot be responsible for the increased incidence of skeletal anomalies. Still, an excessive or excessively rapid ossification of the skeletal elements in fish fed CTP4 2.5% may be at the origin of an in-creased incidence of anomalies in these elements. Fish fed the CTP4 2.5% diet presented the highest degree of mineralization among all groups, and the accessory elements of the abdominal vertebrae had all the highest mineralization index reported. As such, a lower supplementation, for example at 0.5% as tested in this work, may represent a healthier option to provide a better balance between skeletal morphogenic processes and bone mineralization.

Interestingly, the expression of two marker genes of the antioxidant system – the catalase (*cat*) and superoxide dismutase (*sod1*) – was upregulated in fish exposed to both extracts (Supplementary Figure 5). It is important to mention that *Skeletonema costatum* and *Tetraselmis striata* CTP4 recently sparked the interest of the scientific community because of their high content in compounds with antioxidant activity, such as polyphenols[559,565–569]. Ethanolic fractions of *S. costatum* and *T. striata* CTP4 – rich in polyunsaturated fatty acids, alkanes and alkenes, long-chain alcohols, esters, ethers, and sterols – were also found to have in vitro radical scavenging activities[570]. We hypothesize that the pro-osteogenic effects observed in fish exposed to microalgae extracts could also be triggered by the antioxidant compounds present in these extracts, as reactive oxygen species (ROS) are known to negatively affect bone mineral status. Indeed, ROS can affect bone cells through several mechanisms, by inducing osteoclastic differentiation while suppressing osteoblastic differentiation and survival, being at the basis of various bone erosive pathologies including age-related

osteoporosis[52,571–574]. In this regard, the exposure of mammalian and fish models (*in vivo* and *in vitro*) to prooxidant agents inhibited osteoblastic differentiation and activity and increased the incidence of skeletal anomalies, but the supplementation of antioxidants could counteract these negative outputs[575–577]. Recent studies reporting the osteoactive and antioxidative potential of hydroethanolic, methanolic, and dichloro-methane extracts prepared from green (*Cladophora rupestris* and *Codium fragile*) and red (*Ceramium secundatum*, *C. pallidum*, and *Plocamium lyngbyanum*) macroalgae, further support this hypothesis[457,578,579]. Similarly, polyphenol-rich extracts prepared from marine halophyte plants also triggered pro-osteogenic and pro-mineralogenic activities[369].

The increased expression of *cat* and *sod1* in the present study, may also indicate a potentiation of fish antioxidant defenses upon exposure to microalgal extracts. In fact, many antioxidant agents excerpt their protective role against oxidative damage by upregulating the expression of first-line antioxidant enzymes[580–582]. This hypothesis should be further confirmed in a future study (i) by gathering data on protein levels and activity for Cat and Sod1, and on markers of cellular oxidative damage (e.g. DNA damage, lipid peroxidation, and loss in liposome membrane stability), but also (ii) by measuring the production of ROS in fish fed SKLT and CTP4 diets and challenged with pro-oxidant compounds.

Overall, our data provide strong evidence for the presence of osteoactive compounds in both ethanolic extracts, which stimulate bone mineralization in fish by increasing osteoblastic differentiation. Once identified and purified, these compounds may have applications in the treatment of human bone erosive pathologies. Thus, future studies should aim at better characterizing the effect of these extracts in animal models that closely recapitulate the phenotypes of human bone erosive pathologies. In this regard, zebrafish and medaka (*Oryzias latipes*) models of osteoporosis[404], osteomalacia[404], and Paget's disease[406,407] are available. Ovariectomized rats and mice are also commonly used models of post-menopausal osteoporosis[374,375], and rodent models of Paget's disease are also available[396]. In addition, mammalian models resembling human diseases with secondary bone symptoms, such as vitamin D deficiency[383], hyperparathyroidism[387], and chronic kidney diseases[391] have been developed. Finally, chemical characterization of the extracts and identification of the compounds responsible for the osteoanabolic effect will be paramount to the development of future drugs. In this sense, fractionation of the extracts by liquid chromatography and assessment of fraction osteoactivities, coupled to mass spectrometry could represent a suitable approach.

4.5. Conclusions

In the present study, we explored the osteoanabolic potential of ethanolic extracts from two species of commercially available marine microalgae, *Skeletonema costatum* and *Tetraselmis striata* CTP4. Extracts could increase extracellular matrix mineralization in fish bone-derived cell lines and bone formation and mineralization in zebrafish larvae through the stimulation of osteoblastic differentiation. Extracts could also increase skeleton mineralization and reduce the incidence of skeletal anomalies through the modulation of mechanisms underlying bone formation and remodeling and possibly antioxidant system. Overall, our data highlight the presence of promising osteoanabolic compounds in two strains of microalgae, with potential application in the treatment of human bone erosive disorders and widen the spectrum of therapeutic options.

CHAPTER 5.

MICROALGAE EXTRACTS AS A DIETARY SUPPLEMENT TO IMPROVE SKELETAL STATUS IN THE FARMED GILTHEAD SEABREAM (*Sparus aurata*)

Image depicting a specimen of gilthead seabream (Sparus aurata) during the ongrowing phase (about 9 months). Picture by Alessio Carletti.

This section will be part of: J.T. Rosa, A. Carletti, C. L. Marques, H. Pereira, H. Ringeard, I. Borges, M. Barata, P. Pousão-Ferreira, S. Engrola, V. Serra, R. Colen, M.L. Cancela, P. Gavaia, V. Laizé. "Diets supplemented with ethanolic extracts of microalgae improve the skeletal health of gilthead seabream (*Sparus aurata*)". Currently in preparation for submission to *Aquaculture*.

Author's note: This paper is the result of a collective work were different authors contributed to different sections. In detail, I was specifically responsible for the preparation of the extracts, the sampling of vertebrae from gilthead seabream juveniles for mineral content analysis, the preparation of larvae specimens for analysis of skeletal anomalies, the measurement, preparation, and imaging of gilthead seabream adults through x-ray radiophotographs.

Abstract

Skeletal anomalies in farmed fish species are a world-wide issue that cause direct economic losses to the aquaculture companies, impact the customer perception of the aquaculture product, and are at the base of animal welfare issues. Marine microalgae represent an optimal source of highly valuable nutrients and compounds with health beneficial properties and algae-based nutritional supplements could represent an optimal strategy to ameliorate the skeletal health of farmed fish. Here, we have tested diets for the commercially reared species *Sparus aurata* supplemented with two microalgae-derived extracts that were previously shown to possess pro-osteogenic and pro-mineralogenic properties (see **Chapter 4**). Diets were tested in different gilthead seabream life stages - larvae (30-60 DAH) and juveniles during the pre-ongrowing phase (5-18g). Some juvenile fish were maintained for 6 additional months while fed with commercial diets, in order to evaluate whether the short treatment with microalgae extracts could affect fish skeletal status until later on at the ongrowing phase. Overall, we found that the supplementation with microalgal extracts did not affect the incidence of skeletal anomalies in larval stages even though they were able to stimulate molecular mechanisms involved in bone growth and antioxidant mechanisms. In juvenile stages however, supplementation with one of the microalgal extracts promoted fish growth, elevated vertebral mineral content, reduced the incidence of skeletal anomalies and stimulated bone anabolic mechanisms. In addition, after 6 months during which fish were fed commercial diets, fish originally fed with one of the extracts were still larger, less deformed and with a more elongated and slender shape compared to the control fish.

5.1. Introduction

The aquaculture industry is a major and fundamental component of the global food supply machinery, being the main source of fishery products worldwide[408]. However, fish cultured in intensive farming conditions may develop skeletal deformities that affect both their external morphology and welfare. In the case of commercially important species, such as the gilthead seabream (*Sparus aurata*), this is a matter of great concern and a major factor that negatively affects costs, hatchery productivity, and fish market value[409,410].

The problem of skeletal anomalies in farmed fish is not easy to tackle due to its complexity. These anomalies are the result of the interaction of many causative factors contributing to their development, and they are extremely variable in distribution across species and rearing

conditions[409]. However, nutrition is one of the main causative factors of skeletal malformations in cultured fish, due to our lack of knowledge on the species-specific and life stage-specific nutritional requirements for the different species reared in aquaculture[409]. Beyond doubt, an unbalanced larval nutrition, by affecting fish larvae during the processes by which the skeleton is formed and ossified, is one of the key factors on the origin of skeletal anomalies[411,412].

In this scope, among the solutions proposed to reduce the incidence of skeletal deformities in aquacultured fish, the supplementation of diets with natural compounds or extracts that can promote a proper skeletogenesis, is increasingly seen as an economically sound approach to improve the competitiveness of the aquaculture industry and fish health.

In this contest, among the most promising sources of nutrients and nutraceuticals for aquaculture, are marine microalgae. They are characterized by a technologically advanced production industry, and are considered a sustainable alternative source of ingredients for fish feeds including proteins[452], lipids[453], and pigments[454]. Extracts from marine algae were already used as dietary supplements to support fish growth and immune function[452,454], and more recently, various microalgae strains were found to be able to produce pro-osteogenic and pro-mineralogenic compounds[320,323,325,579]. However, no previous studies evaluated if osteogenic extracts from microalgae might be able to improve the skeletal status in a commercially reared species.

Therefore, we have evaluated the potential of feeds supplemented with microalgae extracts obtained from two strains commonly used in aquaculture nutrition - *Skeletonema costatum* and *Tetraselmis striata*, that we have previously demonstrated to possess osteogenic properties in the model species *Danio rerio* (see **Chapter 4**), and for the commercially reared species gilthead seabream (*Sparus aurata*). We have focused on two developmental stages of *S. aurata*: larvae (30-60 days after hatching, DAH), and juveniles (5-18g of dry weight), in order to assess the effects on fish skeletal phenotype. In addition, some juvenile fish were maintained after the end of the experiment and fed for additional 6 months with commercial diets, in order to assess if a dietary supplementation with the microalgal extracts, for a short-time window during the pre-ongrowing phase, could have enduring positive effects on fish until later on the ongrowing phase.

5.2. Materials and methods

5.2.1. Preparation of microalgae extracts and incorporation in experimental diets

Microalgae ethanolic extracts were prepared by macerating dry biomass of *Skeletonema costatum* (Necton, SA, Olhao, Portugal) and *Tetraselmis striata* (Allmicroalgae - Natural Products, SA, Pataias, Portugal) in 96% ethanol, with a biomass-solvent ratio of 1g:40ml, for 18 h under gentle stirring. Macerates were centrifuged for 5 min at 1,000 x g and supernatant was collected. The pellet was washed twice with ethanol 96% and supernatants were pooled then vacuum filtered sequentially with 0.45 µm and 0.22 µm nylon membrane filters (Labbox Labware, S.L., Barcelona, Spain). Filtrates were kept at 4ºC then concentrated by using a rotatory evaporator RV 10 digital (IKA-Werke GmbH, Staufen im Breisgau, Germany) at40 °C. Pressure was initially set to 500 mbar, then gradually dropped below ethanol vapor pressure (178 mbar at 40°C). Extracts were evaporated until obtaining a dense paste-like consistency. Extraction yield was subsequently estimated by collecting 2 mL from each concentrated solution in triplicates. Samples were placed under a gentle flow of 99.8% Nitrogen (N_2) until complete evaporation. Extracts concentration was estimated to be of 372.1 ± 20,3 mg/mL and 400.7 ± 0.5 mg/mL for *S. costatum* and *T. striata*, respectively. Extraction yields were calculated as 37.9 % for *S. costatum* and 19.6% for *T. striata* Experimental diets were prepared by Sparos, Lda (Olhão, Portugal) by vacuum coating of commercial-grade diets for gilthead seabream. Diets were isonitrogenous and isoenergetic and were supplemented with 0.5% of the ethanolic extracts from *T. striata* or 1% of the ethanolic extract from *S. costatum* (CTP4 and SKLT diets, hereon.

5.2.2. Nutritional trial with gilthead seabream larvae

Gilthead seabream larvae were acquired from Mariscos de Estero S.A. (Huelva, Spain) and housed at the Ramalhete marine station (Centre of Marine Sciences, Faro, Portugal) in 100-L cylindroconical tanks in a semi-closed recirculating aquaculture system at an initial density of 52 larvae/L. The experimental rearing system was equipped with a mechanical filter, a submerged biological filter, a protein skimmer, and a UV sterilizer. Photoperiod was programed for 10h light : 14h dark. A daily monitoring of environmental parameters (temperature, salinity and dissolved oxygen in water) and larval mortality was performed; the rearing tanks were cleaned regularly to preserve water quality. Gilthead seabream larvae were fed the experimental diets as illustrated in Figure 5.1. Briefly, fish were fed *ad libitum* 3 to 4 times a day with the supplemented diets from 30 to 60 days after hatching (dah). Dietary treatments were randomly assigned to four tank/replicates per condition.

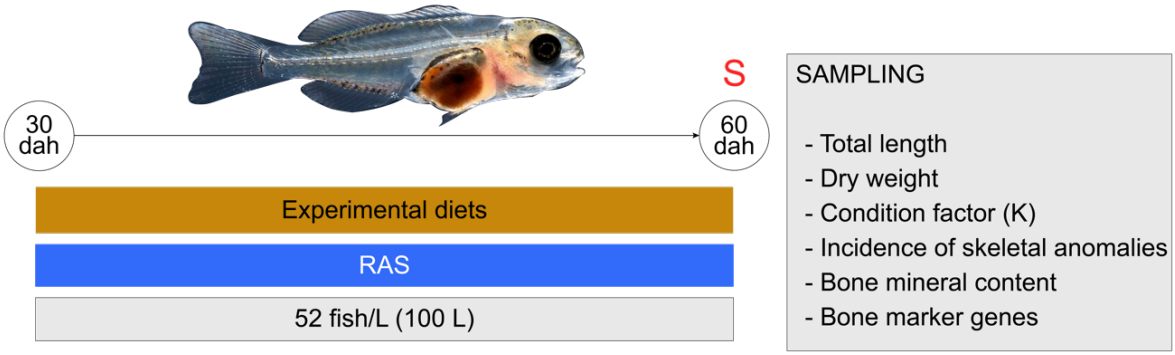


Figure 5.1. Experimental design of the nutritional trial with gilthead seabream larvae. S, sampling; dah, days after hatching; RAS, recirculating aquaculture system.

At the end of the trial larvae were euthanized and sampled to assess biometric parameters: standard length (SL), dry weight (DW), and Fulton´s condition factor (K), bone mineral content (calcium and phosphorus), incidence of skeletal anomalies and expression levels of marker genes of bone formation, extracellular matrix degradation and antioxidant response.

5.2.3. Larval growth parameters

Larvae at 30-dah (n = 30) or 60-dha (n = 60) were randomly selected in each tank, euthanized with a lethal dose of MS-222 Tricaine Methosulphate (500 mg/L), washed twice in distilled water and measured to determine standard length (SL), then snap-frozen in liquid nitrogen and stored at -80ºC. Larvae were then freeze-dried and weighted to determine dry weight (DW). Condition factor (K) was calculated using the Fulton formula (K= 100 x DW/SL)[583]

5.2.4. Whole-mount double staining for bone and cartilage and detection of skeletal anomalies

To assess the occurrence of skeletal anomalies, gilthead seabream larvae were fixed for 18 h in 4% PFA prepared in phosphate-buffered saline (PBS, pH 7.4), washed with PBS then dehydrated using ethanol series (25, 50, 75, 100%). Fish were then stained for detection of bone and cartilaginous structures with an acid-free whole-mount double staining protocol, adapted from[584]. Briefly, specimens were stained with alcian blue 8GX 0.1% (m/v) in ethanolic solution (70% ethanol in ddH_2O) and 60 mM Magnesium Chloride ($MgCl_2$) for 2 h. Subsequently, larvae were re-hydrated in series of ethanol (from 100 to 25%), and then stained for mineralized structures with alizarin red S (AR-S) solution 0.05 % in KOH 1% for 5 h. Specimens were then washed in 1% KOH for 120 h and passed through a series increasing glycerol concentrations (5, 10, 20, 30, 50, 70%). Stained fish were observed and imaged using

a Leica MZ10F stereomicroscope (Leica, Wetzlar, Germany) equipped with a DFC7000T camera (Leica) and observed to determine the occurrence of skeletal deformities.

5.2.5. Nutritional trial with gilthead seabream juveniles

Gilthead seabream juveniles were acquired from the aquaculture farm Mariscos de Estero S.A. Juveniles (4.2 ± 0.9 g and 6.6 ± 0.4 cm at the beginning of the trial) were housed at the Estação Piloto de Piscicultura de Olhão (IPMA- EPPO, Olhão, Portugal) in 250-L rectangular tanks at a density of 1.7 juveniles/L, with 350 L/h water renewal and a temperature of 25.3 ± 1.0ºC. Fish were randomly assigned to 3 tanks/replicates per condition. Gilthead seabream juveniles were fed the experimental diets as illustrated in Figure 5.2. Briefly, fish were fed 3 to 4 times a day with the experimental diets CTP4 or SKLT, and a group with control diet. A sampling (T1), was conducted when the fish tripled their weight (33 days after the beginning of the trial) and growth parameters (total length - TL, total weight, feed conversion rate – FCR), incidence of skeletal anomalies, hematological parameters, and RNA levels of bone-related marker genes were assessed.

After the first sampling, 40 fish/condition were fed with a commercial diet for an additional period of 6 months to evaluate if short-term dietary supplementation during the pre-ongrowing phase could result in an improved quality in the ongrowing phase. A final sampling (T2) was conducted and skeletal quality was assessed from radiographic images through geometric morphometrics.

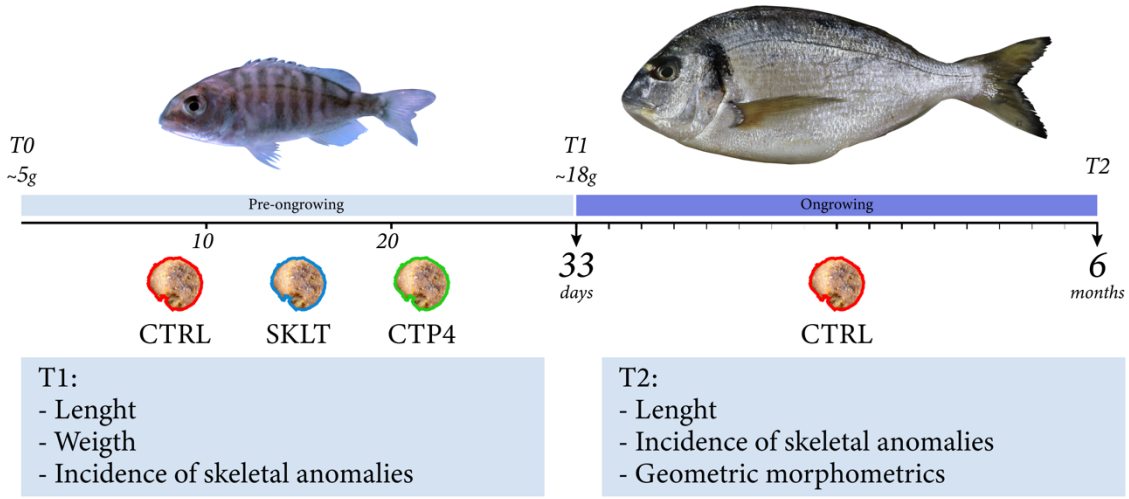


Figure 5.2. Experimental design of the nutritional trial with *S. aurata*.

5.2.6. Growth parameters in S. aurata juveniles

At T1 (33 days) fish were randomly selected in each tank, subjected to deep anesthesia with phenoxyethanol (0.5 ml/L) and subsequently euthanized by post-cranial section. Fish were then measured and weighted (n = 15 per tanks, n = 45 per diet). Feed conversion rate (FCR) was calculated as the ratio between the total amount of food given per treatment unit (n = 3) and the total weight gain.

5.2.7. Haematological parameters

Blood was collected from the caudal vein of gilthead seabream juveniles using a sterile needle/syringe and blood respiratory gasses, electrolytes, and metabolites were determined using an EPOC BGEM test card and the EPOC blood analysis system (Siemens Healthcare, Erlangen, Germany). The following analytes were determined: pH, partial pressure of O_2, partial pressure of CO_2, total CO_2, sodium, potassium, calcium, chloride, glucose, lactate, creatinine, hematocrit and blood urea nitrogen. In addition, cortisol content was determined in the plasma of juveniles fed the experimental diets, using a cortisol ELISA assay (IBL International, Hamburg, Germany).

5.2.8. Radiographic detection of skeletal anomalies

To assess the impact of the diets on the skeletal status of juveniles, >36 fish per treatment (n = 1) were radiographed with a DSX4000 PRO (Carestream, Rochester, USA). The incidence of skeletal deformities was evaluated through the observation of digital radiographs.

5.2.9. Geometric morphometrics analysis

To assess the effect of the supplementation with diets on external shape of the fish, we performed geometric morphometric analysis on digital radiographs of juveniles.

To accelerate the process of landmark detection, a recently developed automatic recognition program based on artificial intelligence (AI) was used with the aim of accurately estimating the percentage and severity of deformed gilthead seabream from digital radiographs in a sound and rapid way, without the need for manual inputs. The software uses a deep learning based Convolutional Neural Network (CNN, U Net 5) to automatically predict the landmark locations. A probability heatmap-based output was produced by the trained CNN which signifies likelihood of the location of each landmark location, which was then converted into a location point (x, y coordinates). Coordinates were then transformed (scaled, translated, and

rotated) using a Procrustes Superimposition (PS) model to adjust landmark configurations for centroid size eliminating potential effects that are irrelevant to shape (Figure 5.3).

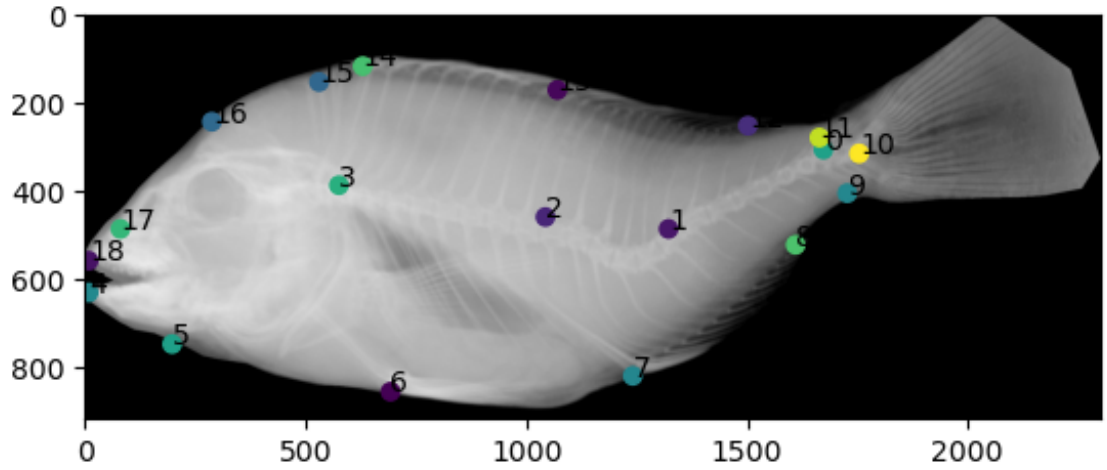


Figure 5.3. Example of landmarks obtained by the use of an automatic landmark detection software.

The transformed consensus matrix of the coordinates was plotted on two ordination models, a between-group Principal Component Analysis (bgPCA) and a Linear Discriminant Analysis (LDA). A Thin Plate Spline (TPS) model was then applied to the consensus configuration to compute the Principal Warps (orthogonal shape configurations). Individual landmark coordinates were then projected onto the principal warps. The model then allows for visualization of shape changes in the form of splines (deformation grids). These deformation grids are predicted by interpolation of shape changes between the landmark coordinates of the individual specimens.

5.2.10. Bone mineral content

Whole larvae (pools of 10) and juvenile vertebrae (clean from soft tissue; pools of 5) samples were dried for 24h at 105°C. Samples of vertebrae were transferred to a muffle furnace and ashed for 24h at 550°C. All samples were then weighed in quartz vessels and digested with 6 mL of nitric acid at 69% (Thermo Fisher Scientific, Waltham, USA) in a Discovery SP-D microwave digestion unit (SP-D80, CEM) for 3 min at 200°C. After digestion samples were diluted in water to obtain a final concentration of 5% nitric acid and mineral contents were measured through Microwave Plasma-Atomic Emission Spectroscopy (MP-AES, Model 4200, Agilent Technologies, Santa Clara, USA) using calibration standards for Ca and P.

5.2.11. Gene expression analysis

Whole larvae (pools of 10) and juvenile vertebrae (clean from soft tissue; pools of 5) were collected, deep frozen and stored at -80ºC. Samples were grinded using a mortar and liquid nitrogen prior to total RNA extraction using NZYol (NZYTech, Lisbon, Portugal), following manufacturer's instructions. RNA quantity and integrity were assessed using a NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific) and through agarose gel electrophoresis, respectively. Total RNA (1 µg) was treated with RQ1 RNase-free DNAse and reverse-transcribed at 37 °C for 1 h using M-MLV reverse transcriptase, RNAse-out and oligo(dT) primers (Thermo Fisher Scientific). Levels of gene expression (see Supplementary Table 2 for genes and primers) were then determined by semi quantitative PCR (qPCR) and normalized using *ß-actin* and *elongation factor 1α* as housekeeping genes. The reaction mixture containing 10 ng of reverse-transcribed RNA, 0.4 µM of qPCR specific forward and reverse primers for each gene and 10 µL of Green Master Mix (NZYTech) was submitted to an initial denaturation step at 95 °C for 15 s and then to 40 cycles of amplification (15 s at 95 °C, 20 s at 56 °C) using a CFX96 real-time PCR (Bio-Rad, Hercules, USA). Levels of gene expression were calculated using the ΔΔCt method[558].

5.2.12. Statistical analysis

Data was analyzed using Prism 9 (GraphPad Software, San Diego, USA). One-way ANOVA followed by Dunnett's multiple comparison test, unpaired *t* test ($p < 0.05$, gene expression), or Chi-square test ($p < 0.0001$, incidence and numbers of skeletal anomalies) were used to detect statistical differences. Geometric morphometric analysis was performed with the free software PAST Ver. 4.02.

5.3. Results

5.3.1. Larval growth parameters

Supplementation with the experimental diets did not dramatically affect the growth parameters of gilthead seabream larvae at 60 DAH (Figure 5.4). A significant reduction of the total length was reported for the group fed with SKLT diet (Figure 5.4A), and an increased condition factor (K) was reported for the larvae fed with the CTP4 diet (Figure 5.4 C).

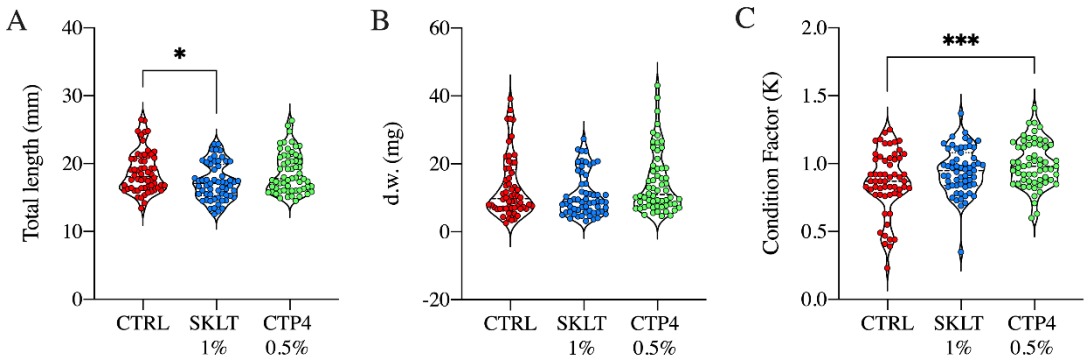


Figure 5.4. Effect of the dietary supplementation of SKLT and CTP4 diets on total length (A), dry weight (B), condition factor K (C). Statistical differences were tested through Student´s *t* test ($p < 0.05$). Significant differences and *p* values are indicated as follows: 0.0332 (*), 0.0002 (***).

5.3.2. Whole larvae mineral content

No significant alterations of the bone mineral contents were reported among the treatments (Figure 5.5 A-C). Both calcium (Ca) and phosphorous (P) contents were increased, although not significantly, by the exposure to both experimental diets compared to the control group.

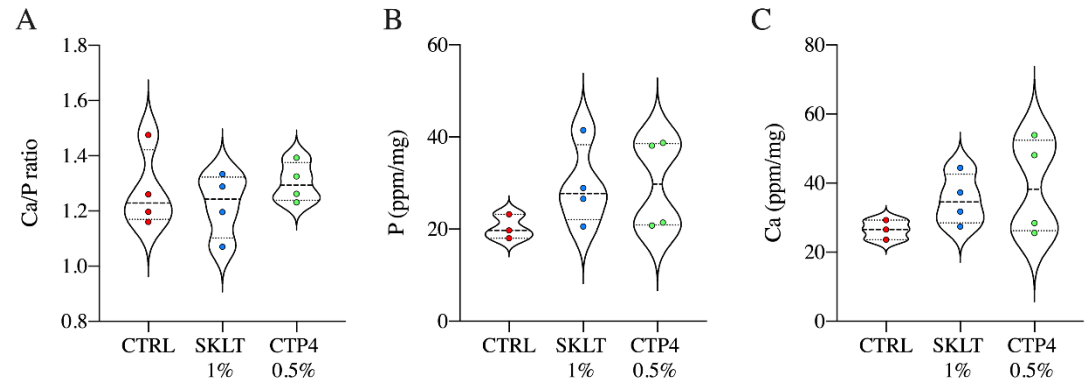


Figure 5.5. Effect of the dietary supplementation of SKLT and CTP4 diets on the total content of calcium (Ca), phosphorous (P), and Ca/P ratio of gilthead seabream larval stages. Statistical differences were tested through Student´s *t* test ($p < 0.05$).

5.3.3. Larval incidence of skeletal anomalies

Overall distribution of skeletal anomalies in *S. aurata* larvae was not affected by the treatments. The most common deformities were associated with the caudal fin complex in all the experimental conditions (Figure 5.6).

5.3.4. Expression of bone marker genes in S. aurata larvae

Osteoblast markers (*oc1*, *spp1*) were upregulated by both experimental diets, while the early osteoblast-associated marker *sp7* was only up-regulated in CTP4 treated larvae (Figure 5.7). Extracellular matrix remodeling marker *mmp9* was also upregulated by both experimental diets (Figure 5.7). Antioxidant enzymes (*grs*, *cat*, *gpx1*, *sod1*) as well as a marker of cellular oxidative stress (*hsp70*) were also evaluated (Figure 5.8). Both microalgae-supplemented diets reduced *grs* and *hsp70* expression.

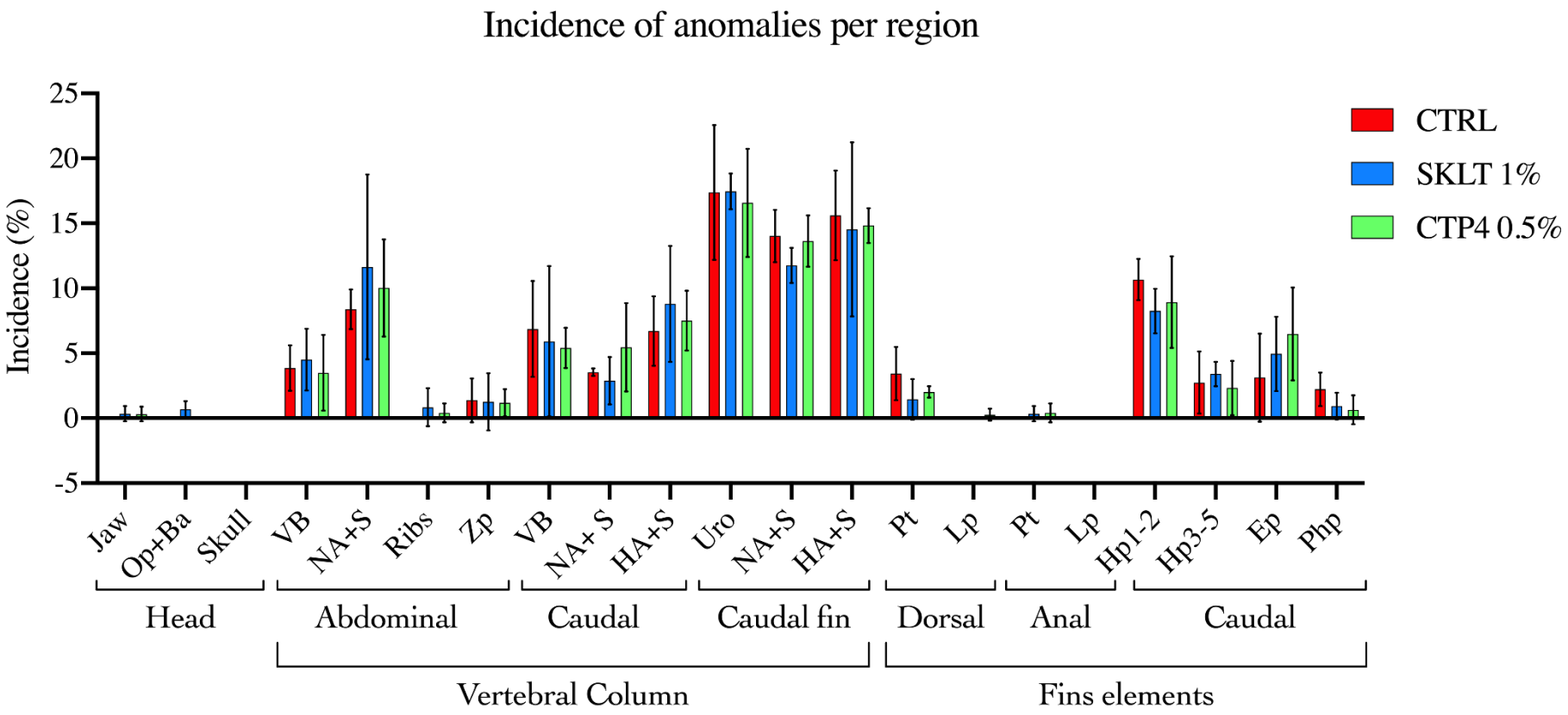


Figure 5.6. Incidence of skeletal anomalies by region in *S. aurata* larvae fed with control, SKLT, and CTP4 diets. Differences were tested though two-way ANOVA followed by Dunnett's multiple comparison test ($p < 0.05$). Op+Ba, Opercular bones and Branchial arches; VB, vertebral bodies; NA+S, neural arches and spines; Zp, zygapophyses; HA+S, hemal arches and spines; Uro, urostyle; Pt, pterygiophores; Lp, lepidotrichia; Hp1-2, hypurals 1-2; Hp3-5, hypurals 3-5; Ep, epural; Php, parahypural.

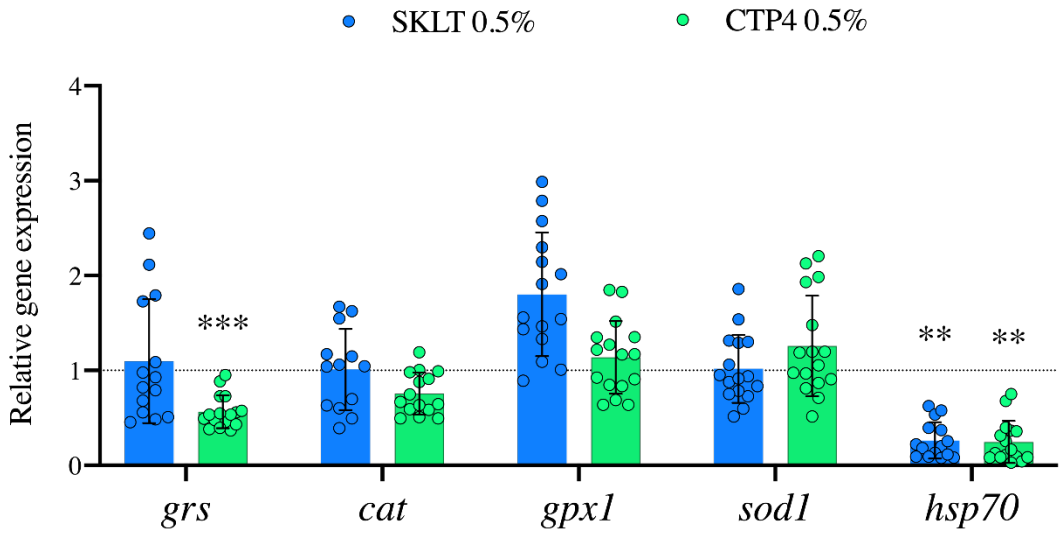


Figure 5.7. Effect of SKLT and CTP4 diets on mRNA levels of bone formation and remodeling gene markers. Data are presented as gene expression relative to the control group. Statistical differences between the control and the treatments were tested through Student´s *t* test ($p < 0.05$). Significant differences and *p* values are indicated as follows: 0.0332 (*), 0.0021. (**), 0.0002 (***).

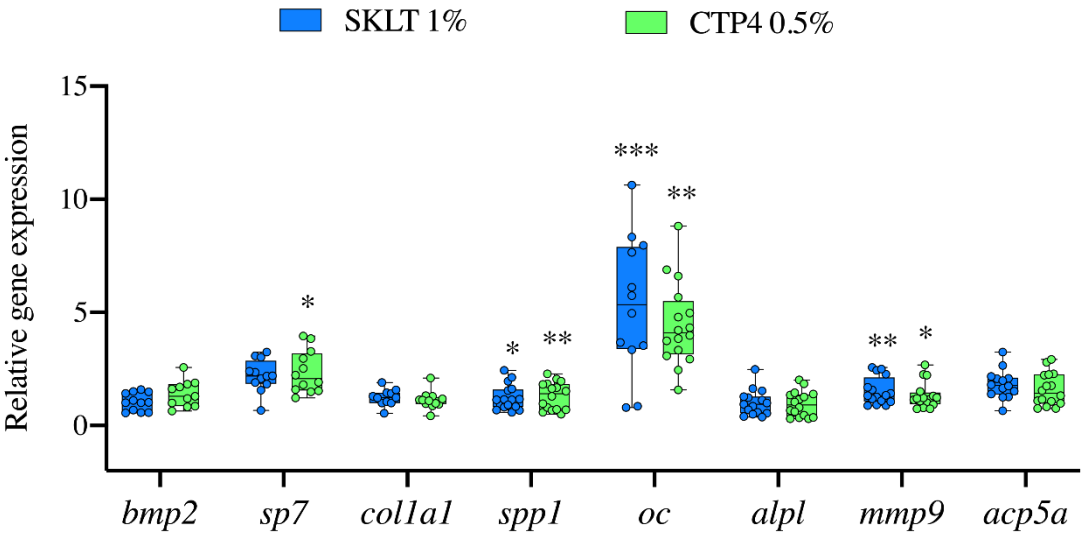


Figure 5.8. Effect of SKLT and CTP4 diets on mRNA levels of gene markers for antioxidant defenses and cellular oxidative stress. Data are presented as gene expression relative to the control group. Statistical differences between the control and the treatments were tested through Student´s *t* test ($p < 0.05$). Significant differences and *p* values are indicated as follows: 0.0021. (**), 0.0002 (***).

5.3.5. Growth parameters is S. aurata juveniles

Juvenile fish fed the CTP4 diet displayed higher growth at 33 days after the beginning of the trial in terms of weight but not length compared to the control (Figure 5.9A, B). A higher food conversion rate (FCR), although not significant, was also observed in fish fed SKLT 1% supplemented diet (Figure 5.9C).

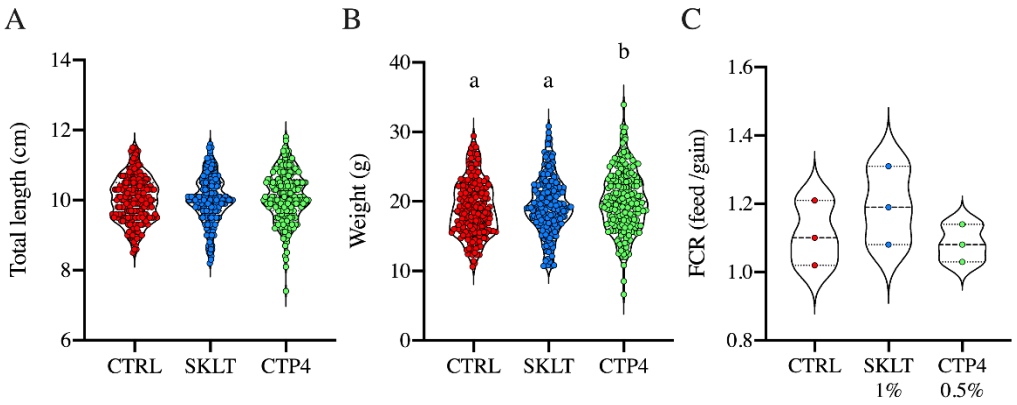


Figure 5.9. Effect of SKLT and CTP4 diets on growth parameters of juvenile *S. aurata* . Statistical differences between the control and the treatments were tested through one-way ANOVA ($p < 0.05$). Significant differences and *p* values are indicated with different letters.

5.3.5. Haematological parameters

Blood analysis was carried out in juveniles fed experimental diets for 33 days to assess the effect of the diets on fish hematological parameters. Food supplementation with microalgae extracts had no impact on fish physiological status and stress levels. Levels of pCO_2 were increased and may indicate a higher cellular activity (Figure 5.10).

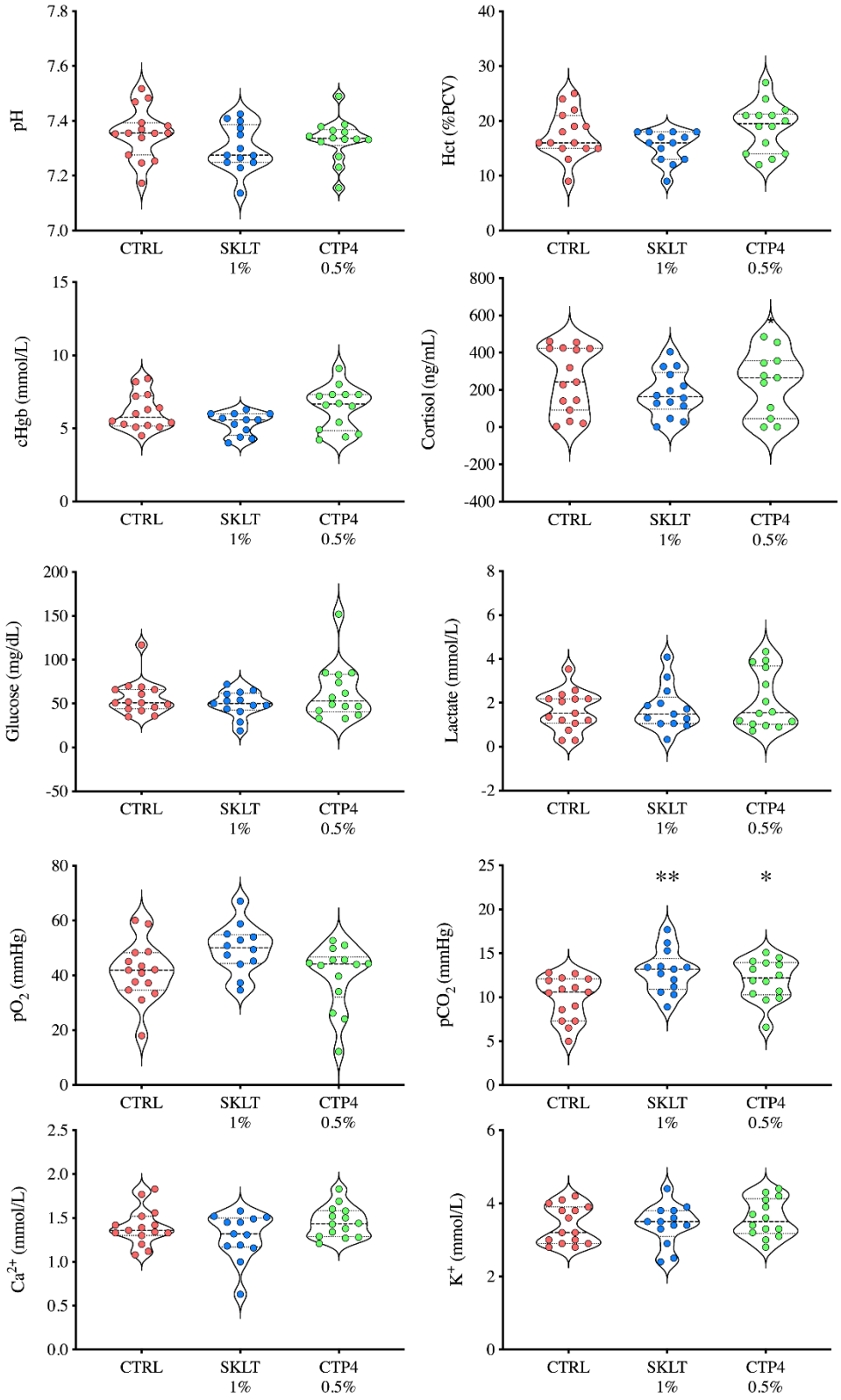


Figure 5.10. Effect of SKLT and CTP4 diets on hematological parameters of *S. aurata* juveniles. Statistical differences between the control and the treatments were tested through one-way ANOVA ($p < 0.05$). Significant differences and p values are indicated as follows: 0.0332 (*), 0.0021. (**).

5.3.6. Vertebrae mineral content

The calcium-phosphate ratio was unaffected in the SKLT group, while it was significantly reduced in CTP4-treated fish (Figure 5.11A). However, when looking at the content of calcium and phosphorous separately, while calcium did not vary between treatments, a higher phosphorus content was observed in the juveniles fed both the supplemented diets (Figure 5.11B, C).

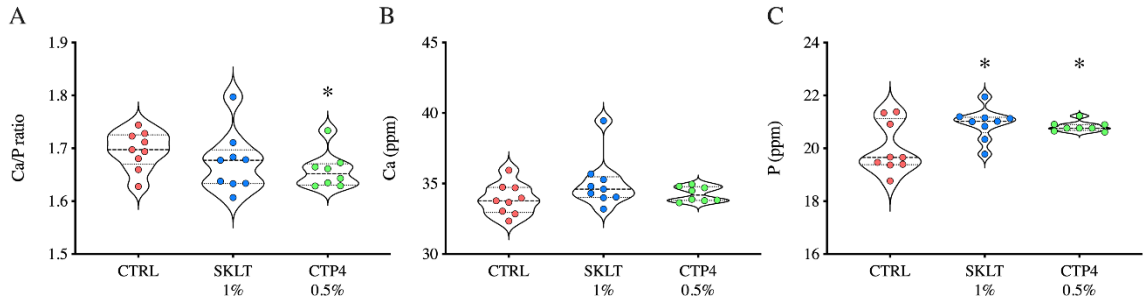


Figure 5.11. Effect of SKLT and CTP4 diets on the vertebral mineral content of gilthead seabream juveniles. Statistical differences between the control and the treatments were tested through one-way ANOVA ($p < 0.05$). Significant differences and p values are indicated as follows: 0.0332 (*).

5.3.7. Incidence of skeletal anomalies

At T1 (33 days after the beginning of the exposure) *S. aurata* juveniles fed the SKLT diet showed, no differences in the total incidence of skeletal anomalies (Figure 5.12A). However, when the deformity charge (number of deformities per individual) was calculated, both experimental diets showed a significantly lower (χ^2, $p < 0.0001$) number of deformities per individual (Figure 5.12B). When focusing on the severe deformities affecting the vertebral column, in all the experimental group the caudal area was the most affected (Figure 5.12C). However, the CTP4-treated fish had a reduction of vertebral anomalies in this (Figure 5.12C).

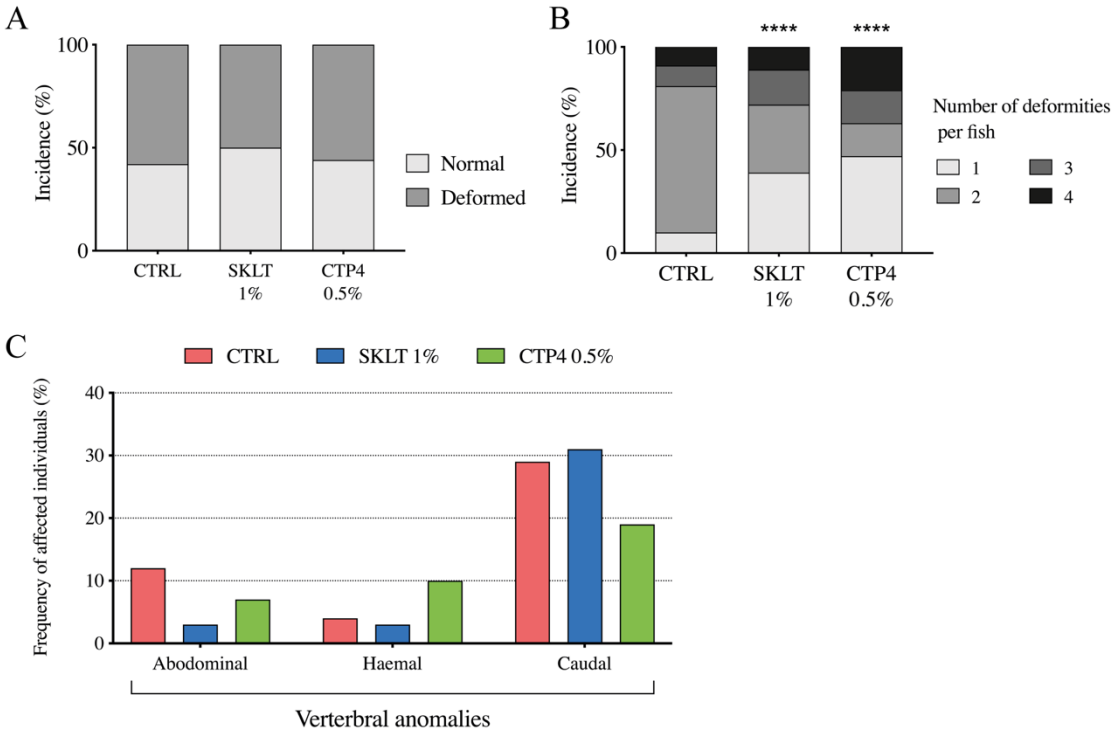


Figure 5.12. Effect of SKLT and CTP4 diets on the incidence and distribution of skeletal anomalies of *S. aurata* juveniles. Statistical differences between the control and each treatment were tested through Chi-square test (χ^2). Significant differences and *p* values are indicated as follows: $p < 0.0001$ (****).

5.3.8. Expression of marker genes (juveniles)

In *S. aurata* juveniles, both experimental diets regulated the expression of gene markers of osteoblastic differentiation (*sp7*), and ECM production (*col1a1*) and remodeling (*acp5)* (Figure 5.13). In addition, both experimental diets induced an overexpression of *glutathione peroxidase* (*gpx1*), suggesting an impact on bone antioxidant mechanisms (Figure 5.13).

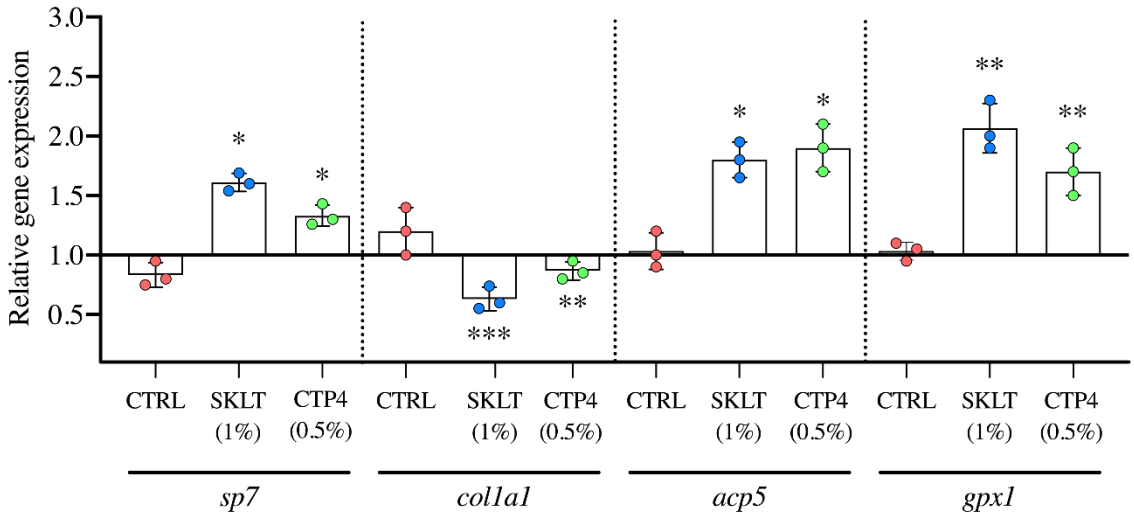


Figure 5.13. Effect of SKLT and CTP4 diets on mRNA levels of selected bone and oxidative stress marker genes in *S. aurata* juveniles. Data are presented as relative gene expression. Statistical differences between the control and the treatments were tested through Student´s *t* test ($p < 0.05$). Significant differences and *p* values are indicated as follows: 0.0332 (*), 0.0021. (**), 0.0002 (***).

5.3.9. Growth and geometric morphometric analysis of S. aurata

In order to assess if the positive effects of this short-timed supplementation with microalgae extracts could endure until later stages of production, 40 fish per condition were fed with a commercial diet for an additional period of 6 months. After this period, fish fed with both enriched diets were longer (Figure 5.14A), while fish fed with CTP4 0.5% diet retained a lower incidence of caudal deformities from the first sampling (Figure 5.14B).

Furthermore, fish shape after 6 months was analyzed by geometric morphometric analyses. Within this scope, two ordination models (LDA and bgPCA) were applied to landmarks corresponding to morphometric shape features detected on radiograph images and revealed shape differences in fish fed with the experimental diets. In detail, The LDA revealed significant differences in fish shape among all the three dietary treatment groups (Wilks' λ, $p < 0.00001$,). Pairwise Mahalanobis squared distances were significantly different between all the conditions (post hoc with Bonferroni corrections, $p < 0.00001$, Figure 5.15).

The bgPCA ordination model, plotting individual fish with respect to the maximum variance explained between the conditions, enables the visualization of condition centroids on the x, y coordinate plane, as well as thin plate splines (TPS) deformation grids along the uniform axes. The TPS grid in the negative direction (in which both algae-supplemented treatments centroids

are located) of the first axis displays a “slendering” effect of morphological features while the TPS grid in the positive direction of the first axis (where the control treatment centroid is placed) shows a “rounding” effect of morphological features with respect to the consensus (Figure 5.16). These data suggest that both treatments with SKLT 1% and CTP4 0.5% enriched diets resulted in fish with a “slenderer” shape on average compared to the CTRL diet fed fish.

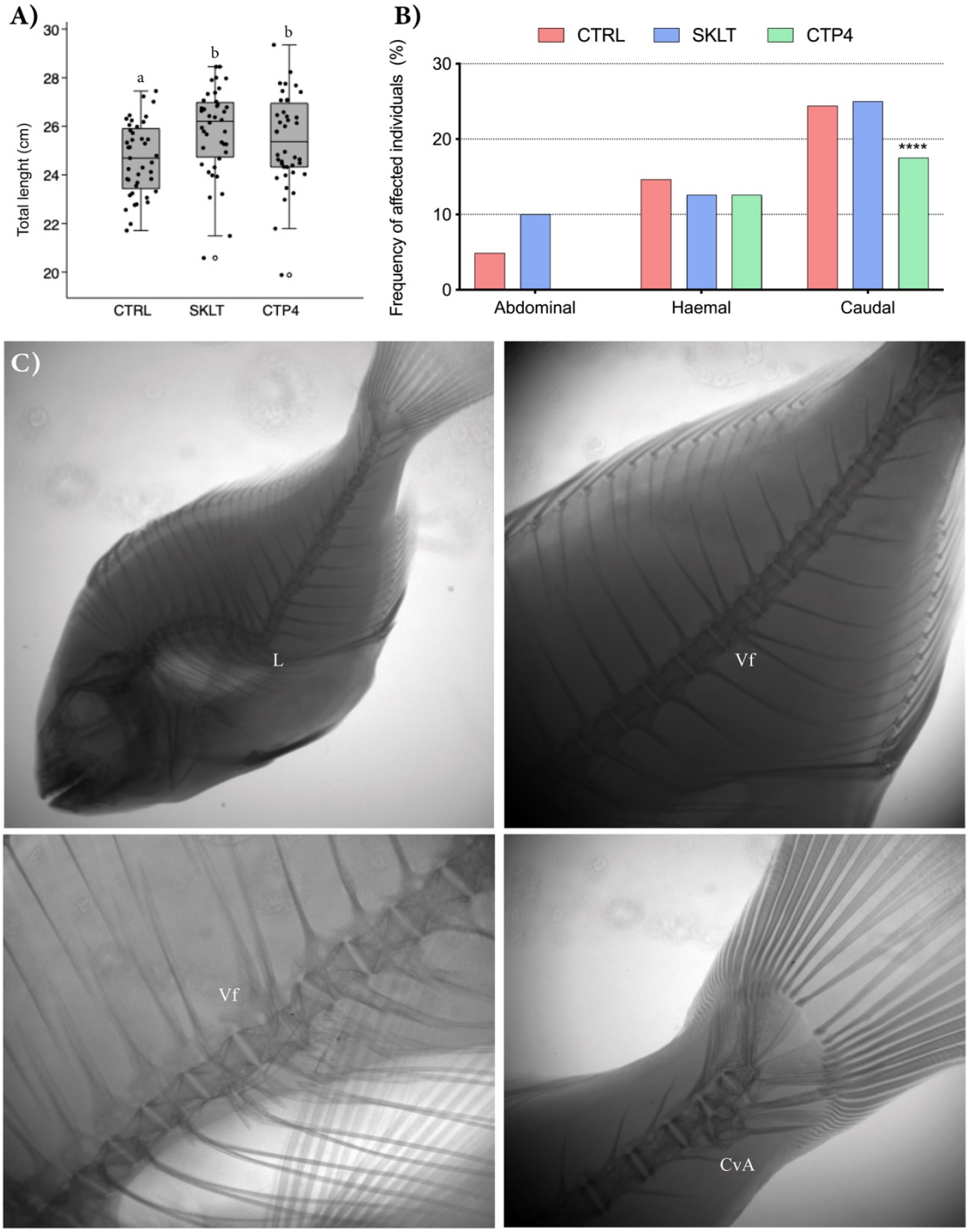


Figure 5.14. Long-term effect of a short-time dietary exposure with SKLT and CTP4 diets on gilthead seabream juveniles growth and incidence of vertebral anomalies. (A) Fish total length were statistical differences were tested through one-way ANOVA ($p < 0.05$) and indicated by different letters and; (B) incidence of severe vertebral anomalies, were statistical differences were tested by a chi-squared (χ^2) test and significant differences indicated as "****" ($p < 0.000001$). (C) Common skeletal anomalies found in juveniles of gilthead seabream. L, lordosis; Vf, vertebrae fusion; CvA, caudal vertebra anomaly.

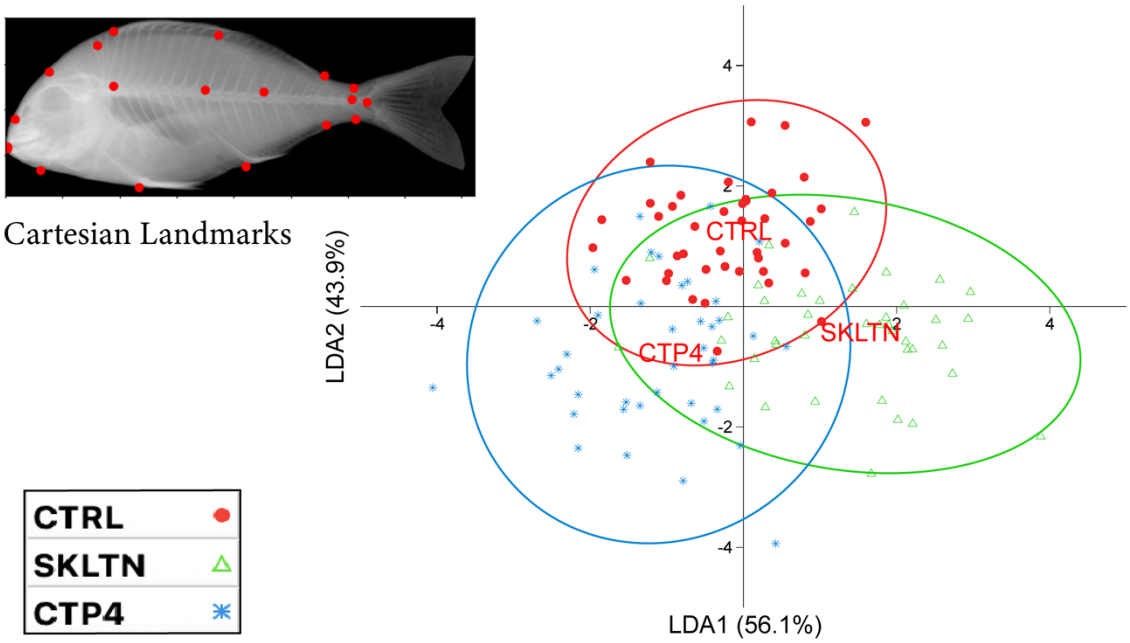


Figure 5.15. Long-term effect of a short-time dietary exposure with SKLT and CTP4 diets on gilthead seabream shape, 6 months after the end of the treatment. Linear Discriminant Analysis (LDA) of geometric landmarks detected on radiograph images.

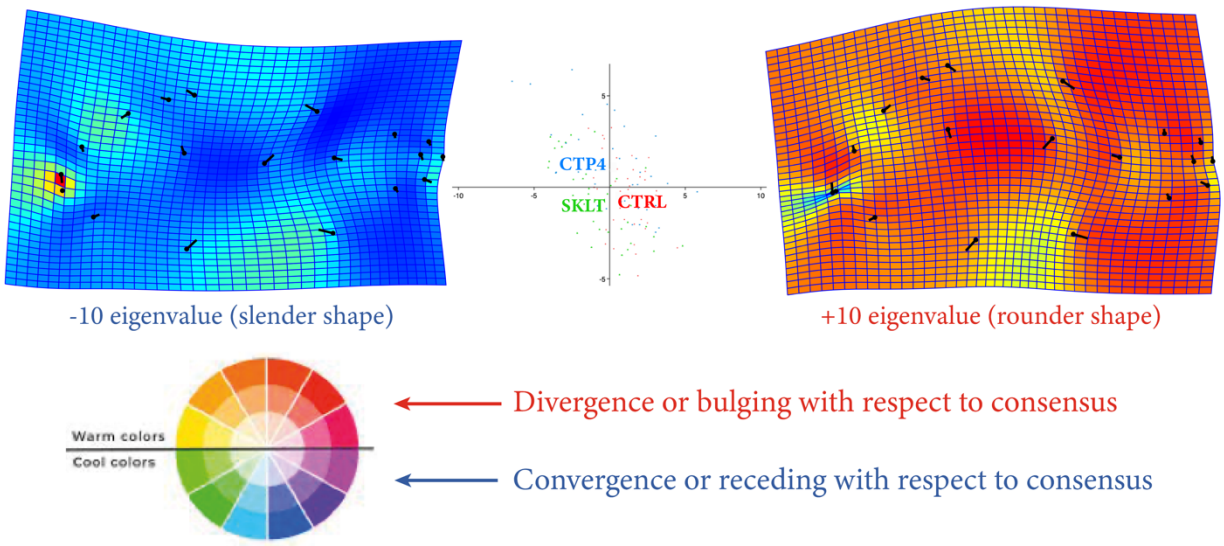


Figure 5.16. Long-term effect of a short-time dietary exposure with SKLT and CTP4 diets on gilthead seabream shape. Between-groups principal component analysis (bgPCA) and thin plate spline (TPS) grids displaying the +10 (on the right) and -10 (on the left) eigenvalues.

5.4. Discussion

Microalgae species belonging to the genera *Skeletonema* spp. and *Tetraselmis* spp. are of particular biotechnological interest due to their potential applications in different fields including aquafeeds, cosmetics, and drug discovery. Ethanolic extracts obtained from species belonging to these two groups were recently described for their high contents in polyunsaturated fatty acids and by a strong anti-oxidant activity *in vitro*[565].

In the scope of this PhD thesis, we have selected ethanolic extracts from *Skeletonema costatum* and *Tetraselmis striata* as a consequence of a large-scale screening activity looking for extracts with pro-osteogenic and pro-mineralogenic properties (**Chapter 2.2**). Both extracts were validated in the model species zebrafish (*Danio rerio*) and were found to be able to induce a better skeletal development, improve bone mineralization, and reduce the incidence of skeletal anomalies in this model species (**Chapter 4**).

Here, we have tested potential application of the two extracts in the aquaculture industry, by evaluating them as dietary supplements to improve gilthead seabream (*Sparus aurata*) skeletal health in larvae and pre-ongrowing juveniles. In larvae, although the supplementation with both microalgae extracts was able to stimulate molecular mechanisms of bone formation and

mineralization, similarly to what observed in zebrafish through **Chapter 4**, no clear effect was observed in terms of reduction of skeletal anomalies nor on larval growth or in mineral content. Overall, the similarities to what was observed in zebrafish provide additional evidence of how molecular mechanisms involved in bone formation and mineralization are highly conserved among teleosts, despite the evolutionary distance and anatomical differences separating a marine sparid such as the gilthead seabream from a freshwater cyprinid like the zebrafish, such as the lack of osteocytic bone in the former, among others[410].

The absence of a clear effect on the skeletal phenotype that we observed in gilthead seabream larval stages might be attributed to the low inclusion of the extracts within the experimental diets, that was chosen to optimize the cost-benefit ratio and considering that the two extracts were bioactive at the same concentrations used in zebrafish (**Chapter 4**). Metabolic, physiological and size differences between these two species might also have contributed in causing the different results here obtained.

In addition, the relatively short duration of treatment might be at the base of the lack of a strong skeletal effect in gilthead seabream larvae. Core events of skeletal development and bone ossification in gilthead seabream are observed within the first 90 days after hatching (DAH)[585–587]. Here, with the aim of avoiding possible early developmental toxicity of the extracts and optimizing the costs associated to the use of the extracts, we have treated the larvae from 30-60 DAH. For the design of future experiments aimed at testing earlier exposures, it should be noted that the weaning of gilthead seabream larvae from live-feeds has been achieved as early as 17 DAH in previous studies[588]. Another way of overcoming the limits imposed by the use of formulated diets could be the use of enriched of live feeds such as rotifers (*Brachionus* spp.) and *Artemia* sp. The enrichment of live preys with lipidic emulsions is a suitable tool to efficiently administer molecules via bioencapsulation to fish early larval stages without the life-stage boundaries imposed by the use of formulated microdiets[589–591].

In this sense, a future experiment aimed at optimizing the use of these microalgae extracts should consider starting the supplementation earlier, by applying them as live feed enrichments, followed by the inclusion into early-weaning microdiets. This strategy might be able to shed a light on the applicability of two microalgal extracts to improve the skeletal phenotype of gilthead seabream larval stages.

Surprisingly, in juveniles, that are in a stage were the developmental processes for the formation of the skeleton are terminated, the treatment with microalgae extracts was particularly beneficial. Not only fish had increased growth, but also a reduced incidence and severity of skeletal anomalies, especially in the most affected areas, the caudal portion of the

vertebral column and the caudal fin complex, which being involved in the swimming activity are particularly prone to develop skeletal deformities.

Investigation of the molecular mechanism involved in bone formation, remodeling and oxidative stress have revelated that bone remodeling process and antioxidant mechanisms were activated in fish treated with the microalgae extracts. These observations indicate that the two extracts might not be only acting on processes involved in the early development of skeletal structures, but also providing a positive nutritional input supporting the following remodeling phase. In this sense, although many skeletal anomalies in fish appear as consequence of events taking place during early development of the skeleton, an important portion of deformities tend to appear later on development and are caused by external stimuli acting on the post-developmental remodeling of fish bones[409,410].

Bone tissues of teleosts is particularly sensitive to oxidative stress. Reactive oxygen species (ROS) inhibit osteoblastic differentiation and stimulate osteoclastic differentiation, thus altering the bone remodeling equilibrium and co-participating in the rise of several skeletal disorders[571–574]. In this sense, the use of nutritional antioxidants to prevent oxidative damage is becoming a valuable alternative and more "prevention-oriented" approach in both human medicine and aquaculture nutrition[190,592]. The capacity of oxidative stress to induce skeletal anomalies and the possibility of these being rescued by treatment with antioxidant agents was recently shown in zebrafish[575] and the gilthead seabream[576].

The extracts here studied might be providing an antioxidant protection against oxidative processes by stimulating the expression of antioxidant mechanisms, as here suggested by the increased expression of the antioxidant enzyme glutathione peroxidase (*gpx1*) in both gilthead seabream juveniles and, although not significantly, in larvae. This hypothesis is supported by the similar results that we obtained in zebrafish fed with experimental diets supplemented with the same extracts (see **Chapter 4**).

However, the hypothesis these microalgae extracts excerpt their positive effect by providing an antioxidant protection is solely based on the observation of an increased expression of antioxidant enzymes, which is not sufficient. Future research should aim at gathering data on GPX protein expression and activity, and whether this correlate with the reduction of markers of cellular oxidative damage (such as DNA damage, lipid peroxidation, peroxisome's membrane stability) in the bone tissue. Nevertheless, both ethanolic extracts from *Skeletonema* spp. and *Tetraselmis* spp. were previously observed to possess a potent anti-oxidant properties[565].

Additional work aimed at correlating the antioxidant activity of these extracts with the positive effects on bone must be conducted to fully elucidate the mechanism of action these microalgae extracts. Interestingly, in gilthead seabream larvae a reduction of the mRNA expression of glutathione reductase *grs* and the molecular chaperone *hsp70* was also reported. Both proteins are important components of the cellular stress response system and their role in the present context is still to be clarified.

Finally, we performed geometric morphometric analysis of juveniles 6 months after suspending the supplementation with microalgae extracts. Geometric morphometric is an approach that studies shape by using cartesian landmark coordinates that provide a proxy for morphologically distinct shape variables[593]. Landmarks are associated to distinct biological features and analyzed based on their relative position and orientation, enabling the evaluation of morphological features and patterns[594]. Our analysis showed how both extracts affected fish external shape by inducing a more slender and elongated profile compared with the control fish, which were shorter and more rounded.

An important part of the marketability of the aquaculture product consists on its alignment with the aesthetic preferences of the costumers, such as color and shape[595,596]. The most paradigmatic example of this is represented by the well-documented costumers' preferences for the pink-reddish color of the wild Atlantic salmon (*Salmo salar,* Linnaeus 1758) flesh, which in aquaculture fish (otherwise lacking this attribute) is induced by dietary supplementation of synthetic astaxanthin[597–599]. In light of the results obtained in the present work, microalgal extracts might represent a possible novel tool to intervene on fish shape with similar proposes. In addition, an interesting future perspective would be to the investigate the effect of the supplementation of microalgal extracts on fish color.

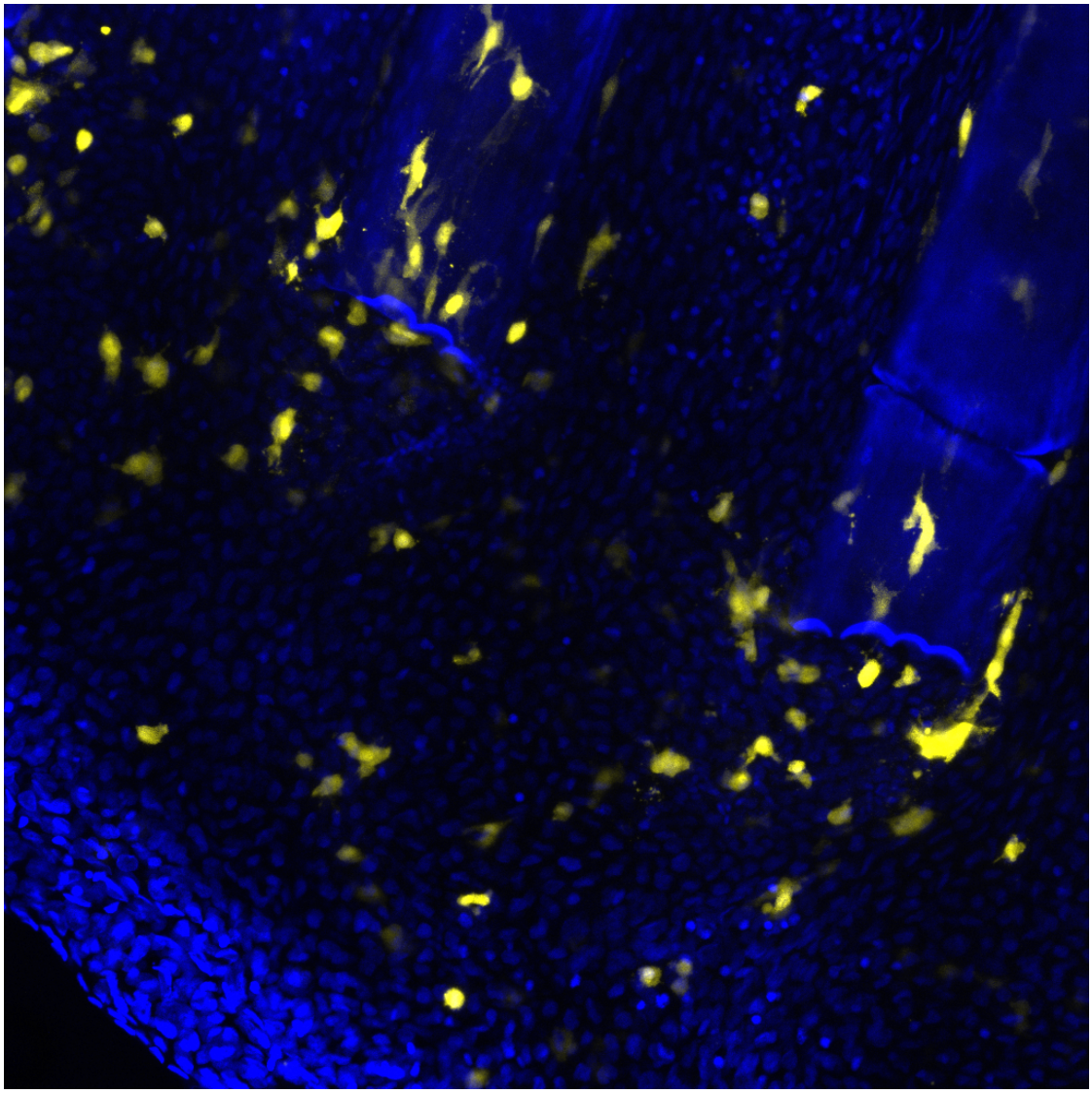
5.5. Conclusions

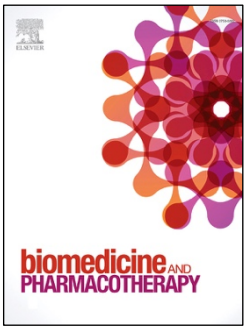
In the present work, we have tested diets for the commercially reared species gilthead seabream (*Sparus aurata*) supplemented with two microalgae-derived extracts that were previously shown to possess pro-osteogenic and pro-mineralogenic properties (see **Chapters 2.2** and **Chapter 4**). Supplementation with microalgal extracts was able to stimulate molecular mechanisms of bone formation and regulated gene associated to antioxidant defenses in gilthead seabream larvae. In juveniles, the supplementation with microalgae had a very positive effect by stimulating growth, bone formation, antioxidant defenses, and by affecting fish shape. Further studies in larval stages are recommended to test if early-supplementation and possible

use as live-feed enrichment might obtain better results. Overall, this data provided a proof of concept of how microalgal extracts can be a potential tool used as nutritional supplement to increase growth and ameliorate skeletal health in the gilthead seabream production pipeline, with positive and long-lasting effects even when implemented in short treatments (1 month).

CHAPTER 6.

THE ANTI-RESORPTIVE ACTIVITY OF *Skeletonema costatum* ETHANOLIC EXTRACT





This section will be part of: A. Carletti, K. Pes1, M. Tarasco, J. T. Rosa, S. Poudel, H. Pereira, M. L. Cancela, V. Laizé, P. J. Gavaia. "Inhibition of osteoclast differentiation and reduction of bone loss in fish models exposed to a microalga extract with anti-inflammatory activity". In preparation for submission to *Biomedicine & Pharmacotherapy*.

(Previous page). Confocal image showing the detail of zebrafish regenerating lepidotrichia at 24 hours post-amputation. Blue, DAPI; Yellow, ctsk^{+} cells (osteoclasts). Picture by Alessio Carletti. To acquire the image, it was used an Andor Technology Benchtop Confocal microscope BC43. The instrument was under beta-testing at the time of the image acquisition (2022).

Abstract

Bone erosive pathologies are the leading cause of fractures worldwide and immediate action should be taken to relief medical and economical burdens. The few therapeutic options available to prevent bone loss have limitations associated with secondary and unspecific effects and short-term efficacy. The pharmaceutical industry is actively searching novel compounds with the potential for treating bone loss and those with anti-inflammatory properties have received a particular attention after a link between inflammation and bone erosive disorders was reported. In this work, the ethanolic extract of the marine microalga *Skeletonema costatum* was evaluated for its anti-osteoclastogenic potential in several fish and mammalian models. Analysis of cellular dynamics revealed that extract inhibited osteoclast recruitment and differentiation during the regeneration of zebrafish caudal fin, and transcriptomics confirmed a strongly downregulation of marker genes for inflammation, macrophages recruitment and osteoclast differentiation. Extract could also partially prevent bone loss in the medaka osteoporotic model and revealed anti-proliferative, immune-suppressive and anti-osteoclastogenic properties in a rodent macrophage cell line. Our results provide strong evidence for the presence of anti-resorptive and anti-inflammatory compounds in *S. costatum* ethanolic extract, and highlight the potential of this microalga for a further development of drugs applicable to human bone erosive disorders.

6.1. Introduction

Bone erosive pathologies represent urgent clinical and pharmacological challenges[1,3]. They are the world leading cause of fractures, and are characterized by an unbalanced bone remodeling leading to mineral loss and causing structural deterioration[4,132]. Among the multiple and complex mechanisms underlying these disorders, inflammation is widely recognized as root causes prompting the progression of many erosive pathologies[471]. In fact, postmenopausal osteoporosis is nowadays considered to be an inflammatory disease[36,600,601], and an increasing amount of evidence has connected chronic inflammation with disorders associated to bone loss as a secondary pathological drift, including renal osteodystrophy, arthritis, inflammatory bowel diseases, and cystic fibrosis[602–604].

Studies conducted in the early 90s provided a mechanistic explanation of how inflammation leads to bone loss[605]. Pro-inflammatory cytokines released by immune cells such tumor necrosis factor α (TNFα), are potent inducers of bone resorption through its interaction with RANKL signaling pathway and the stimulation of osteoclastic differentiation[46,47,606–608].

Therapeutically-wise, disorders that lead to bone loss – e.g. osteoporosis, Paget's disease of bone, hyperparathyroidism, and renal osteodystrophy – are commonly treated with anti-resorptive agents such as bisphosphonates and RANKL-monoclonal antibodies (e.g. Denosumab)[151,546,564], or osteoanabolic drugs such as parathyroid hormone (PTH) analogues (e.g. teriparatide and abaloparatide)[170,172,173]. However, the current antiresorptive agents tend to lose efficacy over time[183] and are associated with both mild and rare yet extremely serious complications, such as the osteonecrosis of the jaw[159,609], while bone anabolic drugs have been associated with the occurrence of osteosarcoma, and a short therapeutic window[170,172,173].

The anti-sclerostin monoclonal antibody Romosumab is a dual-action drug – it promotes bone formation and inhibits bone resorption – that showed promising results in clinical trials. However, positive effects on bone status appear to be limited in time, and its use has been associated to rare cardiovascular secondary effects[175,186].

The limited provision of therapeutics for bone erosive disorders is fueling a strong demand for the discovery of new "osteoactive" agents. The leading role of inflammatory processes in the pathophysiology of osteoporosis and other bone disorders raises the question whether anti-inflammatory-based therapy could be used to prevent bone loss in patient suffering erosive disorders. Surprisingly, little investigation has been conducted in this regard so far, and the anti-inflammatory drugs used by patients suffering osteoporosis or other metabolic bone disorders are primarily to alleviate pain[35].

Nonetheless, data collected over the last decade have highlighted the efficacy of TNFα-inhibitors on preventing bone loss and improving bone mineral density (BMD), when given to patients suffering joint erosive disorders such as rheumatoid arthritis and ankylosing spondylitis, highlighting the anti-osteoclastogenic capacity of an anti-inflammatory drugs[610–612]. The ability of a monoclonal antibody targeting both TNFα and RANKL to inhibit osteoclastogenesis and prevent bone loss in ovariectomized mice[613], suggests that a combined anti-resorptive and anti-inflammatory approach could be an effective strategy for the next generation of drugs to be implemented for the treatment of osteoporosis and other bone erosive diseases.

In this scope, small fish such as the zebrafish (*Danio rerio*) and medaka (*Oryzias latipes*) are becoming increasingly relevant as pre-clinical models in drug development thanks to a series of technical advantages such as low maintenance cost, small size, short life cycle, high fecundity, and the wide availability of transgenic and mutant lines modelling human bone disorders[397,399,401,403].

Recently, marine-derived natural compounds have also become a promising asset in drug discovery[208,210]. An increasing number of studies have established the capacity of marine molecules with anti-inflammatory activity to prevent bone loss in animal models[237–240]. Microalgae are a highly promising group of organisms for the provision of new bioactives. In addition, they are associated with advanced cultivation technologies, can count on a well-established and growing industry, and are the source of a plethora of bioactive compounds[539,541,542]. Anti-inflammatory compounds isolated from marine microalgae such as sulphated polysaccharides[237] and pigments, like fucoxanthin[238–240], could suppress RANKL-induced osteoclastic differentiation and prevent bone loss in mammalian models of osteoporosis. Similarly, polyphenols produced by several microalgae strains[614] have anti-inflammatory and anti-osteoporotic properties[192,615,616]. Microalgae of the genus *Skeletonema* have recently gained momentum in the field of biotechnologies as sources of anti-inflammatory molecules such as polyphenols[559,565–567,570].

Here, we propose to assess the effect of the ethanolic extract from *S. costatum*, previously described for its anti-inflammatory activity *in vitro*[565,570], on osteoclastic cellular dynamics *in vivo* by combining the use of zebrafish transgenic reporter lines and transcriptomics. We then evaluated the anti-osteoporotic activity of the extract in a medaka model of RANKL overexpression-induced osteoporosis[617] and explored the translatability of the results obtained in fish models to a mammalian *in vitro* system.

6.2. Materials and methods

6.2.1. Preparation of ethanolic extracts

Freeze-dried biomass of *Skeletonema costatum* (Necton S.A., Olhão, Portugal) was macerated with 96% ethanol (Laborspirit Lda, Lisbon, Portugal) using a biomass-solvent ratio of 1 g:40 mL (M/V), by gently stirring at 24 °C for 18 h. Macerate was centrifuged for 5 min at 1,000 × *g* using an Allegra 6R centrifuge (Beckman Coulter Inc, Brea, USA) and supernatant was collected. The pellet was washed twice with 96% ethanol and all supernatants were pooled then vacuum filtered sequentially through 0.45 µm and 0.22 µm nylon membranes (Labbox Labware S.L., Barcelona, Spain). Filtrate was concentrated with a rotatory evaporator RV 10 digital (IKA-Werke GmbH & Co. KG, Staufen im Breisgau, Country), with the temperature set at 40 °C and pressure at 178 mbar, until obtaining a dense, paste-like extract. Extraction yield – 37.9 ± 2.4% – was calculated from 2 mL aliquots (n = 3) placed under a gentle flow of 99.8% nitrogen until complete evaporation of the solvent.

6.2.2. Fish maintenance

Zebrafish wild type line AB and transgenic line *Tg(Ola.ctsk:FRT-DsRed-FRT-Cre,myl7:EGFP)*$^{mh201,\ 618}$, hereafter referred to as *Tg(ctsk:DsRed)*, were maintained in a water recirculating system ZebTEC (Tecniplast, Buguggiate, Italy) with the following conditions: temperature 28 ± 0.1 °C, pH 7.5 ± 0.1, conductivity 700 ± 50 µS, ammonia and nitrites at levels below 0.1 mg/L, nitrates lower than 50 mg/L, and a photoperiod of 14:10 h light-dark. Medaka transgenic line *Tg(rankl:HSE:CFP)*$^{TG1135,\ 619}$, hereafter referred to as *Tg(rankl:HSE:CFP)*, were purchased from the National BioResource Project Medaka (NBRP Medaka)[620] and maintained in a home-made water recirculating system with the following conditions: temperature 27 ± 0.1 °C, pH 7.0 ± 0.1, conductivity 300 ± 100 µS, ammonia and nitrites at levels below 0.1 mg/L, nitrates lower than 50 mg/L and a photoperiod of 14:10 h light-dark. For both zebrafish and medaka, system water was prepared by supplementing reverse osmosis treated water with a salt mixture (Instant Ocean, City, USA) and sodium bicarbonate (Sigma-Aldrich, St. Louis, USA). All fish were fed daily with the commercial dry food Zebrafeed (Sparos Lda, Olhão, Portugal).

6.2.3. Zebrafish caudal fin regeneration assay

The caudal fin of wild-type or transgenic adult zebrafish aged 3-4 months was amputated 1-2 segments anterior to the bifurcation of the most peripheral branching lepidotrichia, as described by Cardeira et al. (2016)[621]. After finectomy, fish (n > 14) were placed at 33 ± 1 °C in 3 L-plastic containers at a density of 5 fish/L. Fish were exposed for 5 days (wild-type fish) or up to 10 days (*Tg(ctsk:DsRed)*) fish) to the ethanolic extract (hereafter referred to as SKLT) at 56 µg/mL or to ethanol (vehicle) at 0.1%, supplemented in system water. Treatment was totally renewed daily. Moderate water dynamics and air-water exchanges were facilitated by bubbling fish tanks with an air pump. Water quality parameters were monitored daily and maintained stable for the duration of the experiment as follows: dissolved oxygen 7.0 ± 0.5 mg/L, pH 7.4 ± 0.2 and conductivity 680 ± 20 µS. Wild-type fish were sacrificed at 120 hours post-amputation (hpa) using a lethal anesthesia of 0.6 mM tricaine methanesulfonate (MS-222, pH 7.0; Sigma-Aldrich), then immersed for 30 min in 0.03% alizarin red S (AR-S, pH 7.4; Sigma-Aldrich) and washed 2 times for 5 min with system water. Stained fish were imaged for fin morphometric analysis. Transgenic fish *Tg(ctsk:DsRed)* were sampled ($n \geq 9$) at different timepoints (24, 48, 72, 96, 120 and 240 hpa), then immersed for 30 min in 0.2% calcein (pH 7.4; fluorexon, Sigma-Aldrich), washed twice in system water for 10 min. After the staining, fish were anesthetized for 5 min in tricaine and imaged. For transcriptomic analysis, fin

blastemas were collected at 24 hpa, pooled (n = 5, 10 blastemas per pool), and conserved at -80 °C until further processing.

6.2.4. TRAP activity in caudal fins

Tg(ctsk:DsRed) zebrafish ($n \geq 8$) were sacrificed at 24 and 240 hpa with a lethal dose of tricaine (see above). Caudal fins were amputated at the level of the caudal peduncle, washed once with 1X phosphate buffer saline (PBS, pH 7.4) and fixed for 4 h in 4% paraformaldehyde solution (PFA, solubilized in PBS, pH 7.4) at 24 °C. Tartrate resistant acid-phosphatase (TRAP) staining was performed following a protocol adapted from Blum & Begemann (2015)[622] and fins were imaged as described below.

6.2.5. Morphometric analysis of caudal fins

Fins were imaged under a MZ10F fluorescence stereomicroscope (Leica, Wetzlar, Germany) coupled to a DFC7000T color camera (Leica). Bright-field images were collected to assess the progression of fin regeneration. Fluorescence images were collected to assess (i) *de novo* bone formation in wild type fish stained with AR-S or calcein, and (ii) the involvement of *ctsk*-expressing cells in transgenic fish labelled with DsRed. Bright-field images were acquired with an exposure time of 1 ms. Fluorescence images were acquired with the filter set ET560/40x - ET630/75m and an exposure time of 600 ms for mCherry, and the filter set ET470/40x - ET525/50m and an exposure time of 80 ms for GFP. Other image parameters were as follows: gamma 1.00, image format 1920×1440 pixels, binning 1×1. Fluorescence images were analyzed using ImageJ software version 2.0.0-rc-69/1.52p and processed using ZFBONE toolset for morphometrics[489]. Fin regeneration and mineralization were assessed following the method described by Cardeira et al.[621], by calculating the regenerated area (REG), the stump width (STU), the mineralized area (MIN), and the average width of the rays before the amputation (RAYs). Fin ray patterning was also assessed by calculating the average ray width ratio ($\overline{W}$) and the average bifurcation ratio ($\overline{Bt}$) as shown in Supplementary equations *a* and *b*. For the quantification of *ctsk* signal in transgenic fish, DsRed-positive area was measured using a color threshold on fluorescent images and normalized with REG/STU. For the quantification of TRAP signal, TRAP-positive areas were measured using a color threshold on bright-field images, and subsequently normalized with REG/STU.

6.2.6. RNA preparation

Total RNA was extracted from pools of blastemas at 24 hpa (n = 5) using NZYol (NZYTech, Lisbon, Portugal) and quantified using a NanoDrop OneC spectrophotometer (Thermo Fisher Scientific, Waltham, USA). RNA integrity was confirmed using an Experion Automated Electrophoresis system (Bio-Rad, Hercules, USA). Only RNA with an RNA integrity number (RIN) higher than 7 were used.

6.2.7. RNA sequencing and analysis of differentially expressed genes

RNA sequencing was outsourced to STABVIDA Lda (Caparica, Portugal). DNA libraries were constructed using a Stranded mRNA Library Preparation kit (STABVIDA) and sequenced on a Novaseq platform (Illumina, San Diego, USA) to generate 150 bp paired-end sequencing reads. Raw sequence data was processed using CLC Genomics Workbench 12.0.3[623]. Trimming was done in 3 steps: Quality trimming based on quality scores (error probability 0.01); Ambiguity trimming (ambiguous limit of 2 nucleotides); Length trimming to discard reads shorter than 30 nucleotides. The quality-checked sequencing reads were mapped against zebrafish reference genome GRCz11 (GCF_000002035.6) using length fraction and similarity fraction equal to 0.8. TPM (transcripts per million) and RPKM (reads per kilobase of transcript per million mapped reads) were then determined from mapped data[624].

Principal component analysis (PCA) was performed to assess variation patterns in the gene expression dataset and identify outlier samples for quality control (Supplementary Figure 7). For differential expression analysis a generalized linear model approach influenced by the multi-factorial EdgeR method was used[625]. Only genes with fold change ≥ 1.1 or ≤ -1.1, and false discovery rate (FDR) p-value ≤ 0.05 were considered.

Lists of upregulated and downregulated genes were analyzed with the online resource Database for Annotation, Visualization and Integrated Discovery (DAVID)[626,627] for Gene Ontology – Biological Processes (GO:BP) with $p < 0.05$ and enriched biological processes.

6.2.8. Medaka model of RANKL overexpression-induced osteoporosis

Eggs of the medaka line *Tg(rankl:HSE:CFP)* were produced following an in-house breeding program and maintained in Petri dishes with 40 mL of system water supplemented with 0.0002% (w/v) methylene blue until 8 days post-fertilization (dpf). At 8 dpf, hatched larvae were placed at 39 °C for 2 h (heat-shock) and screened for CFP (cyan fluorescent protein) signal using a fluorescence microscope (see parameters below). CFP-positive fish were distributed into a 6-well plate at the density of 5 larvae/well. Each well was filled with 10 mL

of system water supplemented with SKLT at 56 µg/mL or 0.1 % ethanol (vehicle). Treatment was renewed 100% daily.

At 6 days after heat-shock (dahs), larvae were sacrificed with a lethal dose of tricaine (see above), stained with AR-S and imaged as described above. For each fish, bright-field and fluorescence images were collected. Bright-field images were acquired using an exposure time of 2 ms. AR-S fluorescence images were acquired using the filter set ET560/40x - ET630/75m and an exposure time of 700 ms. CFP fluorescence images were acquired using the filter set ET436/20x - ET480/40m and an exposure time of 200 ms. Other image parameters were set as follows: gamma 1.00, image format 1920×1440 pixels, binning 1×1. Images were analyzed using ImageJ using two *ad hoc* macros.

CFP fluorescence images were transformed to 8-bit images and CFP-positive area was measured using a color threshold (min intensity 7 and max intensity 255). Pixel mean intensity inside the CFP-positive area was calculated and used as a proxy for CPF fluorescence intensity. AR-S fluorescence images were transformed to 8-bit images and AR-S positive areas were measured using a color threshold (min intensity 5 and max intensity 255). AR-S positive area was normalized using the total body area (determined manually from bright-field images) to correct for inter-specimen size variation. AR-S positive area in abdominal and caudal vertebrae was used as a proxy of the mineralization of the vertebral column. The nomenclature proposed by Di Biagio et al. (2022)[628] was used to identify vertebrae from 4 to 29. The number of mineralized neural arches was manually counted in each fish from AR-S images.

6.2.9. Culture of mouse RAW 264.7 macrophages

RAW 264.7 cells were cultured in 10 mL cell culture dishes with Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich), 1% penicillin–streptomycin, 1% L-glutamine, and 0.2% fungizone at 37 °C in a 5% CO_2-humidified atmosphere. Pre-confluent cell cultures were sub-cultured 1:4 every other day using trypsin-EDTA solution (0.2% trypsin, 1.1 mM EDTA, pH 7.4). All cell culture reagents were from GIBCO-ThermoFisher Scientific, unless otherwise stated.

6.2.10. Cytotoxicity and cell proliferation

LDH Cytotoxicity Assay kit and XTT Cell Proliferation Assay kit (Canvax Biotech, Córdoba, Spain) were used, respectively, to evaluate the effects of SKLT extracts on cellular toxicity and proliferation. For cytotoxicity, RAW 264.7 cells were seeded in a 96-well plate at 1.0×10^4 cells/well ($n = 4$) in 100 µL of culture medium supplemented with either the SKLT extract at

50, 75, 100, 200 µg/mL or 0.1% ethanol (vehicle) and cultured for 24 and 48 h. Relative cytotoxicity was calculated as a percentage of LDH lysis control. For cell proliferation, RAW 264.7 cells were seeded in a 96-well plate at 500 cells/well ($n = 6$) in 100 µL of culture medium supplemented with either the SKLT extract at (50, 75, 100, 200 µg/mL or 0.1% ethanol (vehicle) and cultured for 1, 3, 5 or 7 days. Culture medium was renewed every other day. Relative cell proliferation was calculated as a percentage of the negative control.

6.2.11. Osteoclast differentiation

RAW 264.7 cells were seeded in 12-well plates at 1.5×10^4 cells/well in 1 mL of culture medium (described above) supplemented with either SKLT extract at 50 µg/ml or 0.1% ethanol (vehicle) and treated for 6 days with 50 ng/mL of RANKL (prepared in 0.1% BSA) to induce their differentiation into multinucleated osteoclasts. Experimental conditions were: Undifferentiated control (Ethanol); differentiated control (RANKL+Ethanol); differentiated cells exposed to SKLT extract (RANKL+SKLT). Culture medium was freshly prepared and replaced daily. Osteoclast differentiation was assessed by counting the number of tartrate-resistant acid phosphatase (TRAP) positive cells and the number of nuclei following 4′,6-diamidino-2-phenylindole (DAPI) staining. For this, cells were fixed in 4% PFA at 24 °C (RT) for 10 min and stained with a protocol adapted from Blum & Begemann (2015)[622]. Cells were subsequently stained with DAPI for nuclei detection and count. Cells were imaged using an Axio Vert.A1 inverted microscope (ZEISS, Jena, Germany) coupled with (i) an Axiocam 202 monocolor camera (ZEISS) for DAPI images, or (ii) a VWR VisiCam 5 Plus (VWR, Radnor, USA) for bright-field TRAP images. Number of multinucleated osteoclasts per field (5.0X magnification; $n = 9$) was calculated considering only $TRAP^+$ cells with at least 2 nuclei.

6.2.12. Statistical analysis

For all the experiments, normality was tested with a D'Agostino-Pearson omnibus normality test or with an Anderson-Darling test ($p < 0.05$). Homoscedasticity was tested through the Brown-Forsythe test ($p < 0.05$). When the distribution of the data of all the experimental groups resulted normal and homogeneous, statistical differences between the control and the extract were tested with either an Unpaired *t* test or a one-way ANOVA followed by Dunnett's multiple comparison test ($p < 0.05$). If the distribution of the data of any of the experimental conditions resulted non-normal or nonhomogeneous, statistical differences between control and the extract were tested with a Mann-Whitney test or a non-parametric test followed by

Dunn's multiple comparison test ($p < 0.05$). Statistical analyses were performed using Prism version 9.00 (GraphPad Software Inc., La Jolla, United States).

6.3. Results

6.3.1. Skeletonema extract induces the distalization of ray bifurcation by inhibiting osteoclast recruitment

Adult zebrafish were exposed to the ethanolic extract of *Skeletonema costatum* (SKLT) for 5 days after caudal fin amputation to evaluate the effect on the regenerative and mineralogenic performances, and on the ray patterning (Figure 6.1A, B). While no effect was found on the regenerated (Figure 6.1C), SKLT-treated fish exhibited an increase of the mineralized area in AR-S stained fish (Figure 6.1D), suggesting the presence of pro-mineralogenic or antiresorptive compounds in the extract. SKLT also affected the patterning of the regenerating fin rays. Although no effect was found on ray width (Figure 6.1E), SKLT induced the distalization of the bifurcation point in the proximo-distal axis (Figure 6.1F). To further characterize SKLT effect, osteoclast dynamics were monitored in transgenic fish *Tg(ctsk:DsRed)* (Figure 6.2A). A peak in *ctsk*$^{+}$ cells was observed in control fish (exposed to ethanol) between 24 and 48 hpa (Figure 6.2B, D), following which their density in the regenerated area declined over time. In contrast, fewer *ctsk*$^{+}$ cells were observed in SKLT-treated fish at both 24 and 48 hpa (Figure 6.2C, D). At 72 hpa, no differences were detected between SKLT-treated and control fish. This reduction in *ctsk*$^{+}$ cells translated into strong reduction of osteoclast activity at 24 hpa, as detected by TRAP staining (Figure 6.3A, B). These results indicate that SKLT extract either inhibited osteoclast precursor recruitment or osteoclast differentiation in the early stages of fin regeneration.

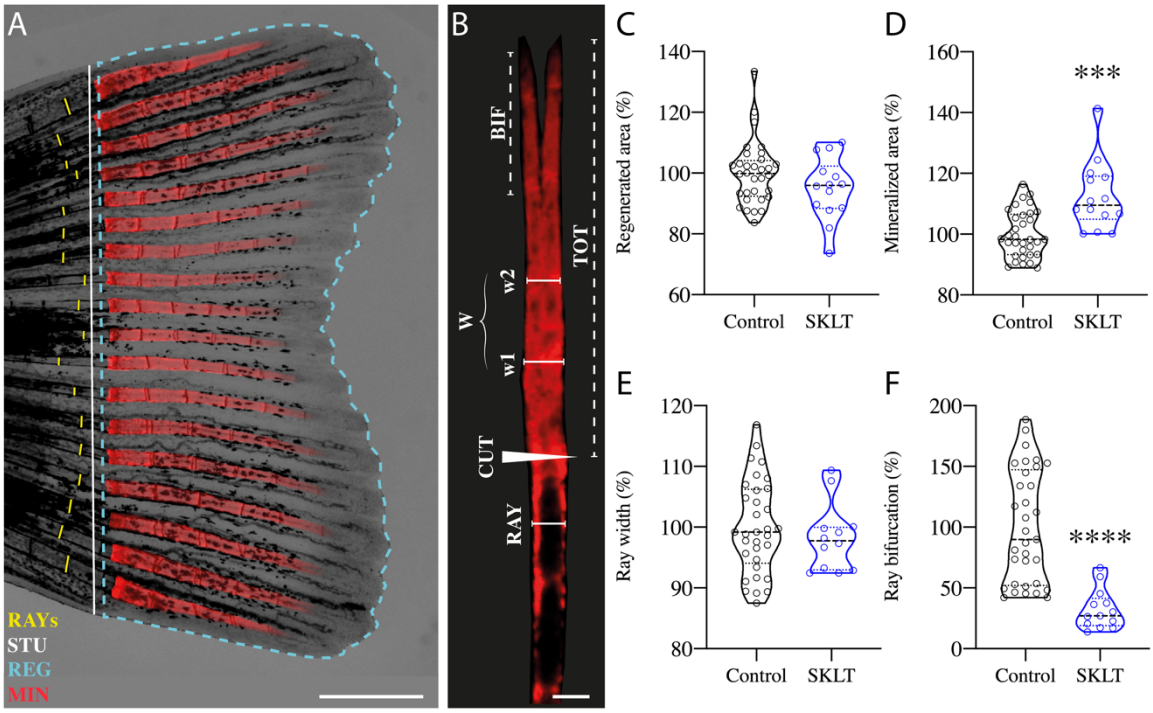


Figure 6.1. Effect of the waterborne exposure to the ethanolic extract of *Skeletonema costatum* (SKLT) or 0.1% ethanol (Control) on caudal fin regeneration and mineralization in zebrafish young adults. (**A**) Representative image illustrating the morphometric measurements used for the analysis of regenerative and mineralogenic performances. (**B**) Detail of an AR-S stained fin ray highlighting the morphometrics used for the evaluation of the effect over ray patterning. Amputation plan (CUT); Ray width before the amputation (RAY); Average ray width after amputation (W); Bifurcation distance (BIF); Total regenerated ray length (TOT). Effect of the exposure to the extract on the regenerated area (**C**), Mineralized area (**D**), Average ray width (**E**), and ray bifurcation (**F**). Normality was tested though Anderson-darling test ($p < 0.05$). Statistical differences were tested through unpaired *t* test ($p < 0.05$), or by a non-parametric Mann-Whitney test ($p < 0.05$) whenever the data distribution resulted non-normal. Asterisks indicate values statistically different. $p < 0.0002$ (***), $p < 0.0001$ (****). Scale bars = 1000 µm (**A**), and 250 µm (**B**).

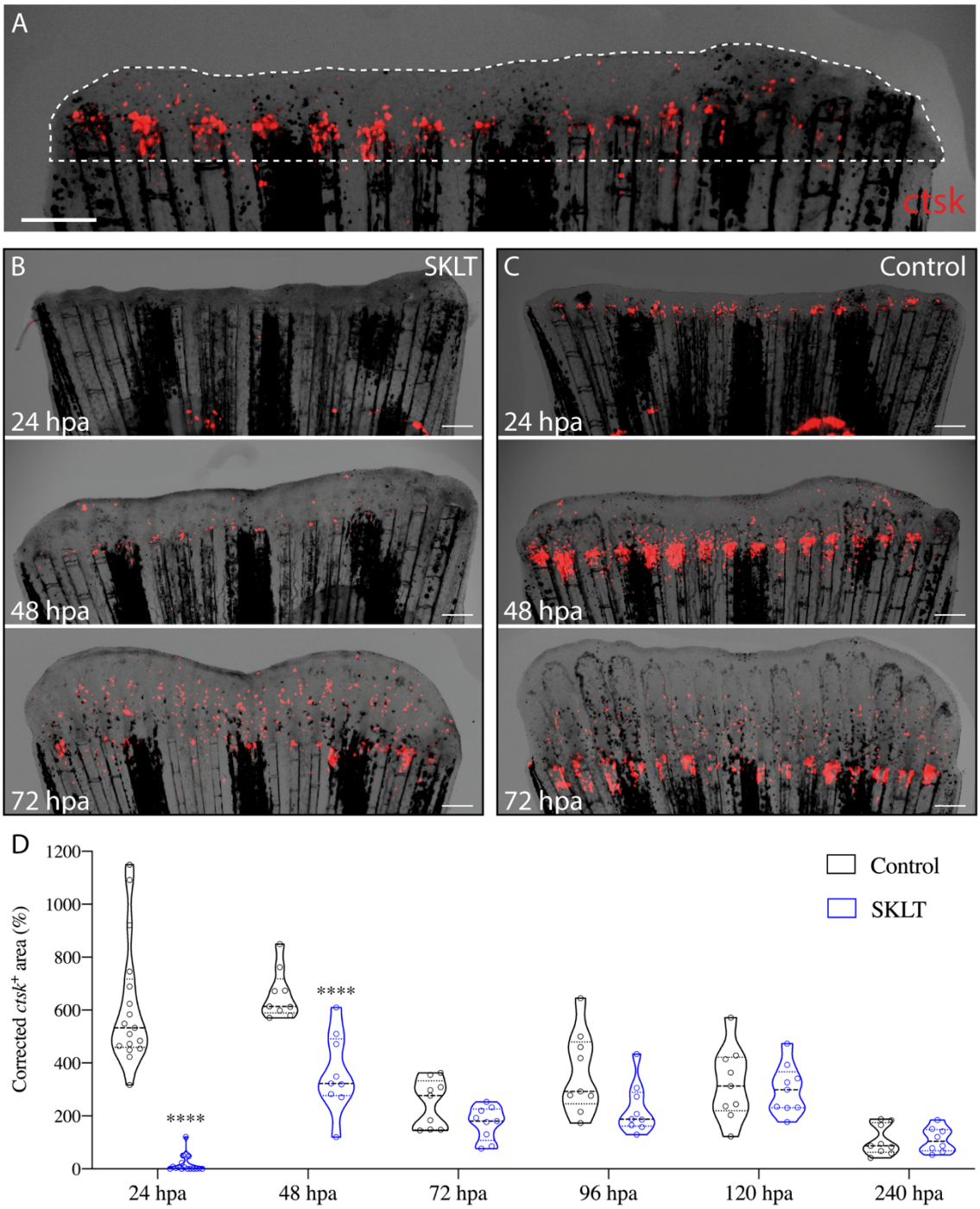


Figure 6.2. Effect of *Skeletonema costatum* ethanolic extract (SKLT) on the recruitment of *ctsk*$^{+}$ cells to the regenerating caudal fin. (**A**) Representative image illustrating how the regenerated area (dotted line) and *ctsk*$^{+}$ area (red signal) were measured. (**B**, **C**) Details of *ctsk*$^{+}$ area in regenerating fins at 24, 48- and 72-hours post-amputation (hpa) in SKLT treated (**B**) and control fish (**C**). (**D**) Quantification of the *ctsk*$^{+}$ area in regenerating fins exposed to SKLT and its control fish from 24 to 240 hpa. At each timepoint, statistical differences were tested through unpaired t test ($p < 0.05$), or by a non-parametric Mann-Whitney test ($p < 0.05$)

whenever the data distribution resulted non-normal. Asterisks indicate values statistically different. $p < 0.0001$ (****). Scale bars = 500 µm.

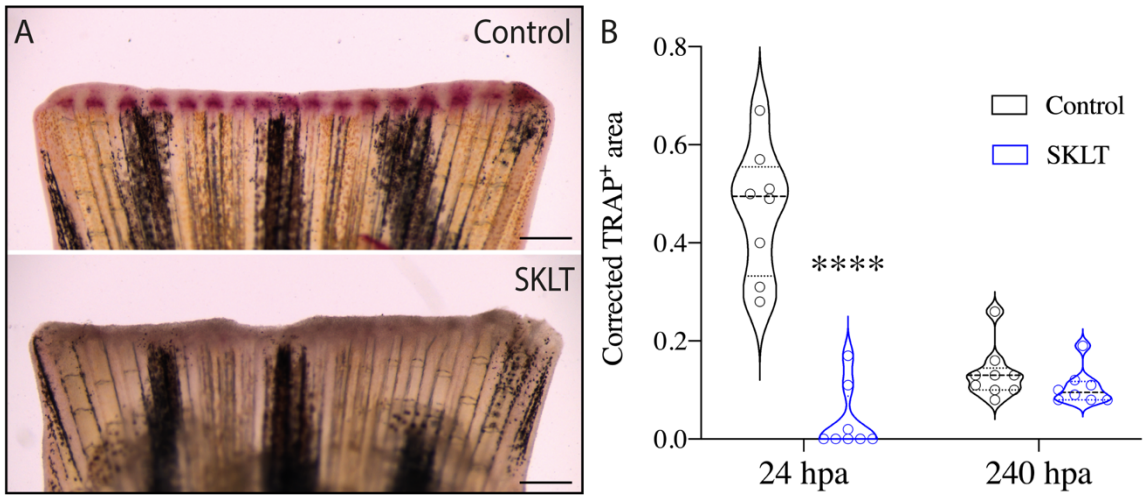


Figure 6.3. Effect of *Skeletonema costatum* ethanolic extract (SKLT) on osteoclast activity (TRAP staining) in regenerating caudal fin at 24- and 240-hours post-amputation (hpa). Representative images showing TRAP staining at 24 hpa in control and SKLT-treated (**A**) fish. **(B)** At each timepoint, statistical differences were tested through unpaired *t* test ($p < 0.05$). Asterisks indicate values statistically different. $p < 0.0001$ (****). Scale bars = 500 µm.

6.3.2. Skeletonema extract reduces the regeneration of the caudal fin

Caudal fin regeneration was estimated in amputated zebrafish exposed to SKLT extract by measuring the corrected regenerated area (REG/STU) over time. SKLT-treated fish showed a significant reduction of the regenerated area starting from 72 until 240 hpa (Figure 6.4), suggesting that the extract contains compounds with the capacity to inhibit processes involved in regenerative programs.

6.3.3. Skeletonema extract inhibits the expression of genes involved in inflammation, immune response, and osteoclast differentiation

Analysis of RNA sequencing data identified 228 upregulated and 321 downregulated genes in the blastema of SKLT-treated fish at 24 hpa. Of these, 175 upregulated (76.8%) and 205 downregulated (63.9%) were assigned a Gene Ontology (GO) terms by DAVID. 17 GO terms were found to be upregulated (FDR < 0.05), and after being filtered for redundancy, 9 were selected. Of these, biological processes important for photoperiodism including: Circadian

regulation of gene expression (GO:0032922), Response to light stimulus (GO:0009416), Photoperiodism (GO:0009648) were found to be significantly enhanced, together with Vacuolar protein processing (GO:0006624), and Positive regulation of heart rate (GO:0010460). Interestingly, processes involved in energy production from fatty acid metabolism, including Tricarboxylic acid cycle (GO:0006099), Lipoxygenase pathway (GO:0019372), and Lipid oxidation (GO:0019372) were found to be stimulated (Figure 6.5A). Similarly, 55 GO terms were found to be downregulated (FDR < 0.05), and after being filtered for redundancy, 14 terms were selected. Most biological processes downregulated were related to inflammation and immune response: Immune response (GO:0006955), Inflammatory response (GO:0006954), Neutrophils migration and chemotaxis (GO:1990266, GO:0030593), Monocyte and Lymphocyte chemotaxis (GO:0002548, GO:0048247), T cell activation (GO:0050870), Antibodies production and adaptive immune response (GO:0002381, GO:0002250), Macrophages cytokine production (GO:0060907), Chemokine-mediated signaling pathway (GO:0070098), Cellular responses to inflammatory cytokines such as IL-1 and TNF (GO:0071347, GO:0071356). Interestingly, Cell proliferation (GO:0051301), and ERK1/2 MAPK cascade (GO:0070374) were also inhibited (Figure 6.5A).

A panel of genes involved in recruitment macrophages, immune response, and osteoclastic differentiation were selected from the list of differentially expressed genes (DEGs) to get clues on the involvement of possible an anti-inflammatory effect on the reduction of *ctsk*$^{+}$ expressing cells in the fins blastemas. Markers of inflammation (*tnfa* and *il6*) were significantly downregulated in fish exposed to SKLT, as well as markers of early-responding immune cells, i.e. neutrophils (*mpx*) and macrophages (*mpeg1.3*, *mpeg 1.2*, *cxcr4b*, *irf8*). Genes involved in macrophages recruitment and commitment to osteoclastogenesis (*cxcr 3.2*, *cxcr 3.1*, *cxcl11.1*, *cxcl11.5*) were also downregulated. Finally, important markers of osteoclast differentiation and activity (*csf2rb*, *ctsk*, *rank*, *acp5b*, *mmp9, mmp13b*) were consistently downregulated across all replicates in fish exposed to SKLT (Figure 6.5B). These results suggest a mechanistic link between the anti-inflammatory and the anti-osteoclastogenic properties of SKLT.

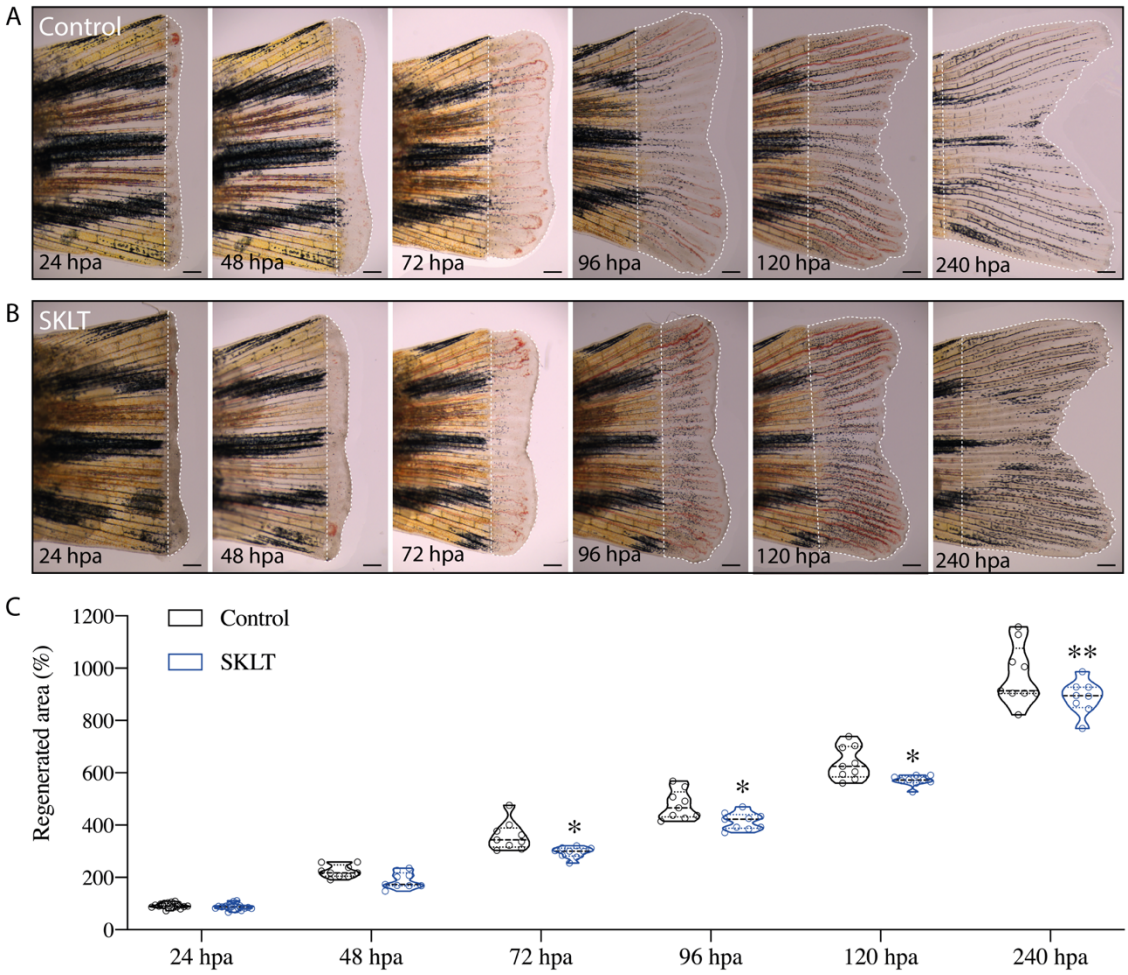


Figure 6.4. Effect of *Skeletonema costatum* ethanolic extract (SKLT) on the regenerative performances of amputated caudal fins in zebrafish. Representative images depicting the time course of the regenerated area in fish treated with negative control (**A**) and SKLT (**B**). (**C**) Quantification of regenerated area. At each timepoint, statistical differences were tested through unpaired *t* test ($p < 0.05$), or by a non-parametric Mann-Whitney test ($p < 0.05$) whenever the data distribution resulted non-normal. Asterisks indicate values statistically different. $p < 0.05$ (*), $p < 0.01$ (**). Scale bars = 500 µm.

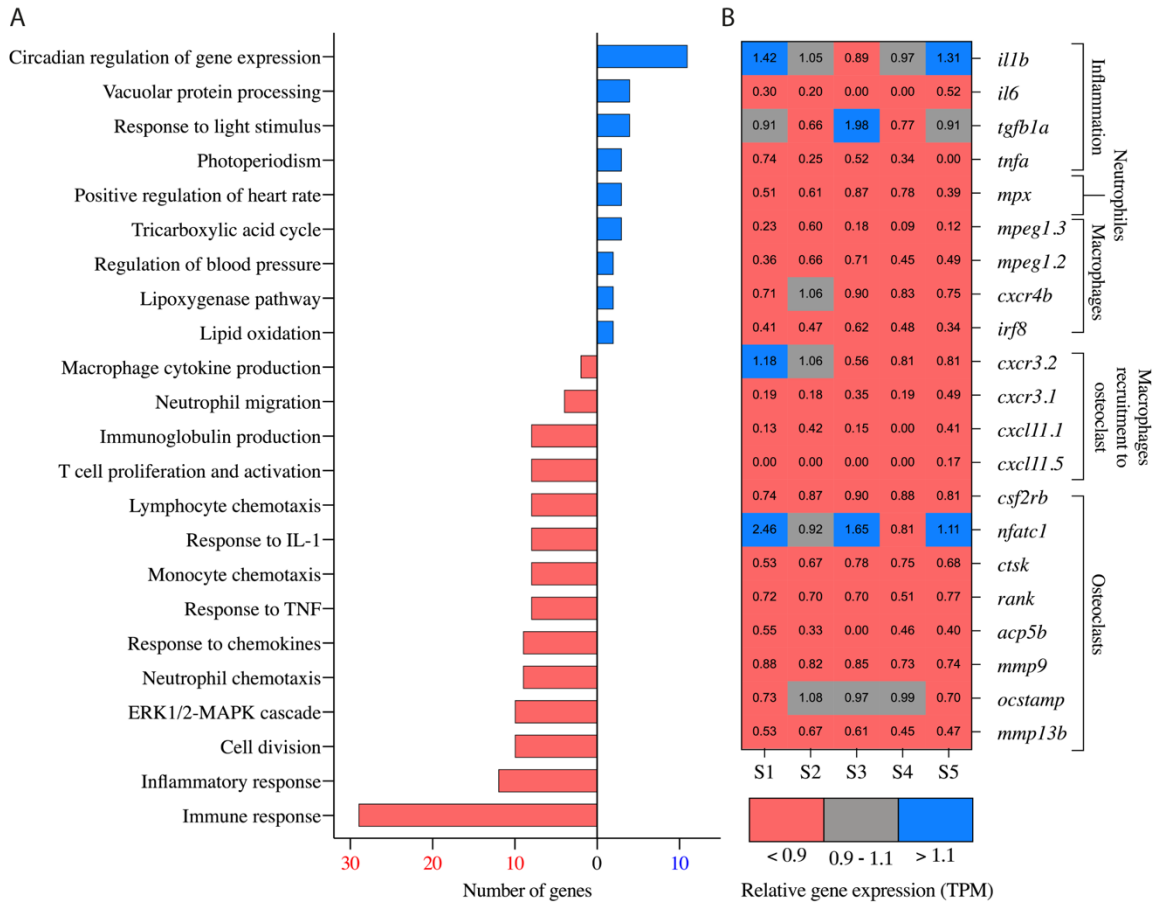


Figure 6.5. (**A**) Visualization of the enriched gene ontology terms falling under the classification of Biological Processes (GO:BP, FDR < 0.05) in regenerates of fish treated with *Skeletonema costatum* ethanolic extract (SKLT) at 24 hpa. (**B**) Selection of genes markers of osteoclast, macrophages and neutrophils. Data are represented in terms of TPM (transcripts per million) relative to the control group (ethanol). FDR = Corrected *p* value.

6.3.4. Skeletonema extract reduces bone loss in a medaka model of osteoporosis

Fish size (total body area) and intensity of CFP expression (mean pixel intensity) were assessed in heat shocked medaka *Tg(rankl:HSE:CFP)* exposed for 6 days to SKLT extract or ethanol (Control) (Figure 6.6A, B). Frequency distribution of fish size and CFP intensity was tested through an Anderson-Darling test ($p < 0.05$) and normality was not met in the Control group (Total body area, $p < 0.0001$; CFP intensity, $p = 0.0035$). Similarly, normality was not met for total body area in the SKLT group ($p = 0.0008$), but distribution of CFP intensity was normal ($p = 0.220$). Data distribution for total body area was bimodal for both groups (Figure 6.6C), while data distribution for CFP intensity was right skewed (median < mean) for the control

group and bimodal for SKLT group (Figure 6.6D). These finding demonstrated that a clustering was necessary to proceed with the statistical analysis.

Fish were therefore grouped according to their size and CFP intensity into "Big fish" (total body area > 200000 px^2), "Small fish" (total body area < 200000 px^2), "High CFP" (mean pixel intensity > 50) and "Low CFP" (mean pixel intensity < 50) (Figure 6.6C, D). Joint distribution of the two variables was studied by assigning the X axis to the body area, and the Y axis to CFP intensity (Figure 6.6E). As a result, individual fish were clustered in 4 groups, based on their combined distribution, into "Big fish-High CFP", "Big fish-Low CFP", "Small fish-High CFP", and "Small fish-Low CFP".

Quantification of the mineralization of the vertebral column within each group is reported in Figure 6.6 F-I. No differences were observed between Control and SKLT for "Small fish" groups (Figure 6.6F, G) and for "Big fish-Low CFP" (Figure 6.6H). Mineralization of the vertebral column of fish of the group "Big fish-High CFP" was significantly increased upon exposure to SKLT extract (Figure 6.6I). A higher number of mineralized neural arches was also observed (Figure 6.6L). These data indicate that bone loss was reduced upon exposure to SKLT extract in fish with the strongest osteoporotic phenotype.

6.3.5. Skeletonema extract inhibits proliferation and differentiation of RAW 264.7 macrophages

While no cytotoxicity was observed in RAW 264.7 cells exposed for 24 and 48 h to SKLT (Figure 6.7A), cell proliferation was significantly reduced at 1, 3, 5 and 7 days of exposure in a dose-dependent manner (Figure 6.7B). Osteoclast differentiation was assessed in cells exposed to 50 µg/mL of SKLT and stained with DAPI and TRAP. While no multinucleated (> 2 nuclei) $TRAP^+$ cells, i.e. osteoclasts, were observed in the control group (Figure 7C, D), 10-20 osteoclasts/field were counted in RANKL-treated group. Co-treatment with SKLT (50 µg/mL) significantly reduced the number of differentiated osteoclasts to 0-2 osteoclast/field (Figure 7C, E). These data highlight the anti-proliferative and anti-osteoclastogenic effect of SKLT in mouse macrophages.

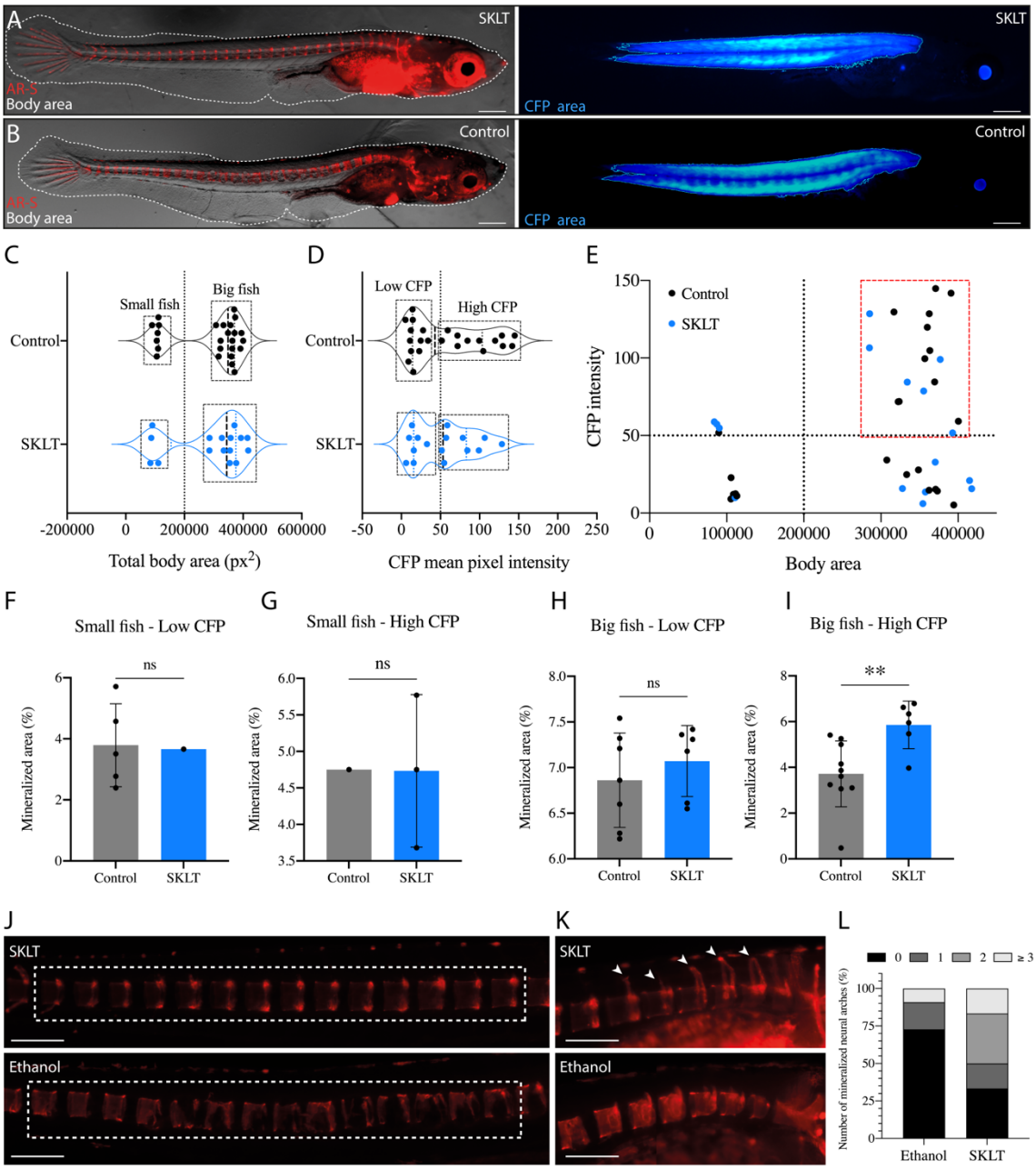


Figure 6.6. Effect of *Skeletonema costatum* ethanolic extract (SKLT) in a RANKL-induced osteoporosis medaka model. (**A**, **B**) Representative images illustrating how the total body area (dotted line), and CFP^{+} area, were measured in SKLT (**A**) and control fish (**B**). (**C, D**) Violin plot illustrating the frequency distribution for the total body area (**C**), CFP mean intensity (**D**). (**E**) Joint distribution (total body area vs CFP mean intensity) and clustering of individual fish (red dotted line, "Big fish - High CFP intensity" group). (**F-I**) Quantifications of the mineralized area of the vertebral column in the different fish clusters. (**J**) Representative images depicting AR-stained vertebral column from SKLT (upper image) and Ethanol (lower image) fish. (**K**) Representative images depicting the count of mineralized neural arches in SKLT (upper image) and Ethanol (lower image) fish and its quantification in the group "Big

fish-High CFP" (**L**). Difference between Ethanol and SKLT fish were tested through unpaired *t* test ($p < 0.05$). Asterisks indicate values statistically different. $p < 0.01$ (**). Scale bars = µm in (**A**, **B**), and 730 µm (**J**, **K**).

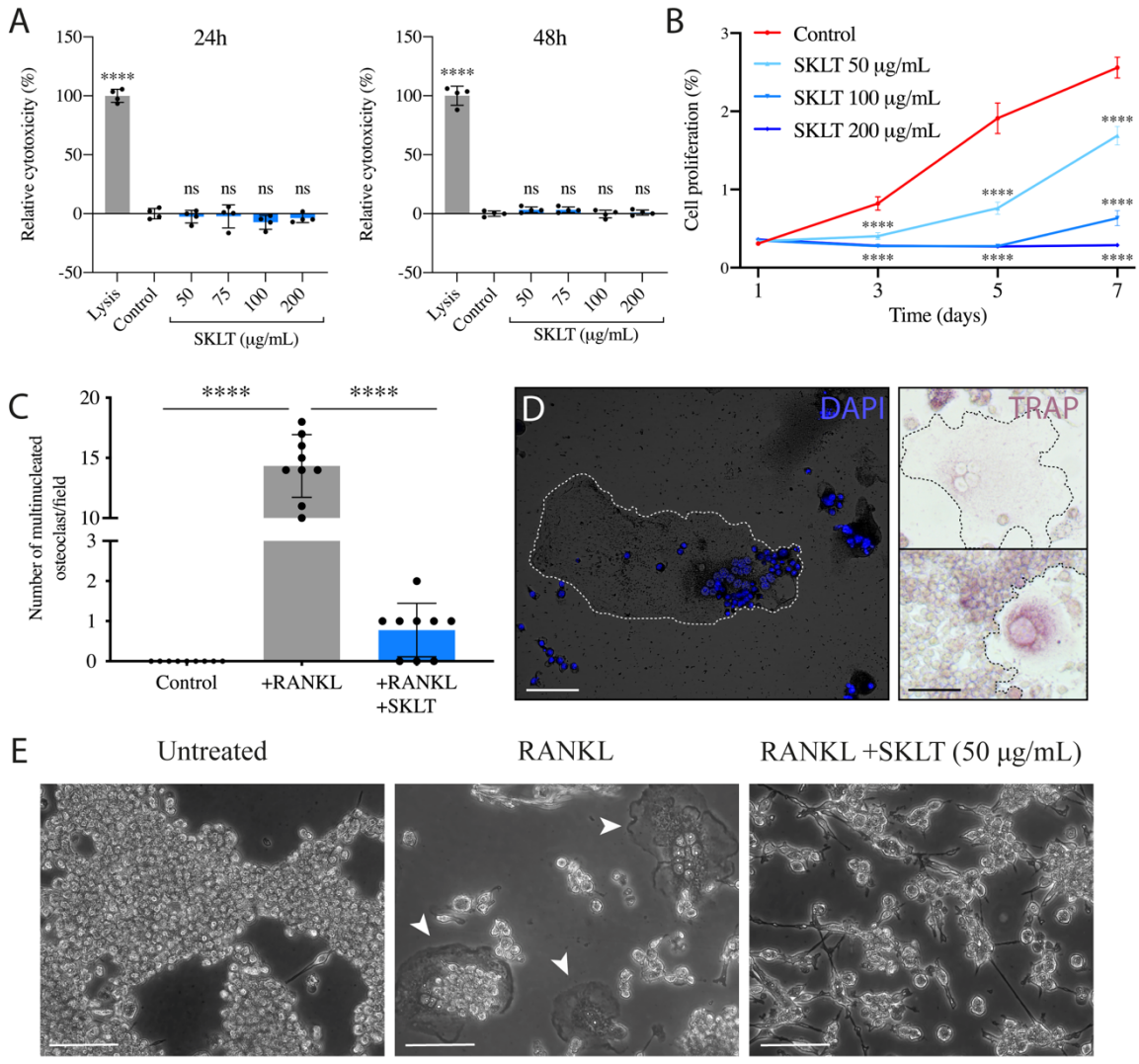


Figure 6.7. Effect of *Skeletonema costatum* ethanolic extract (SKLT) on cytotoxicity, proliferation and osteoclastic differentiation of RAW 264.7 cells. (**A**) Results of LDH assay of cells treated for 24h (left) and 48h (right) with different concentration of SKLT. (**B**) Effect of SKLT extracts on cell proliferation assessed through XTT assay. (**C**) Count of the number of multinucleated osteoclasts in 5.0x fields. (**D**) Representative fluorescence image of DAPI stained multinucleated osteoclasts (left) and brightfield images of $TRAP^{+}$ osteoclasts (right). (**E**) Representative phase-contrast images of the 3 experimental groups at 5.0x magnification. Differences between each treatment and the control were tested though one-way ANOVA followed by Dunnett's multiple comparison test ($p < 0.05$). Asterisks indicate values statistically different. $p < 0.0001$ (****). Scale bars = 150 µm.

6.4. Discussion

This work provided evidences that an ethanolic extract prepared from *Skeletonema costatum* – a microalgae commonly cultivated in Europe and approved for human consumption[629] – can positively impact on bone formation in zebrafish and medaka models of bone regeneration[630,631] and osteoporosis[617], respectively.

In zebrafish, this extract promoted the mineralization of regenerating fin rays, and also affected the patterning of the newly formed rays through the distalization of the bifurcation point.

Previous works have shown that during fin regeneration, ray branching is controlled by morphogenetic programs inducing the de-differentiation and subsequent re-differentiation of resident osteoblasts[632–634]. A recent study revealed the role of osteoclast-like cells – in the form of osteolytic tubules – in this process, through a timely anti-stitching action that precisely determine the position of the bifurcation point along the proximal-distal axis[635]. The same study showed that fish exposure to pro-osteogenic or anti-resorptive pharmaceuticals can modulate the proximo-distal positioning of the bifurcation point. As such, the distalization of ray bifurcation in regenerating fins treated with SKLT is likely to result from compounds with bone anabolic or anti-resorptive activity.

In the light of recent studies reporting anti-inflammatory activity in *S. costatum* extracts[566,570], we hypothesized a possible action of compounds present in the ethanolic extract on osteoclast function. This hypothesis was validated by a weaker expression of cathepsin k – a marker of osteoclast differentiation – and a weaker activity of the tartrate-resistant acid phosphatase – a marker of osteoclast function – in fish exposed to SKLT up to 48 h post amputation, consistent with data previously reported[635]. Because Ctsk expression was back to levels comparable to control fish at 72 hpa, we propose that SKLT contains compounds that have the capacity to delay the recruitment and/or differentiation of osteoclast precursors at early stages of caudal fin regeneration.

Fin regeneration was reduced in SKLT-treated fish and we propose that it may be the result of an anti-inflammatory action of SKLT. Indeed, early stages of fin regeneration are characterized by a quick immune response in which immune cells invade the wound and massively release pro-inflammatory cytokines[636,637]. This results in an early inflammatory flare, which is not only a consistent event in all regenerative processes across different organs including fin[637], heart[638,639], and spinal cord[640], but also a necessary step enabling and somehow triggering the activation of pro-regenerative molecular programs[641]. In this regard, it has been shown that zebrafish larvae with impaired inflammatory signaling exhibit a reduced regenerative capacity, i.e. the regenerated area of the larval fin fold is reduced[642,643]. Thus, the reduced regenerative

capacity of fish exposed to SKLT may be related to the presence of anti-inflammatory compounds in the extract that would delay the activation of regenerative programs. Similar results were observed in adult zebrafish, where genetic ablation of macrophages not only reduced regenerative performances but also inhibited the bifurcation of bony rays[644].

We also propose that both effects – i.e. impaired fin regeneration due to SKLT anti-inflammatory effect and altered fin ray mineralization and patterning due to SKLT anti-resorptive effect – may be corelated. Indeed, inflammation has been shown to have a role in enabling macrophages, as osteoclast precursors, to become responsive to pro-osteoclastogenic signaling in a medaka model of osteoporosis. In this study, impaired *tnfa* expression inhibited macrophage recruitment in the bone tissue and their differentiation into *ctsk*$^{+}$ osteoclasts by RANKL, and prevented bone loss[645]. Our hypothesis is that compounds in SKLT delay osteoclast recruitment to the caudal fin blastema by blocking inflammation during the early step of fin regeneration. A recent work demonstrated the anti-inflammatory action of ethanolic extracts prepared from *Skeletonema costatum*[565], and our transcriptomic analysis further supported this effect with several genes involved in inflammation (*il6* and *tnfa*) and macrophages markers (*mpeg1.2*, *cxcr4b* and *irf8*) significantly downregulated in fish exposed to SKLT, and biological processes associated with both adaptive and innate immune responses negatively regulated.

Recent studies conducted in the medaka RANKL-induced osteoporosis model unveiled the important role of chemokines produced by osteoblast progenitors in the recruitment and commitment of macrophages towards osteoclastogenesis. In details, the chemokine Cxcl9l (orthologous to human CXCL9) is produced by pre-osteoblasts and guides, in response to RANKL signaling, the recruitment of a subset of macrophages expressing the chemokine receptor Cxcr3.2. (orthologous to human receptor CXCR3[646]) toward osteoclastogenesis. A following case-control study in humans patient revealed that serum CXCL9 level efficiently predict for the risk of osteoporosis-related hip fracture, highlighting the conservation of these molecular controls between medaka and humans[647]. In zebrafish, the chemokine receptor Cxcr3 is coded by 3 genes – *cxcr3.1*, *cxcr3.2* and *cxcr3.3* – and the cytokine Cxcl9 appears to be coded by seven *cxcl11*-like genes[648,649]. In the present scenario, *cxcr3.1*, *cxcr3.2*, *cxcl11.1* and *cxc.11.5* were downregulated in fish exposed to SKLT, which could indicate a reduction of a specific population of macrophages responsive to pro-osteoclastogenic signaling. Whether the CXCR3-CXCL9 role in the commitment of macrophages to osteoclastogenesis is conserved between zebrafish and medaka has yet to be experimentally demonstrated, although the whole chemokine system was described to be fairly conserved across all vertebrates[650].

Another osteoblast-secreted paracrine factor that recently emerged as important for osteoclastic function is the matrix-metalloproteinase 13[651]. The analysis of medaka transcriptome in osteoporotic conditions revealed that *mmp13b* was strongly expressed by osteoblast progenitors, and that *mmp13b* loss of function resulted in osteoclasts being immature, inactivated, and unable to resorb the extracellular matrix[651]. In the present work, fish exposed to SKLT had a reduced expression of *mmp13b*, as well as to other genes important for osteoclastic maturation and activity (*acp5b*, *mmp9* and *ctsk*), and transcriptomic analysis revealed that ERK1/2-MAPK axis was significantly inhibited. Previous studies highlighted the role of the ERK pathway in the positive regulation of osteoclast survival, activation, and chemotaxis in response to different endogenous stimuli including inflammatory clues[652–655]. This further support a possible effect of *S. costatum* ethanolic extract on osteoclast differentiation and/or function.

In medaka, the extract could limit the extent of bone loss in the vertebral column, i.e. bone mineralization was higher in SKLT-treated fish, although only in a subgroup of fish, those with high CFP expression thus with the most severe osteoporotic phenotype. This suggest that compounds in SKLT have the capacity to limit bone loss that would result from a genetically induced osteoclastic differentiation and, once purified and identified, could be used to develop drugs for osteoporosis.

Finally, we could evidence a dose-dependent anti-proliferative effect and an anti-osteoclastogenic effect of SKLT in murine macrophages cultured *in vitro*. Combined effects are in agreement with the fact that cell division must be inhibited for macrophages to fuse and give rise to osteoclasts, and RANKL itself, here used to stimulate osteoclastic differentiation, possess anti-proliferative properties[656]. It is worth to highlight the fact that SKLT almost completely suppressed the formation of multinucleated osteoclast when co-treated with RANKL at the lowest concentration tested and that no cytotoxicity was observed, important criteria for a possible application on drug development of the bioactive(s) once identified.

Altogether, data presented here provide strong evidence for the presence of compounds with anti-inflammatory, anti-osteoclastogenic and immuno-suppressive activities in the ethanolic extract of *Skeletonema costatum*, which open up to possible future implementation of compounds contained in this extract for the treatment of bone erosive disorders.

Macrophages are direct precursors of osteoclasts and their role in the development of various bone disorders such as osteoporosis is widely recognized, thus any compounds targeting macrophages represent promising options for novel therapeutic approaches[657]. Whether the anti-inflammatory activity and the anti-osteoclastogenic activity of SKLT extracts are a result

of the action of a single compound or the synergistic effect of different molecules was not addressed here; the identification of this/these compound(s) must be a priority to further the exploitation of this microalgae for pharmacological applications. This study also provided evidence that an anti-inflammatory approach may be suitable to efficiently protect patients from bone loss in a disease setting. Further pharmaceutical development for this extract should be evaluated by testing it with mammalian systems modeling human bone erosive disorders. In this regards, beside the well know ovariectomized mouse or rat[375] mimicking post-menopausal osteoporosis, other models for age-related osteoporosis[376,377], renal osteodystrophy[390–392], inflammatory induced osteolysis[297], and various forms of arthritis[261,263] are available.

6.5. Conclusions

The present work explored the anti-osteoclastogenic and anti-osteoporotic potential of compounds present in the ethanolic extract of the marine microalgal species *Skeletonema costatum*, which was previously reported for its anti-inflammatory activities. We showed that the extract promotes *de novo* bone formation and inhibits ray bifurcation during the regeneration of the zebrafish caudal fin by inhibiting the recruitment and differentiation of osteoclast precursor cells. We also provided evidence for a causal relation between the anti-inflammatory property of the extract and its anti-osteoclastogenic activity, showing how fish exposed to the extract had biological processes associated with inflammation, immune response, macrophages recruitment and osteoclastic differentiation downregulated at the gene level. We validated the capacity of the extract to limit bone loss in an *in vivo* medaka model of osteoporosis and confirmed that our data collected using fish *in vivo* models can be translated to a mammalian *in vitro* model of osteoclast differentiation. Altogether, our data provide strong evidence for the presence of druggable compounds in the ethanolic extract of *Skeletonema costatum* and highlight the potential of microalgae as a source of osteoactive compounds.

CHAPTER 7.

CONCLUSIONS AND FINAL REMARKS

7.1. Skeletal disorders: A point of contact between biomedicine and aquaculture

Despite outstanding advancements in biomedical and pharmaceutical research happened over the latest decades, bone erosive disorders are, more than ever, a pressing medical challenge[2,3]. This diverse set of conditions share a common pathophysiological feature: they alter the equilibrium of bone remodeling towards resorptive processes and lead to mineral loss from the bone tissue, increasing its susceptibility to fractures[4,5]. To exacerbate the problem is the poor choice of therapeutic options to treat these diseases, often characterized by limited efficacy and associated side effects[163,546].

On the point of view of animal production, skeletal anomalies in cultured fish are a burden to the aquaculture industry, a primary source of seafood for human consumption[410]. These skeletal defects are present in all species of reared fish, and appear to be largely caused by factors intrinsically related to the condition of captivity, including the lack of an adequate and species-specific nutrition[409].

In this context, marine-based pharmacology, the branch of pharmaceutical research focalized on the screening and characterization of marine natural compounds, can contribute to find solutions for both these otherwise very different research fields.

Many groups of marine organisms have been studied as sources of "osteoactive" compounds, some of which were described for their highly promising pharmacological potential[215]. In **Chapter 1**, we have reviewed previous studies that focused on the description of marine-derived molecules with osteoactive properties, with the aim of providing an updated state of the art on the advancement of the implementation of such compounds for drug development, as well as identifying promising and underexploited taxonomic groups of marine organisms suitable for the tasks developed in the frame of this PhD project. In addition, we have discussed the role of some of these compounds with osteoactive properties to be applied as dietary supplements, in the form of raw extracts or of purified compounds for incorporation in aquaculture nutrition, to promote a better skeletal health in cultured fish.

7.2. A medium-scale screening project identified promising extracts from microalgae and invertebrates with pro-osteogenic activity

In **Chapter 2**, we have conducted a screening for bioactivity in a total of 148 extracts and fractions obtained from different groups of marine organisms, by applying previously validated screening system to evaluate the pro-osteogenic activity of natural compounds and drugs, which is based on the morphometric analysis of the developing opercular bone in the zebrafish larva[478]. The analysis of extracts from seven species of marine invertebrates led us to identify

3 hydroethanolic extracts from tunicates belonging to the family Polyclinidae characterized by a strong pro-osteogenic activity. A further characterization of the bioactive potential of these extracts allowed us to correlate their pro-osteogenic activity with their *in vitro* anti-oxidant and anti-inflammatory activities as well as the high content of polyphenols (**Chapter 2.1**). These findings add up to a growing body of literature highlighting the role of antioxidants and anti-inflammatory compounds in bone metabolism, and how these can be used with nutraceutical and pharmaceutical applications[36,575–577]. In a second, more extensive work (**Chapter 2.2**), we analyzed 133 extracts and fractions from marine cyanobacteria, actinobacteria, planctomycetes, and microalgae by using the same methodologies. All these groups of marine microorganisms have high potential for the isolation of osteoactive compounds due to the large number of bioactives already isolated. Nevertheless, these taxa are yet relatively unexplored for their bioactive potential[214–219].

As a result of this screening, ethanolic extracts from four marine microalgae, one cyanobacterium, and one tunicate increased the size of the operculum in more than 40% compared with their control. This strong pro-osteogenic activity highlighted the potential of these species for the future isolation of natural compounds with possible pharmacological and nutraceutical applications. Two of these extracts, from the macroalgae *Skeletonema costatum* and *Tetraselmis striata* CTP4, were chosen as promising candidates to be characterized, in **Chapters 4**, **5**, and **6** for better understanding their molecular mechanisms and modes of action, and for validation toward applications in aquaculture as dietary supplements, and for biomedicine, as potential source of molecules to treat human bone erosive pathologies.

7.3. A bioassay-guided identification pipeline for identifying marine osteoactive compounds

Following the screening of microalgae extracts performed in **Chapter 2**, in the **Chapter 3** the ethanolic extract from *Tetraselmis striata* CTP4 was selected as best the candidate to perform its chemical characterization, aiming at identifying putative compounds candidate to be responsible for the pro-osteogenic effects induced by the raw extract. A bioassay-guided identification pipeline was developed for this task, consisting in the fractionation of the extracts by high performance liquid chromatography (HPLC) followed by the re-screening of purified fractions.

Two fractions retaining the pro-osteogenic effects at lower concentration compared to the raw extract and were selected and further analyzed by tandem mass spectrometry (MS^n). This analysis allowed for the identification of 15 potential candidates compounds, 7 of which were

commercially available, and were purchased and tested separately in zebrafish larvae. As none of the tested molecules resulted positive for bioactivity, at the end of **Chapter 3**, we discussed the possible sources of hits rejection and compound misidentification, as well as the potential future strategies to adopt for correcting the protocol developed. As such, we proposed an optimized strategy to be adopted in future research, aiming at identifying the osteogenic compounds in the *Tetraselmis striata* CTP4 extract.

7.4. Ethanolic extracts from *Skeletonema costatum* and *Tetraselmis striata* CTP4 promoted osteoblastogenesis and bone mineralization in *in vitro* and *in vivo* fish models

In **Chapter 4**, we have characterized more in-depth the biological activity of the two selected ethanolic extracts from the microalgae *Skeletonema costatum* (*SKLT*) and *Tetraselmis striata* (CTP4) by taking advantage of the availability of *in vitro* fish-derived cell lines and zebrafish transgenic lines labelling specific populations of bone cells. Both extracts stimulated ECM mineralization in a mineralogenic cell line derived from the gilthead seabream (*Sparus aurata*). The characterization of the different populations of osteoblastic cells using zebrafish reporter lines provided evidence supporting the hypothesis that the mineralogenic effect of these extracts is due to the stimulation of osteoblastic maturation, evidenced by the increased population of immature and mature osteoblastic cells but not osteoprogenitor cells, and the upregulation of gene markers of osteoblastic differentiation and ECM production.

In addition, to evaluate the potential of these extracts to be used as dietary supplements towards ameliorating skeletal health, we prepared experimental diets for zebrafish on which extracts were spray-coated, and evaluated the effect of these supplemented diets on fish skeletal development and on the incidence and severity of skeletal deformities. Following the dietary exposure of zebrafish, we showed a consistency in the pro-mineralogenic effect to all the zebrafish skeleton as well as a reduction of the incidence of skeletal anomalies. Gene expression analysis revealed that fish fed diets supplemented with both extracts presented an overall increased expression of genes involved in bone anabolic and remodeling processes. Furthermore, as the two microalgal species evaluated in this study previously sparked the attention of researchers in the field of pharmaceutical sciences due to their *in vitro* anti-oxidant and anti-inflammatory activities[559,565–570], we decided to evaluate in parallel the effect of dietary exposure on the expression of gene markers for antioxidant defense activation in the bone tissues of treated fish, and we found these to be upregulated.

These data highlighted the potential of these extracts to ameliorate skeletal health in fish models, and provided a first insight on the molecular pathways that are being modulated,

pointing to osteoanabolic mechanisms, especially for the ethanolic extracts from *Tetraselmis striata* CTP4. Future studies should consider testing these extracts in animal models of bone disorders, to validate these data in an environment better resembling human pathologies. At the same time, to validate if these extracts might have applications in the aquaculture field, they should be tested in species relevant to the aquaculture industry. In the following chapter, we provided a clue in this direction, by testing the same two extracts as dietary supplements for the gilthead seabream (*Sparus aurata*), one of the most important marine species for the European aquaculture industry.

7.5. Ethanolic extracts from *Skeletonema costatum* and *Tetraselmis striata* CTP4 promoted bone formation and reduced skeletal anomalies in gilthead seabream juveniles

In **Chapter 5**, we have tested diets for the gilthead seabream (*Sparus aurata*) that were supplemented with the ethanolic extracts from either *Skeletonema costatum* or *Tetraselmis striata* CTP4 by vacuum coating. To explore at which stage of production these extracts could have the most useful application and optimize the cost-benefit ratio, diets were tested in different life stages of gilthead seabream: larvae from 30-60 days after hatching (DAH), and juveniles during the pre-ongrowing phase (5-18g). In addition, to evaluate whether a short treatment with diets supplemented with microalgae extracts could ameliorate fish skeletal health until later stages of the ongrowing phase, some juvenile fish were maintained for 6 additional months while fed with commercial diets. The supplementation with the two microalgal extracts did not increased bone mineralization nor reduced the incidence of skeletal anomalies in gilthead seabream larvae. However, the observation that marker genes of bone formation and remodeling were upregulated, providing a clue that bone anabolic mechanisms were stimulated in a similar manner to what was previously observed in both zebrafish and gilthead seabream bone derived cell lines in **Chapter 4**.

We argued over the possibility that interspecific variation might account for the differences observed here and raised the hypothesis that testing these extracts in higher supplementation levels might lead to higher effects on the skeletal phenotypes. Surprisingly, in juvenile gilthead seabream the supplementation with both microalgal extracts increased the phosphorous content of fish vertebrae, significantly reduced the incidence of skeletal anomalies and, in the case of CTP4 extracts, also significantly increased fish growth. After 6 months, during which fish were fed commercial diets, fish originally fed CTP4-supplemnetd diets for only 33 days during the pre-ongrowing phase resulted larger, less deformed, and with a more elongated and slender shape compared to the control fish. These results highlight that, if optimized in terms of

concentration, life stage, and duration of exposure, diets supplemented with CTP4 ethanolic extracts could represent a useful asset to improve growth, skeletal health, and promote a wild-type like shape in the culture of gilthead seabream.

7.6. *Skeletonema costatum* extract has anti-osteoclastogenic and anti-resorptive properties

In **Chapter 6** we provided evidence for an anti-resorptive and anti-inflammatory mechanism of the ethanolic extracts from *Skeletonema costatum* (SKLT), confirming its potential for drug development with future applications in the treatment of human bone erosive pathologies. In a first approach, we performed treatment with the extracts in a zebrafish model of bone regeneration using transgenic lines labelling osteoclastic cells and transcriptomics, and showed that the anti-resorptive effect observed in the caudal fin is probably related to its anti-inflammatory activity, being it at the basis of the capacity of this extract to inhibit macrophage recruitment and differentiation into osteoclastic cells.

By testing the extracts in a medaka model of induced osteoporosis, we showed how the treatment with SKEL can reduce bone loss and ameliorate osteoporotic phenotype in this model. This provides insights on the possible applicability of this extract in a disease context.

Finally, in order to validate the osteoactivity of this extract in a mammalian model, we applied the extracts to rodent-derived cell line RAW 264.7 of macrophages and showed how the extract possesses anti-proliferative properties and is able to strongly suppress RANKL-induced osteoclastic differentiation. These results provided strong evidence for: (1) The presence of anti-inflammatory compounds in *S. costatum*, as also supported by previous *in vitro* studies, (2) The anti-resorptive potential of these compounds. These evidences make the microalga *Skeletonema costatum* a very interesting candidate for the isolation of compounds with potential applications for the development of drugs to treat human bone erosive pathologies, and future research must address this by chemically characterization of the extracts and isolation of the compounds responsible for the observed effects.

7.7. Final remarks

Our aim in the scope of this research project was to screen extracts obtained from selected marine groups as sources of osteoactive compounds and explore their potential applications for both the biomedical sector, as sources of future drugs to treat human bone erosive pathologies, and for the aquaculture industry, as nutraceuticals to be incorporated into fish feeds with the aim of improving the skeletal health in produced fishes. With this aim, we have put in place a

medium-scale screening project which evaluated about 150 extracts and fractions from various marine groups, that recent pharmacological research has identified as promising sources of bioactive compounds, including holothurians, tunicates, cyanobacteria, marine fungi and microalgae. Several of these marine extracts were characterized by having a pro-osteogenic activity, including three ethanolic extracts from tunicates that were especially interesting due to the correlation between their osteogenic activity and their anti-inflammatory properties. Few extracts from bacterial and cyanobacterial species were also characterized by high osteogenic activities. Overall, the results of this collaborative screening project provided a starting point for future isolation of compounds with potential biotechnological applications.

We then decided to focus on ethanolic extracts from two marine microalgae, *Tetraselmis striata* CTP4 and *Skeletonema costatum*, that we considered the most promising for their pro-osteogenic and mineralogenic activities. We have further characterized them by testing in *in vitro* cellular models and *in vivo*, in the model organism zebrafish, used as platform to perform a deeper characterization of the molecular mechanism and modes of action of the two extracts. By doing this, we revealed that the ethanolic extracts from *Tetraselmis striata* CTP4 acts through an osteoanabolic mechanism, while *Skeletonema costatum* extract possess osteoanabolic, anti-resorptive, and anti-inflammatory properties.

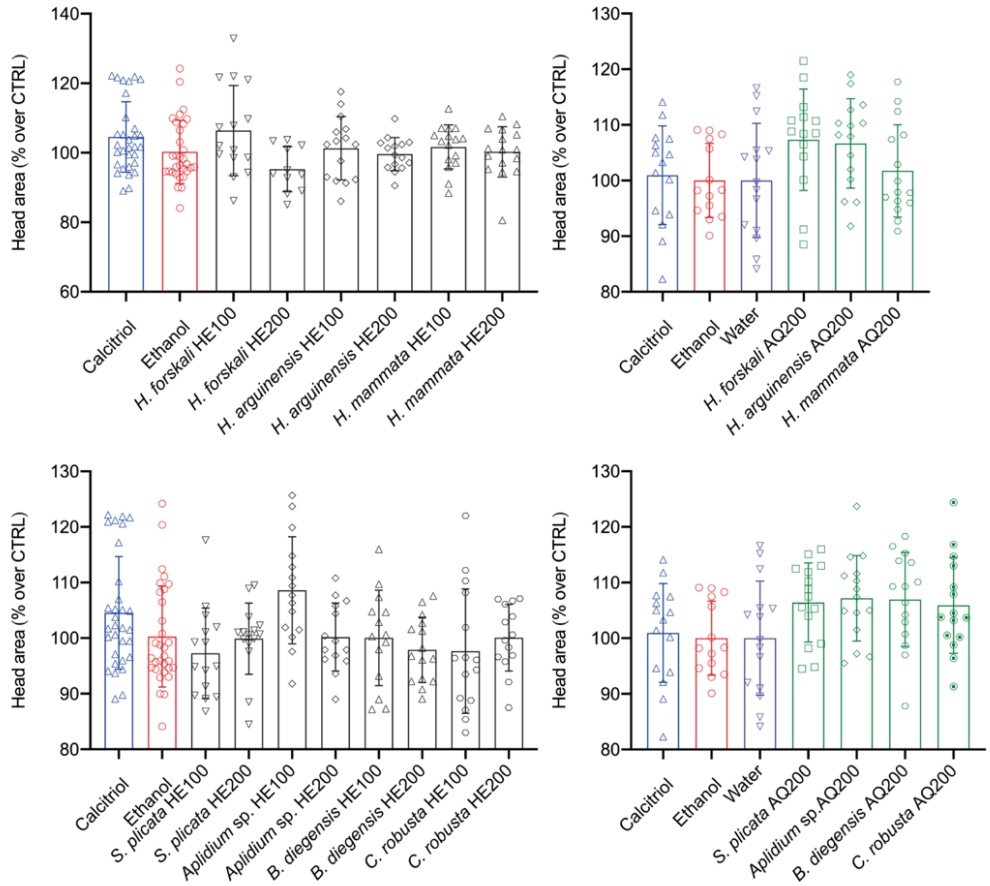
We then validated the applications of these two extracts in the aquaculture industry, by exploring their potential to be used as dietary supplements for the commercially reared species *Sparus aurata*, and found that *Tetraselmis striata* CTP4 is the most promising extract in this sense, in light of its capacity to promote fish growth, bone mineralization, and reduce the incidence of skeletal anomalies. Finally, we validated the potential application of the ethanolic extracts from *Skeletonema costatum* for the treatment of human bone erosive disorders, by dissecting the molecular mechanism involved in its anti-resorptive activity, demonstrating how its application can limit bone loss in a medaka model of osteoporosis, and by showing how its anti-osteoclastogenic properties are conserved in a mammalian *in vitro* model.

Overall, with the experimental work developed in the frame of this PhD project, we have provided a contribute to the identification of marine extracts with the potential for isolation of compounds with applications in human medicine as well as dietary supplements for aquaculture nutrition.

SUPPLEMENTARY MATERIAL

Supplementary Table 1. GenBank accession numbers for the sequence data obtained for the identification of four species of Tunicates.

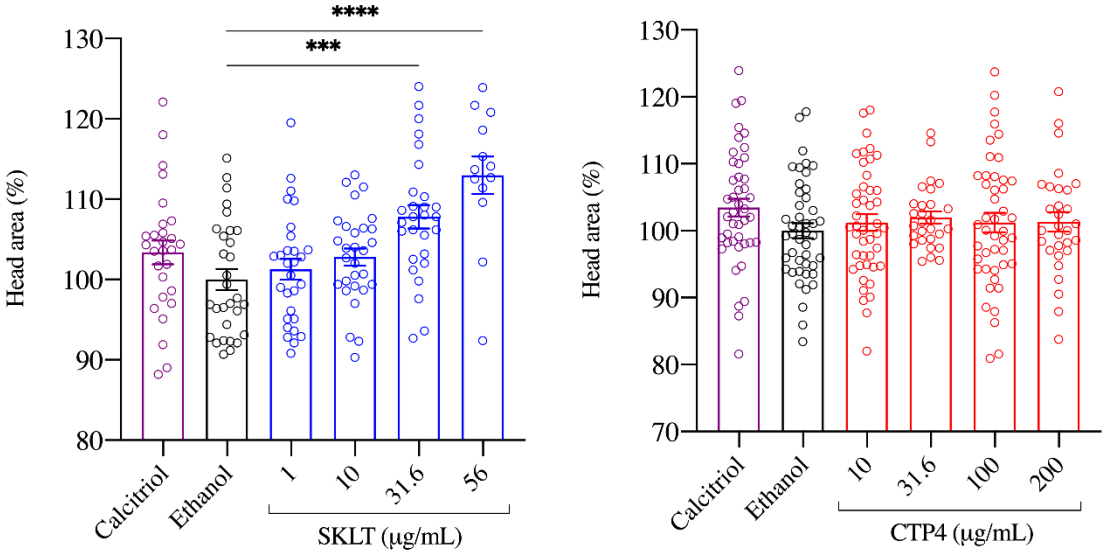
Species	BOLDSystems Process ID	GenBank accession number
Aplidium sp.	SCUTU005-22	ON059139
Aplidium sp.	SCUTU006-22	ON059140
Botrylloides diegensis	SCUTU008-22	ON059141
Ciona robusta	SCUTU010-22	ON059142
Ciona robusta	SCUTU009-22	ON059143
Holothuria (Roweothuria) arguinensis	SCUTU001-22	ON059144
Holothuria (Panningothuria) forskali	SCUTU002-22	ON059145
Holothuria (Holothuria) mammata	SCUTU004-22	ON059146
Holothuria (Holothuria) mammata	SCUTU003-22	ON059147
Styela plicata	SCUTU007-22	ON059148



Supplementary Figure 1. Effect of the treatment with different extracts on the area of the head of zebrafish larvae for holothurians hydroethanolic (**A**) and aqueous extracts (**B**) and for tunicates hydroethanolic (**C**) and aqueous extracts (**D**) respectively. Statistical differences among the means were tested through One-way ANOVA followed by Dunnett's multiple comparison test ($p < 0.05$) or, whenever normality and homoscedasticity weren't met, through a non-parametric test followed by Dunn's multiple comparison test ($p < 0.05$). HE - hydroethanolic extracts, AQ – aqueous extracts, 100 – 100 µg/mL, 200 – 200 µg/mL.

Supplementary Table 2. qPCR primers used for gene expression in zebrafish larvae and juveniles.

Gene acronym		**Primer sequence (5′ →3′)**
ef1a1l1	Fw	TTGAGAAGAAAATCGGTGGTGCTG
	Rev	GGAACGGTGTGATTGAGGGAAATTC
actb1	Fw	GATGCGGAAACTGGCAAAGG
	Rev	GAGGAGGGCAAAGTGGTAAACG
rps18	Fw	AACACGAACATTGATGGAAGACG
	Rev	ATTAGCAAGGACCTGGCTGTATTT
runx2a	Fw	AGCCGACCCAGCCCAGTTTGAG
	Rev	TGGGGTGTAGGTGAATGTTGCTGGATA
runx2b	Fw	TCAGGAATGCCTCAGGGGTTATG
	Rev	CTTGCGGTGGGTTTGTGAATACT
oc1	Fw	CACTCCTGCTCCTCATGTGC
	Rev	GTGTAAGCCGCTACGATCCC
oc2	Fw	TGCTGCCTGATGACTGTGTG
	Rev	GTGCAGTTCCAGCCCTCTTC
sp7	Fw	AGCCTCGCATCTGAAAGCCCAC
	Rev	GTTTTCTGGTGTTTGCTGAGGTGGTC
alpl	Fw	TTCCTCTGCGGTGTCAAAGCCA
	Rev	AAGCAGCACTCGGGGTGGCAT
col10a1	Fw	TGGAGCGCCTGGAGTTGGTT
	Rev	GGCCCAGATTCCCCATCACGG
col1a1a	Fw	GCTTCATTGCCCAGCCACAGGA
	Rev	GCAGGGTTCTTCTTGGTGCCGTCT
ctsk	Fw	GGATTCCCTCTGCTGGTGCTG
	Rev	GCATGTTCTTCTCCCAAATCGTCC
acp5a	Fw	ATAGAGACCGCTACAGCCCGCA
	Rev	TGCCAGCAATGACGTACCAAGG

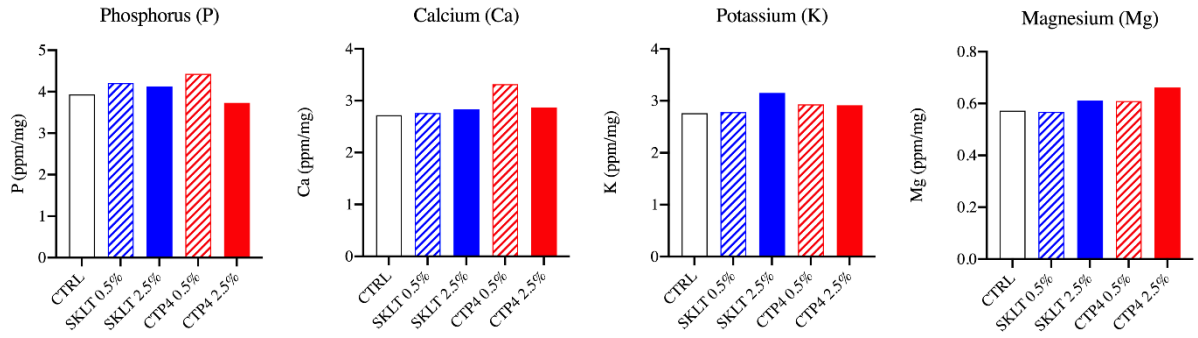


Supplementary Figure 2. Effect of the waterborne exposure of ethanolic extracts of *Skeletonema costatum* (SKLT) and *Tetraselmis striata* CTP4 (CTP4) on the area of the head in 6-dpf zebrafish larvae. Values are presented as mean ± SE and are percentages over the control group. Normality was tested through Anderson-Darling test ($p < 0.05$). Statistical differences were tested through one-way ANOVA followed by post-hoc Dunnett's or Kruskal-Wallis test ($p < 0.05$). Asterisks indicate values statistically different. $p < 0.0002$ (***), $p < 0.0001$ (****).

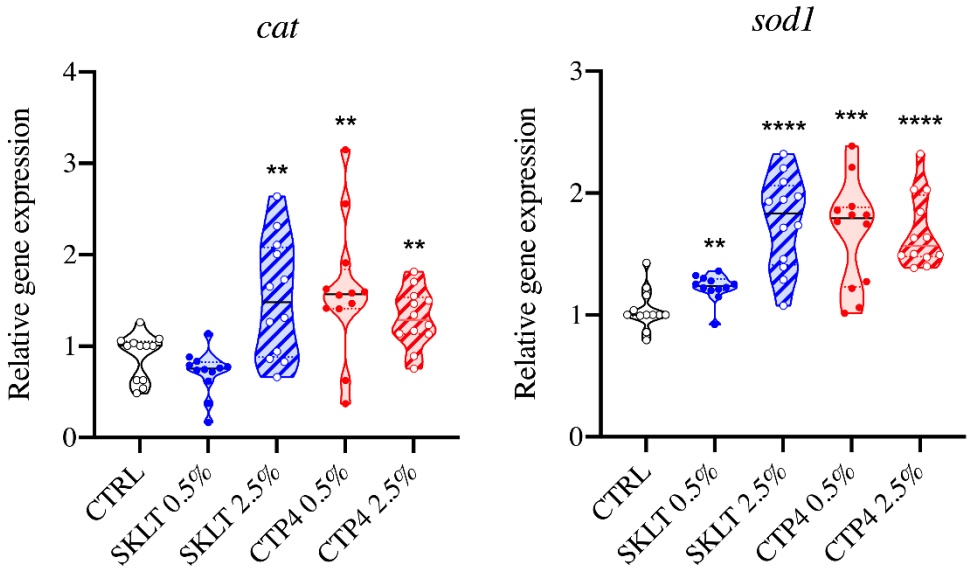
Supplementary Table 3. Ingredients used, and proximal composition of the control diet used as base for the production of experimental diets by vacuum coating.

Ingredients	**Relative amount (%)**
Fish meal	10.00
Fish gelatin	4.00
Soy protein isolate	6.00
Soy protein concentrate	10.00
Corn gluten meal	8.50
Soybean meal	50.00
Vitamins and minerals premix	1.00
Monoammonium phosphate	1.50
Fish oil	2.00
Palm oil	7.00

Composition	Relative amount (%)
Crude protein	50.2
Crude fat	12.3
Fiber	2.5
Starch	4.4
Ash	7.5
Gross energy (MJ/kg feed)	20.0

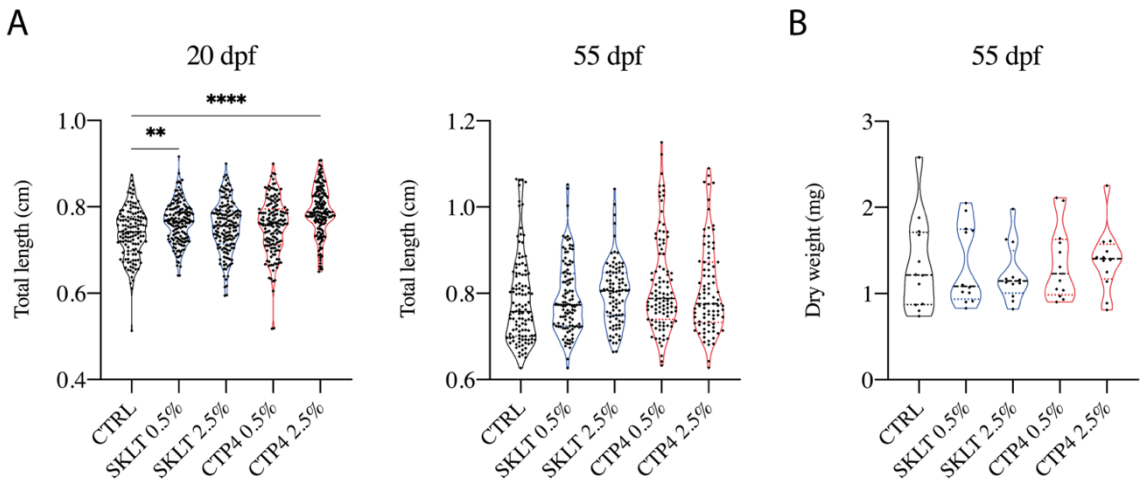


Supplementary Figure 3. Content of phosphorus (P), calcium (Ca), potassium (K), and magnesium (Mg) in the five experimental diets used for the feeding trial.

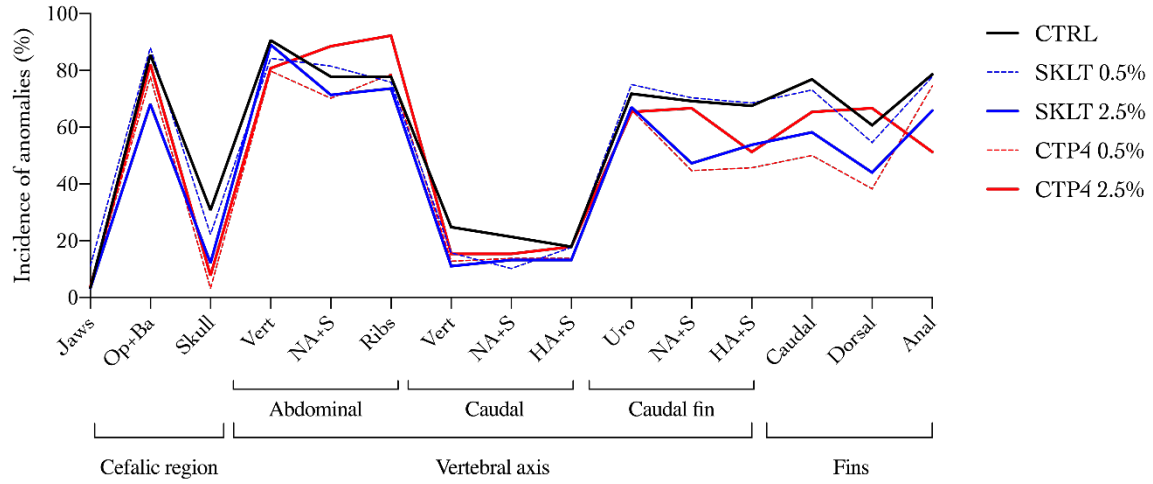


Supplementary Figure 4. Effect of ethanolic extracts of *Skeletonema costatum* (SKLT) and *Tetraselmis striata* CTP4 (CTP4) on fish total length at 20 and 55 dpf (**A**), and dry weight at 55 dpf (**B**). Normality was tested though Anderson-Darling test ($p < 0.05$). Statistical differences were tested through one-way ANOVA followed by post-hoc Dunnett's or Kruskal-

Wallis test ($p < 0.05$). Asterisks indicate values statistically different. $p < 0.002$ (**), $p < 0.0002$ (***).



Supplementary Figure 5. Effect of ethanolic extracts of *Skeletonema costatum* (SKLT) and *Tetraselmis striata* CTP4 (CTP4) on the expression of antioxidant response markers in juvenile zebrafish (55 dpf). *cat*, catalase; *sod1*, superoxide dismutase 1, soluble. Statistical differences between each group and the control (CTRL) were tested through Student's *t* test ($p < 0.05$). Asterisks indicate values statistically different. $p < 0.002$ (**), $p < 0.0002$ (***), $p < 0.0001$ (****).



Supplementary Figure 6. Effect of the ethanolic extracts of *Skeletonema costatum* (SKLT) and *Tetraselmis striata* CTP4 (CTP4) on the relative distribution of skeletal anomalies in juvenile zebrafish (55 dpf) for each skeletal structure. Op+Ba, operculum and branchial arches; Vert, vertebral bodies; NA+S, neural arches and spines; HA+S, haemal arches and spines; Uro, urostyle and caudal vertebrae bodies.

Supplementary Table 4. List of the qPCR primers used in the analysis of the gene expression during the gilthead seabream dietary trails.

Gene name (acronym)	Accession Number	Sequence (5'-3')
superoxide dismutase 1 (*sod1*)	XM_030439011.1	Fw: TGACGCTCACAGGAGAAATCAAAGGG Rev: CAGTAGGACCGCCATGATTCTTACCA
catalase (*cat*)	Q308823.1	Fw: TGGTCGAGAACTTGAAGGCTGTC Rev: AGGACGCAGAAATGGCAGAGG
glutathione-disulfide reductase (*grs*)	XM_030396245.1	Fw: TTCAGCCACCCACCCATCGG Rev: GCGTGATACATCGGAGTGAATGAAGTC
glutathione peroxidase 1 (*gpx1*)	KC201352.1	Fw: GAAGGTGGATGTGAATGGAAAAGATG Rev: CTGACGGGACTCCAAATGATGG
heat shock protein 70 kDA (*hsp70*)	XM_030408740.1	Fw: GCTGATTGAGAAGTGTGACGAGACCA Rev: GGCGTTCCTCCCTGATACAACCTG
tartrate-resistant acid phosphatase type 5 (*acp5a*)	XM_030423913.1	Fw: GTCAAAGCCCAGATCGAGTACAG Rev: AGTCGTCAGAGTTGCCACAC
matrix metallopeptidase 9 (*mmp9*)	XM_030424484.1	Fw: CACCACAAGCAACTTTGACACCGA Rev: TGCCATCTCCTCTTCCCTCACTG
osterix (*sp7*)	XM_030420335.1	Fw: TGTGGGAAGGTCTACGGCAAGG Rev: CTCTCCAGTTCATCTGAGCGGGT
osteocalcin 1 (*oc1*)	AF048703.1	Fw: ACTCTGGCCTTCCTGGTTCTCTG Rev: CCCTCTCCACGAACATACCCTC
osteopontin (*spp1*)	AY651247.1	Fw: TACCATCGTCACGGACACAGAGACAG Rev: GCTCGTAGGACTTGTAGGGAACAGG
bone morphogenetic protein 2 (*bmp2*)	JF261172.1	Fw: CTCCAGAGACAACAGAACCAGCGA Rev: TCAAAGAGTCCTGCACTAGCCGT
collagen type I alpha 1 chain (*col1a1*)	XM_030407011.1	Fw: GGTTCTTCTGGTTCTCCTGGTCTG Rev: GTCCCTGCTTTCCAACCTCTC
alkaline phosphatase (*alpl*)	AY266359.1	Fw: GTGTGGACAGAGACTGGTACTCCGA Rev: CCTCCACCCATAATCACATCAATGTTGG
ß-actin (*actb*)	AF384096.1	Fw: CTTCCTCGGTATGGAGTCCTGCGG Rev: TCCTGCTTGCTGATCCACATCTGCT
elongation factor 1α (*ef1α*)	AF184170.1	Fw: TAACGGAACCACACTGCTGGAG Rev: GCTTCAGGACACCAGTCTCAACAC

Supplementary equations *a* and *b*

(a) Ray width ratio (W) = $\frac{\sum_1^n w1,w2,wn}{n}$, Average width ratio ($\overline{W}$) = $\frac{\sum_1^M Wm}{RAYs}$ where,

w = width of the segments after the amputation

n = number of segments after the amputation

$RAYs$ = average width of the first segment before the amputation

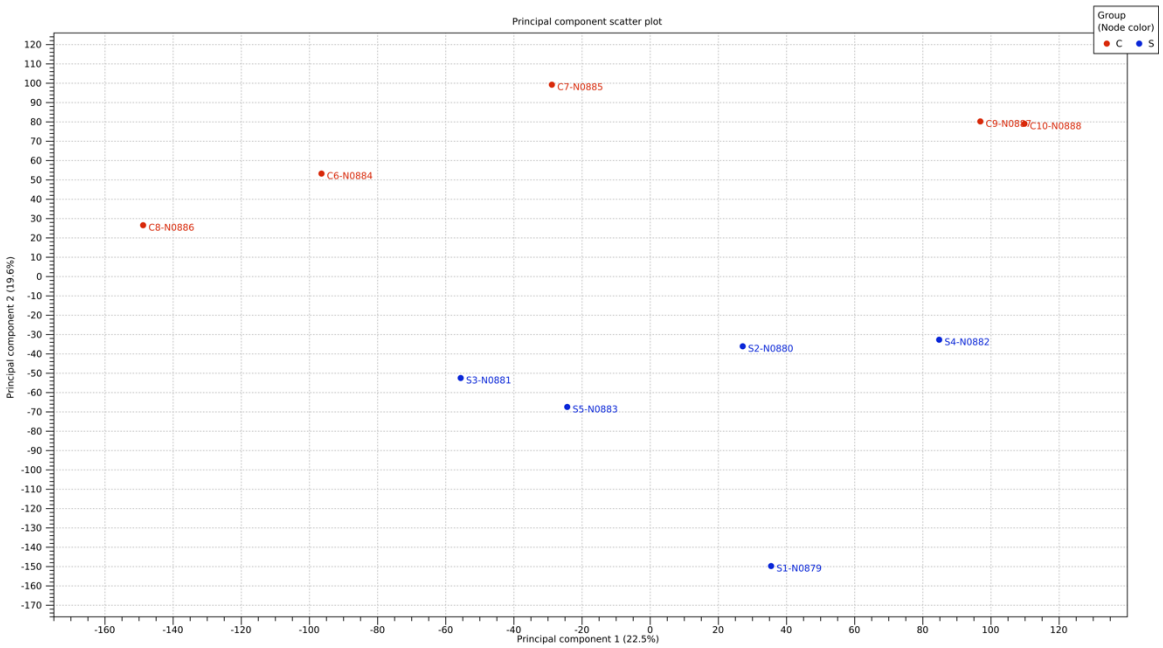
m = number of rays

(*b*) Ray bifurcation ratio (Bt) = BIF/TOT, Average width ratio ($\overline{Bt}$) = $\frac{\sum_1^M Bt1,Bt2,Btm}{m}$ where,

BIF = bifurcation length

TOT = regenerated ray length

m = numbers of rays



Supplementary Figure 7. PCA corresponding to the gene expression of the groups of samples. The first principal component is shown on the X axis and the second principal component is shown on the Y axis. The value after the principal component identifier displays the amount of variance explained by this particular principal component.

SCIENTIFIC OUTPUTS

This PhD project led to the production of 7 scientific articles (between already published and the ones in preparation), and 14 communications (oral presentations and posters) to international conferences. In addition, collaborative projects in which I have been participating during the 4 years of this PhD, have led to the production of 3 more scientific articles.

Scientific articles (part of this PhD project)

- **Carletti A**, Cardoso C, Lobo-Arteaga J, Sales S, Juliao D, Ferreira I, Chainho P, Dionisio M A, Gaudencio M J, Afonso C, Lourenco H, Cancela M L, Bandarra N M & Gavaia P J (2020). "Biopotential of sea cucumbers (Echinodermata) and tunicates (Chordata) from the western coast of Portugal for the prevention and treatment of chronic illnesses". *Proceedings* 61, 13. https://doi.org/10.3390/IECN2020-06994
- **Carletti A**, Cardoso C, Lobo-Arteaga J, Sales S, Juliao D, Ferreira I, Chainho P, Dionisio M A, Gaudencio M J, Afonso C, Lourenco H, Cancela M L, Bandarra N M & Gavaia P J (2022). "Antioxidant and anti-inflammatory extracts from sea cucumbers and tunicates induce a pro-osteogenic effect in zebrafish larvae". *Frontiers in Nutrition*, 9, 888360 (IF 6.008; Endocrinology, Diabetes and Metabolism - Q1). https://doi.org/10.3389/fnut.2022.888360
- **Carletti A**, Gavaia P J, Cancela M L, Laizé V. "What can the oceans do for metabolic bone disorders? The state of art on marine osteoactive compounds" (2023). In preparation for submission to *Marine Drugs*.
- J.T. Rosa, **A. Carletti**, T. Varela, I. Vitorino, O. M. Lage, G. Lopes, V. Gonçalves, T. Santos, H. Galvão, M.L. Cancela, P. Gavaia, V. Laizé. "Phenotypic-based zebrafish screening pipeline for the identification of bone anabolic compounds". In preparation for submission to *Molecules*.
- **A. Carletti**, J.T. Rosa, K. Pes, I. Borges, L. Barreira, T. Santos, J. Varela, H. Pereira, M.L. Cancela, P. Gavaia, V. Laizé. "The osteogenic and mineralogenic activity of microalgae Skeletonema costatum and Tetraselmis sp. CTP4 assessed in fish-based models". Currently submitted to *Biomolecules*.
- J.T. Rosa, **A. Carletti**, C. L. Marques, H. Pereira, H. Ringeard, I. Borges, M. Barata, P. Pousão-Ferreira, S. Engrola, V. Serra, R. Colen, M.L. Cancela, P. Gavaia, V. Laizé. "Diets supplemented with ethanolic extracts of microalgae improve the skeletal health of gilthead seabream (*Sparus aurata*)". In preparation for submission to *Aquaculture*.
- **A. Carletti**, K. Pes, M. Tarasco, H. Pereira, M.L. Cancela, V. Laizé, P. Gavaia. "Anti-inflammatory, anti-osteoclastogenic, and anti-osteoporotic activity of the microalga *Skeletonema costatum*: A potential for the treatment of bone erosive disorders". In preparation for submission to *Biomedicine & Pharmacotherapy*.

Scientific articles (Results of collaborations over the time of this PhD project)

- Sojan J M, Gundappa M K, **Carletti** A, Gaspar V, Gavaia P J, Maradonna F & Carnevali O (2022). "Zebrafish as a model to unveil the pro-osteogenic effects of boron-vitamin D3 Synergism". Frontiers in Nutrition, 9, 868805 (IF 6.008; Endocrinology, Diabetes and Metabolism - Q1). https://doi.org/10.3389/fnut.2022.868805.
- Kumar N, Carletti A, Gavaia P J, Muller M, Cancela M L, Geurts P & Maree R (2021). "Deep learning approaches for head and operculum segmentation in zebrafish microscopy images". *International Conference on Computer Analysis of Images and Patterns* pp. 154-164. Springer. https://doi.org/10.1007/978-3-030-89128-2_15.
- Pes K, **Carletti A**, Dellacqua Z, Martins G, Tarasco M, Diogo P, Gavaia P J. "Establishing the best dietary dose of rotifers for optimal growth and skeletal development in zebrafish.". In preparation for submission to *Animals*.

Communications to scientific conferences

- (Oral presentation) **Carletti A**, Rosa J T, Pes K, Borges I, Barreira L, Santos T, Pereira H, Cancela M L, Gavaia P J, Laizé V. "Microalgae extracts stimulate mineralogenesis during zebrafish development and regeneration". Interdisciplinary Approaches in Fish Skeletal Biology (IAFSB). November 9th-12th, 2022. Olhao, Portugal.
- (Oral presentation) **Carletti A**, Rosa J T, Dellacqua Z, Kumar N, Pes K, Marques C L, Barata M, Pousão-Ferreira P, Engrola S, Serra V, Colen R, Cancela M L, Gavaia P J, Laizé V. "Microalgae extracts with osteoprotective properties for aquaculture nutrition". Aquaculture Europe 2022. September 27th-30th, 2022. Rimini, Italy.
- (Oral presentation) **Carletti A**. "Ethanolic extracts of marine microalgae induce a pro-osteogenic and pro-mineralogenic effect in zebrafish ontogenetic and regenerative models". MAR2020 Workshop: Sustainable Aquaculture of Emerging Species. December, 14th, 2021. Olhao, Portugal.
- (Oral presentation) **Carletti A**, Rosa J T, Pes K, Engrola S, Serra V, Colen R, Cancela. M L, Laizé V, Gavaia P J. "Dietary supplementation of microalgae extracts on the skeletal health of zebrafish (Danio rerio) and gilthead seabream (Sparus aurata) larval stages: a comparative study". Aquaculture Europe 2021. Funchal, Madeira. October 6th, 2021.
- (Oral presentation) **Carletti A**, Gangadhar K N, Cristofoli NL, Barreira L, Pereira H, Varela J, Cancela M L, Laizé V, Gavaia P J. "The potential of Tetraselmis sp. CTP4 as a source for bone anabolic compounds in zebrafish". 11th European Zebrafish Meeting. Prague. October 26-27th 2020.
- (Oral Presentation) Cancela M L, Laizé V, Gavaia P J, Rosa J T, Roberto V, Varela T, **Carletti A**. "Development of in vivo zebrafish systems for identification of molecules with pro/ anti osteogenic properties and bone regenerative capacities". 1st Ocean4Biotech Conference, Piran, Slovenia. 5-6th February 2020.

- (Poster) Pes K, **Carletti A**, Dellacqua Z, Martins G, Tarasco M, Diogo P, Gavaia P J. "Establishing the best dietary dose of rotifers for optimal growth and skeletal development in zebrafish.". Interdisciplinary Approaches in Fish Skeletal Biology (IAFSB). November 9th-12th, 2022. Olhao, Portugal.

- (Poster) Florio Furno M, Spina F, Ferrero D, **Carletti A**, Varese GC, Gavaia P J, Laizé V. “The biopotential of marine fungi associated to microplastics as possible source of mineralogenic and osteogenic compounds”. Interdisciplinary Approaches in Fish Skeletal Biology (IAFSB). November 9th-12th, 2022. Olhao, Portugal.
- (Poster) **Carletti A**, Dellacqua Z, Rosa J T, Kumar N, Tarasco M, Marques C L, Barata M, Pousão-Ferreira P, Cancela M L, Gavaia P J, Laizé V. “Dietary supplementation with osteogenic extracts during the pre-ongrowing phase improves the skeletal quality of gilthead seabream at the ongrowing phase”. XX International Symposium on Fish Nutrition 2022. June 5th-9th 2022. Sorrento, Italy.
- (Poster) Pes K, Martins G, **Carletti A**, Tarasco M, Diogo P, Gavaia P J. “Dietary rotifers requirement for zebrafish optimal larval growth and skeletal development”. Aquaculture Europe 2021_Funchal, Madeira. October 4th-8th, 2021.
- (Poster) Rosa J T, **Carletti A**, Pes K, Borges I, Engrola S, Serra V, Colen R, Cancela M L, Gavaia P J, Laizé V. “Microalgae extracts as a dietary supplement to improve skeletal status in zebrafish Danio rerio and gilthead seabream Sparus aurata”. Aquaculture Europe 2021_Funchal, Madeira. October 4th-8th, 2021.
- (Poster) Rosa J T, **Carletti A**, Marques C L, Ringeard H, Borges I, Barata M, Pousão-Ferreira P, Gavaia P J, Cancela M L, Laizé V. “Microalgae-supplemented diet improves the skeletal health of gilthead seabream Sparus aurata juveniles”. Aquaculture Europe 2021_Funchal, Madeira. October 4th-8th, 2021.
- (Poster) Rosa J T, **Carletti A**, Marques C L, Ringeard H, Barata M, Pousão-Ferreira P, Gavaia P J, Cancela M L, Laizé V. "Microalgae-supplemented diets to improve skeletal status of gilthead seabream juveniles". 5th AquaImprove Workshop. Online. November 6th, 2020.
- (Poster) **Carletti A**. “Osteogenic and Mineralogenic Effect of Marine Derived compounds”. BIOMEDAQU 2nd Summer School on “Monitoring of fish Skeletal Anomalies”. University of Rome Tor Vergata, Rome, Italy. 27th September 2019.

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