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Expression of the nuclear receptors NR1J1, CYP and ABCB1 genes in marine mussel *Mytilus galloprovincialis*, mediated by food regime and Okadaic Acid exposure

Juliana Lourenço Rodrigues

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FACULDADE DE **CIÊNCIAS**

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Dissertação de Candidatura ao grau de Mestre em Toxicologia e Contaminação Ambientais submetida ao Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto.

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Abstract

Mytilus galloprovincialis production has a low environmental impact and has the benefit of contributing to the global food supply improvement. However, because this is a filter-feeding species, it is capable of accumulating and tolerating a great variety of compounds, such as the diarrhetic shellfish toxin, the okadaic acid, which can lead to diarrhetic shellfish poisoning in humans.

The defensome comprises the nuclear receptors responsible for recognizing these toxicants and regulating the response of a repertoire of enzymes and transporters involved in xenobiotic metabolism. NR1J1 genes are the invertebrate orthologs of the vertebrates NR1I genes, thought to be involved in the regulation of expression of genes from phases I, II, and III, such as cytochrome P450 (CYP450) and ATP-binding cassette B member 1 gene (ABCB1). Despite this, there's still limited information available about the NR1J group in marine invertebrates and in particular in mussels.

Thus, the present work aimed to study the influence of food intake (the microalgae *Tetraselmis* sp. and *Isochrysis* sp.) and the presence of okadaic acid in the expression of NR1J1, CYP3, and ABCB1 genes in the marine mussel *Mytilus galloprovincialis,* following a previous hypothesis sustained with *in vitro* (cell line) studies.

In the analysis of the food intake influence on the regulation of the referenced genes, the gills had higher levels of expression than the digestive gland, which showed a predominant down-regulation of NR1J1 genes and phase I genes. The gills revealed an important role in conducting xenobiotic metabolism reactions since they were susceptible to the intake of microalgae, leading to the upregulation of NR1J1 and phase I genes. These results agree with other studies that show that food supply can contribute to toxin elimination since food intake can accelerate metabolic mechanisms and expel the toxicants through fecal matter.

Exposure to okadaic acid led to different gene expression responses since the organ that displayed higher gene expression levels is the digestive gland. In fact, in this tissue, NR1J1, CYP3, and ABCB1 genes were upregulated, supporting the hypothesis that NR1J1 members are involved in the biotransformation and elimination of toxicants through the regulation of the tested genes. In the gills, the expression of CYP3 genes decreased, indicating that CYP3 members could be involved in early toxin metabolism.

This study demonstrated that the toxin okadaic acid and food supply modulate NR1J1 and other key genes of the defensome of the marine mussel *M. galloprovincialis*, which opens new opportunities to reduce the toxin load in contaminated shellfish. Nevertheless, more studies are required to better understand the mechanisms involved in okadaic acid

metabolism and detoxification in bivalves, namely in mussels, to develop efficient toxin depuration strategies.

Resumo

A produção de *Mytilus galloprovincialis* tem baixo impacto ambiental, e contribui para a melhoria do abastecimento alimentar global. No entanto, uma vez que é um organismo filtrador, esta espécie é capaz de acumular e tolerar uma grande variedade de compostos, como a toxina diarreica de moluscos, o ácido ocadáico, que pode provocar a contaminação diarreica por consumo de moluscos, em humanos.

O defensoma compreende os recetores nucleares, responsáveis por reconhecer essas substâncias tóxicas e regular a resposta de enzimas e transportadores moleculares. Os genes NR1J1 são os ortólogos de invertebrados dos genes NR1I de vertebrados e especula-se que estes estejam envolvidos na regulação da expressão de genes das fases I, II e III, como o citocromo P450 (CYP450) e um gene dos transportadores ABC (ABCB1). Apesar disso, as informações disponíveis sobre o grupo NR1J1 em invertebrados marinhos e, em particular, nos mexilhões são ainda limitadas.

Assim, o presente trabalho teve como objetivo estudar a influência da ingestão alimentar (das microalgas *Tetraselmis* sp. e *Isochrysis* sp.) e a presença de ácido ocadáico na expressão dos genes NR1J1, CYP3 e ABCB1 no mexilhão marinho *Mytilus galloprovincialis*.

Na análise da influência da ingestão alimentar na regulação dos genes referenciados, as brânquias apresentaram níveis de expressão mais elevados do que a glândula digestiva, onde os genes NR1J e as enzimas da fase I sofreram uma diminuição da expressão. Por sua vez, nas brânquias foi notável o importante papel que desempenham na condução das reações do metabolismo de xenobióticos, uma vez que apresentaram ser muito sensíveis à ingestão de microalgas, levando à regulação positiva dos membros NR1J1 e dos genes da fase I. Estes resultados apoiam o uso de *Tetraselmis* sp. e *Isochrysis* sp. na eliminação de toxinas, servindo de alimento a animais contaminados, acelerando mecanismos metabólicos e expelindo os tóxicos através da matéria fecal.

Quando o ácido ocadáico está presente, a expressão é diferente, uma vez que o órgão que apresenta níveis mais elevados de expressão génica é a glândula digestiva. De facto, neste último tecido, os genes NR1J1, CYP3 e ABCB1 foram regulados positivamente, apoiando a hipótese de que os recetores NR1J1 estão envolvidos na biotransformação e eliminação de tóxicos, através da regulação dos genes testados. Nas brânquias, a expressão dos genes CYP3 diminuiu, indicando que estes poderiam estar envolvidos no metabolismo precoce da toxina.

Este trabalho demonstrou que a exposição ao ácido ocadáico e o suplemento alimentar podem direcionar a expressão de genes do defensoma do mexilhão marinho *M. galloprovincialis*, abrindo novas oportunidades para o desenvolvimento de estratégias de depuração e redução do teor de toxinas nos bivalves moluscos. No entanto, mais estudos sobre o tema são necessários, para melhor compreender os mecanismos envolvidos no metabolismo e desintoxicação de ácido ocadáico em bivalves, nomeadamente em mexilhões.

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Abbreviations

ATP Binding Cassette (ABC)

ATP binding cassette B member 1 gene (ABCB1)

Cytochrome P450 (CYP450)

Constitutive androstane receptor (CAR)

Diarrhetic shellfish poisoning (DSP)

Diarrheic shellfish toxins (DSTs)

Dinophysis toxins (DTX)

Dinophysistoxin-1 (DTX1)

Dinophysistoxin-2 (DTX2)

Dinophysistoxin-3 (DTX3)

Dinophysistoxin-4 (DTX4)

Dinophysistoxin-5 (DTX5)

DNA-binding domain (DBD)

Dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1 (DAX1)

Farnesoid X receptors (FXR)

Germ cell nuclear factor (GCNF)

Glutathione transferases (GST)

Hepatocyte nuclear factor-4 (HNF4)

Ligand binding domain (LBD)

Liver receptor homolog-1 (LRH-1)

Liver X receptors (LXR)

Nuclear receptors (NRs)

Okadaic acid (OA)

Peroxisome proliferator activated receptors (PPAR)

P-glycoproteins (PGP)

Pregnane X receptor (PXR)

Response elements (REs)

Retinoic acid receptors (RAR)

Retinoic acid related receptors (ROR)

Retinoid X receptors (RXR)

Reverse-Erb receptors (REV-ERB)

Small heterodimer partner (SHp)

Steroid receptors (SRs)

Steroidogenic factor 1 (SF-1)

Thyroid hormone receptors (TR)

Vitamin D receptors (VDR)

1. Introduction

1.1. Mytilus galloprovincialis

The mussel *Mytilus galloprovincialis* is a species of bivalve mollusk, first described by Lamarck in 1819 [1].

This species of bilateral symmetry has dark-colored shells and bluish nuances, with a characteristic oval and elongated shape and an umbo curved downwards (Fig. 1) [2-4]. Mussels reach dimensions varying between 5 and 9 centimeters, depending on the habitat, being smaller in coastal areas and larger if inhabiting deep areas [2,3].

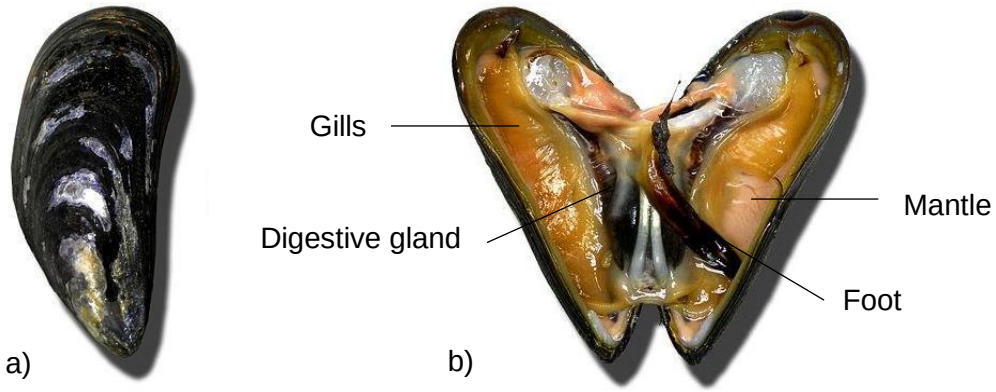


Fig. 1. Morphology of *Mytilus galloprovincialis*: a) appearance of the shell, b) appearance of the interior and body organs. Adapted from [3]

M. galloprovincialis has been reported in the Mediterranean, Black, and Adriatic Seas and the Atlantic Ocean, and its geographic distribution is dependent on water temperature (Fig. 2) [1,3]. There are also other relevant factors in characterizing the ideal habitat for mussel growth: relatively strong currents, the richness of phytoplankton in suspension, the existence of other bivalve populations that have suffered massive mortality and whose

shells serve as a stratum, and the presence of filamentous algae [4,5]. It is often found on rocky coastlines and estuaries with high water flow [2,6].

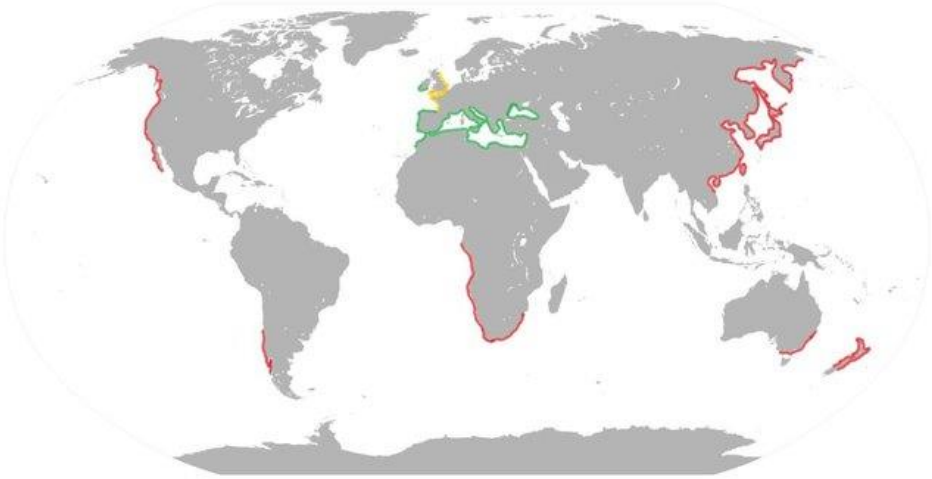


Fig. 2. Distribution of *Mytilus galloprovincialis*. Green and yellow areas are those where the species is native, and red areas are regions of invasion. Image from [3]

With the continuous increase in the human population and concern for a healthy lifestyle, there is a greater demand for fish and seafood for consumption [7,8]. Thus, the aquaculture of these animals has received increasing attention and is an important evolving activity to overcome the food needs of the human population [8]. In fact, the significant decrease in the mussel harvest was accompanied by an increase in the production rate of these animals, with the harvesting reaching 3,114,561 tons and the production reaching the overwhelming amount of 94,350,976 tons in 2021 [2].

Mussel production, mainly in coastal regions, has a low environmental impact, with the benefit of contributing to the global food supply improvement [7,8]. However, this activity also faces risks associated with environmental changes, pollution, diseases, and the presence of toxic algal blooms and biotoxins, making it necessary to find effective mitigation measures to secure food safety and the economic viability of this activity [5,9].

The body of these animals is made up of a foot that allows them to move on the substrate and adhere to it through the filaments (byssal threads) production with bioadhesive properties [1,10]. The gills, which are filamentous and have ciliary movements, are

responsible for respiration, filtering the food from the water, sorting, transporting, and expelling food particles [1,10]. Phytoplankton comprises the main diet of bivalve mollusks and *M. galloprovincialis*. The digestion takes place in the digestive gland [1,2,6]. In contact with the shell are the mantle and the reproduction organs [10]. In fact, the specific characteristics of this organism make it an excellent colonizer of the shorelines. *M. galloprovincialis* displays high growth rates, tolerance to exposure to air and variations in environmental conditions, fast dispersal, and high competitive capacity [3,11,12].

Furthermore, the sedentary lifestyle, filter-feeding, and low metabolism contribute to the selection of this animal as an indicator of pollution and the health condition of the environment. *M. galloprovincialis* is capable of accumulating and tolerating a great variety of natural and anthropogenic compounds [1,5,12,13]. Therefore, this organism is a preferred model species for developing aquatic ecotoxicological studies [1,13,14].

Despite being persistently in contact with chemical substances dispersed in the water, pathogenic microorganisms, and endogenous toxic compounds, mussels are able to maintain homeostasis and tolerate adverse environments, thanks to the development of efficient immune and xenobiotic defense systems that confer protection against biological and chemical hazards. The “defensome” comprises multiple molecular pathways that allow the recognition, metabolizing, and elimination of toxic compounds [5,14-16].

In *M. galloprovincialis*, the “defensome” functions and composition are limited. The pangenome nature of this species has revealed a high intraspecific diversity, characterized by a wide range of genes and receptors expansion [5]. In fact, the genome of this mussel comprises 20,000 genes that can be present or not in some individuals [17].

These animals can accumulate biotoxins produced by microalgae in their body, mainly in the digestive gland [18,19]. An example of these toxins is okadaic acid (OA), which is easily accumulated but hardly eliminated [18]. Despite several studies investigating behavioral, physiological, and immunological effects caused by diarrhetic shellfish toxins (DSTs) in bivalves, it is not yet well established whether these toxins threaten the survival of mussels [18].

1.2. Diarrhetic shellfish toxins

Okadaic acid (Fig. 3) belongs to the diarrhetic shellfish toxins (DSTs) group, described for the first time in 1981, responsible for diarrhetic shellfish poisoning (DSP) [20,21]. These toxins are lipophilic substances produced, for instance, by dinoflagellates of the genera *Prorocentrum* and *Dynophysis*, the latter being predominant in the intoxication process by OA due to its planktonic nature, which allows higher ingestion of these organisms by aquatic filter feeders, such as mussels [18,20,22-24].

In turn, despite their benthic nature, dinoflagellates of the genus *Prorocentrum*, namely the species *Prorocentrum lima*, are often involved in symbiotic relationships with marine invertebrates, thus contributing to the ingestion of OA by these animals [23,24]. This genus is considered cosmopolitan since it is widely distributed from temperate to tropical and subtropical regions. It is also commonly found on the Portuguese coast [23-25].

In dinoflagellates of this genus, a group of fat-soluble esters was found, the OA diol-esters, in which the carboxylic group of OA is conjugated with diols of 6- to 10-carbons, to form allylic diol-esters [23,26]. For instance, in *Prorocentrum lima* and *Dinophysis acuta*, it has been reported a C9-triol ester and a hydroperoxide derivative of a C9-diol ester of OA [27]. Another example of these derivates is the cis-isomer of a C8-diol ester of okadaic acid extracted from *D. acuta* [28].

In bivalves like *M. galloprovincialis*, which can ingest toxic dinoflagellates, it was discovered another type of OA derivative, the Dinophysistoxin-3 (DTX3), which is a mixture of 7-O-acyl ester derivatives of OA, Dinophysistoxin-1 (DTX1) and Dinophysistoxin-2 (DTX2), also responsible for DSP symptoms [26,29]. *Prorocentrum* is also responsible for producing water-soluble derivatives, in which the diol-esters are conjugated to a polar side chain: dinophysistoxin-4 (DTX4) and dinophysistoxin-5 (DTX5) [26].

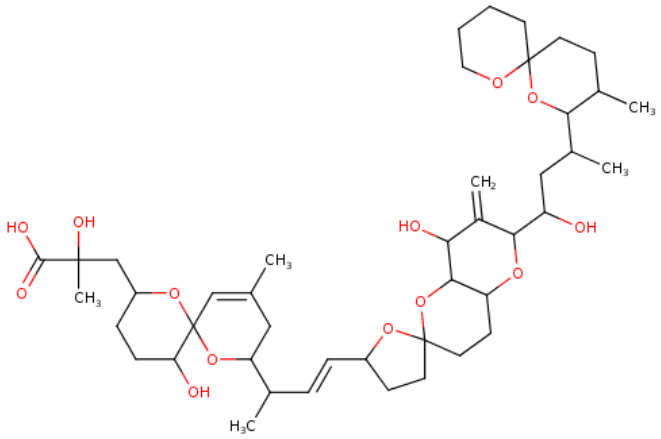


Fig. 3. Chemical structure of okadaic acid [30].

Filter-feeding bivalves, such as mussels, tend to accumulate OA and its derivatives in their bodies, which can be transferred along the trophic chain and poisoning humans. In humans, poisoning by these toxins causes gastrointestinal diseases [18,22,31]. These toxins act at low concentrations, and in the long term, their mode of action includes the inhibition of serine/threonine protein phosphatases PP1 and PP2 through their interactions with the catalytic domains of these enzymes. Thereby, OA and its derivatives may affect multiple cellular functions, impairing the growth and division of cells and the cytoskeletal structure and ultimately causing cell death [18,20,22,32,33]. It was concluded that inhibition of protein phosphatases PP1 and PP2A also happens with OA derivatives, but only with those with a free acid group. On the other hand, OA diol-esters can be hydrolyzed to produce the active parent OA [26]. OA also stimulates the secretion of sodium in intestinal cells and increases their cellular permeability, causing diarrhetic poisoning symptoms [34].

Symptoms associated with this food poisoning appear thirty minutes after ingesting contaminated shellfish, including diarrhea primarily, nausea, vomiting, and abdominal pain [18,20,22,33]. Additionally, these toxins can also cause neurotoxicity, hepatotoxicity, cytotoxicity, embryotoxicity, and genotoxicity, and eventually can evolve into cancer [20,32]. The intensity of these symptoms is associated with the amount of toxin ingested, with 0.33 µg of OA equivalents per kg body weight being the minimum dose necessary to cause poisoning effects [35].

Due to the global distribution of DSTs, DSP events have been reported worldwide, but no fatalities related to this contamination have been observed to date [22,31]. The first

confirmed incidence of this poisoning in Portugal occurred in 1998, in the Algarve, resulting from a DSP toxins detection method implemented in 1994 [36,37]. Six hours after ingesting contaminated clams, symptoms of diarrhea, vomiting, abdominal pain, weakness, and headaches were reported, and their intensity was proportional to the amount consumed by each person. In this case, it was observed that both raw and cooked seafood caused the same effects [37]. This is due to the properties of these toxins, which are resistant to cooking or freezing. Furthermore, they are difficult to detect without testing, as they do not alter the seafood flavor[18,22,31]. In 2001, another outbreak of DSP occurred with clams from Aveiro that, combined with studies of contamination of bivalves from these areas, made it possible to improve methods of identifying the toxin and discovering more about its nature in different species, either as the active parent or as derivatives [29,38].

In mussels, as in other bivalve mollusks, these toxins are mainly accumulated in the digestive gland, but their presence is also found in non-visceral tissues, such as the gills [19]. Unlike several bivalve species, mussels accumulate mostly OA-free forms and less esterified forms of this toxin. The accumulation occurs mainly in the digestive gland, where the producers of this toxin are most present intact or recently digested and whose toxins have not yet undergone the esterification process and may present more intense effects [29,38]. Therefore, it is safe to say that depending on the species and where this toxin is stored, OA can differentially affect humans.

More studies of the anatomical distribution of DSTs in mussels are needed, in particular, to develop effective mitigation and elimination strategies for these substances [19]. One strategy that has been tested was the transfer of contaminated animals to a clean environment, with favorable conditions, to allow them to depurate the toxins [9]. In addition, increasing the water temperature during depuration and/or feeding mussels with non-toxic food, such as microalgae of the genus *Isochrysis* sp*.* and *Tetraselmis* sp*.*, may contribute to toxin elimination through fecal matter and accelerate metabolic mechanisms [9,39]. Associated with the low esterification rates of these toxins in *M. galloprovincialis,* there is, therefore, a need to better understand the detoxification mechanisms in this mussel species [14,18].

1.3. The chemical defensome

Within the defensome system, the “chemical defensome” consists of a network of genes and pathways that protect the organism against a diverse range of toxicants, like microbial products, metals, phytotoxins, and other natural compounds, most of them hydrophobic [40]. The chemical defensome works through receptors and ligand-activated transcription factors capable of recognizing the toxins, enzymes that transform chemicals into less toxic and easily excreted metabolites, and transporters that export substrates from the cells [40].

This defense starts with toxicants expulsion by efflux proteins, the ATP Binding Cassette (ABC), like the p-glycoproteins (PGP), members of the ABCB family. If these steps fail and the toxicants enter the cytoplasm, then oxidative modifications are built up. This is known as phase I, carried out by cytochrome P450 (CYP) enzymes, which are involved in compound transformation into more hydrophilic metabolites, facilitating the excretion process [40].

The proteins responsible for recognizing and activating this cascade of reactions comprise the nuclear receptors (NRs), chemical sensors that regulate the transcription of enzymes (like CYP2s, CYP3s, and CYP4s), and transporters [40].

1.4. Nuclear receptors

Nuclear receptors are crucial regulators of the endocrine systems in animals, being involved in several processes, namely in metabolic pathways and pathological processes, such as lipid and glucose metabolism, cell differentiation and apoptosis, gonad development, organogenesis, reproduction, and immunity [41-43]. A clear example of the action of NRs is the regulation of xenobiotic metabolism through the gene expression modulation linked to CYP450 [14,15,44].

NRs are transcription factors that recognize and directly bind to specific elements in the DNA, activating, repressing, or blocking the expression of genes, mainly modulated by binding to ligands, which include endogenous or exogenous lipophilic molecules. Thus, toxic substances can induce responses in the contaminated organism as after NR activation, a cascade of biological processes is triggered [14,16,45-48]. Depending on the type of cells, NRs can regulate different genes, affecting different pathways [47].

NRs are characterized by a structural organization composed of 5 domains: the amino-terminal region, AF1 (A/B domain); the DNA-binding domain, DBD (C domain); the hinge region (D domain); the ligand-binding domain, LBD (E domain); and the carboxyl-terminal domain (F domain) (Fig. 4) [47].

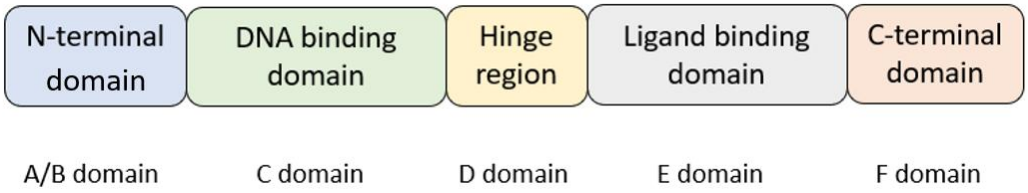


Fig. 4. Representative model of the structural organization of NRs and their domains. Adapted from [46].

In the LBD, a moderately conserved domain, ligand binding occurs, activating the NRs. In turn, the DBD, a highly conserved domain, is the region that mediates interactions with DNA through the recognition of response elements (Res), controlling the specificity of the transcriptional response [16,46,49]. These receptors are divided into two classes: NRs with known ligands and orphan NRs, whose ligand does not exist or is not yet known [46]. NRs are often linked to co-repressors that are then replaced by co-activators upon binding to ligands, resulting in NR activation and subsequent transcription of target genes. Others are constantly associated with co-activators, inducing gene expression, which is interrupted when the ligand is present. Finally, ligand-independent NRs can repress or activate gene transcription [48].

The conservation of the DBD and LBD domains helps the NRs identification in genomic sequences and the phylogenetic reconstruction of this protein superfamily [16,48]. This superfamily can be divided into nine subfamilies (0, 1, 2, 3, 4, 5, 6, 7, 8), which are thought to have originated from a single ligand-activated ancestral NR and gave rise to the multiple NR subfamilies and groups through a process of genetic expansion, linked to the function of endocrine systems [46,50].

NR0 is considered a heterogeneous subfamily, as it contains all nuclear receptors whose structure differs from the typical conformation of NRs, lacking the LBD (NR0A) or the DBD

(NR0B) [46,50]. It encompasses DAX1 (dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1) and SHP (small heterodimer partner) [51].

In the human NR repertoire, the members of the NR1 subfamily are modulated by lipophilic signaling molecules. This subfamily includes thyroid hormone receptors (TR), retinoic acid receptors (RAR), peroxisome proliferator-activated receptors (PPAR), reverse-Erb receptors (REV-ERB), retinoic acid-related receptors (ROR), farnesoid X receptors (FXR), liver X receptors (LXR), pregnane X receptor (PXR), constitutive androstane receptor (CAR) and vitamin D receptors (VDR) [51]. This subfamily comprises the majority of ligand-activated receptors in vertebrates, which then establishes heterodimeric complexes with retinoid X receptors (RXR) [46]. The NR2 subfamily includes orphan receptors, of which only hepatocyte nuclear factor-4 (HNF4) and RXR have known activating ligands (fatty acids and 9-cis retinoic acid, respectively) [51]. Steroid receptors (SRs) constitute the NR3 subfamily and are regulated by cholesterol-derived hormones such as cortisol and estrogen. They play a role in metabolic, reproductive, and developmental processes [51]. The NR4 subfamily receptors act in the development and maintenance of neurons. In turn, the NR5 subfamily encompasses steroidogenic factor 1 (SF-1) and liver receptor homolog-1 (LRH-1), which are involved in the development and metabolism [51]. The NR6 subfamily contains only one receptor with a significant difference in the LBD, the germ cell nuclear factor (GCNF), playing a key role in development [51].

The NR7 subfamily is characterized by a different structure than the rest of the others since it has two DBD, constituted with a P-box sequence in the first DBD, which is unique to this subfamily [52]. Lastly, the NR8 subfamily, the third-oldest subfamily, is involved in transcriptional regulation, being able to bind to conserved DNA core motifs [53]. These two subfamilies have not been identified in vertebrate genomes.

NRs are not present in plants, fungi, algae, or protozoa. However, the NR gene repertoires of metazoans have been characterized, reporting a species-dependent pattern with variable content and number of NR genes[42,43], like about 2 members in sponge, 4 in placozoan, 48 in mammals (e.g., 48 in humans, 49 in mouse), 66-137 in teleost (e.g., 73 in zebrafish, 74 in tilapia), 250 in nematodes (e.g., 119 in flatworms)[42,43].

The vast diversity of NR genes and the differences reported between gene subfamilies are due to moments of replication (by whole genome duplication, segmental duplication, or tandem duplication) or loss of genes during animal evolution [42,43,46]. For instance, a study with flatworms found NR genes from the NR0-NR5, NR7, and NR8 subfamilies, with the largest subfamily, NR1, counting 27 members [43]. In another study involving

cartilaginous fish, the subfamilies NR0-NR6 were identified, and it was possible to assess their orthology with humans, which implies a conservative evolutionary relationship [42].

Despite the stability of the NR repertoire, we cannot infer functional conservation [42]. An example of this statement is the fact that despite the orthologous relationship between the mollusk NR1B and human RAR, the mollusk ortholog was not able to bind or respond to retinoids due to the evolutionary plasticity of its LBP [54]. On the other hand, in a study with the rotifer *Brachionus manjavacas*, NR function conservation between this species and mammalians was reported [55]. This could indicate that, due to a common ancestor of the Bilateria, hormonal signaling pathways and endocrine systems are probably conserved across most animal taxa.

The nuclear receptors from subfamily 1, groups I and J (NR1I and NR1J) present similarities in the LBD and are distinguished from the rest of the subfamilies. Phylogenetic analyses inferred that NR1J are the invertebrate orthologs of the vertebrate NR1I group (Fig. 5), meaning that both groups share the same ancestral genetic line, which underwent distinct evolutionary processes during vertebrates and invertebrates split [14,45].

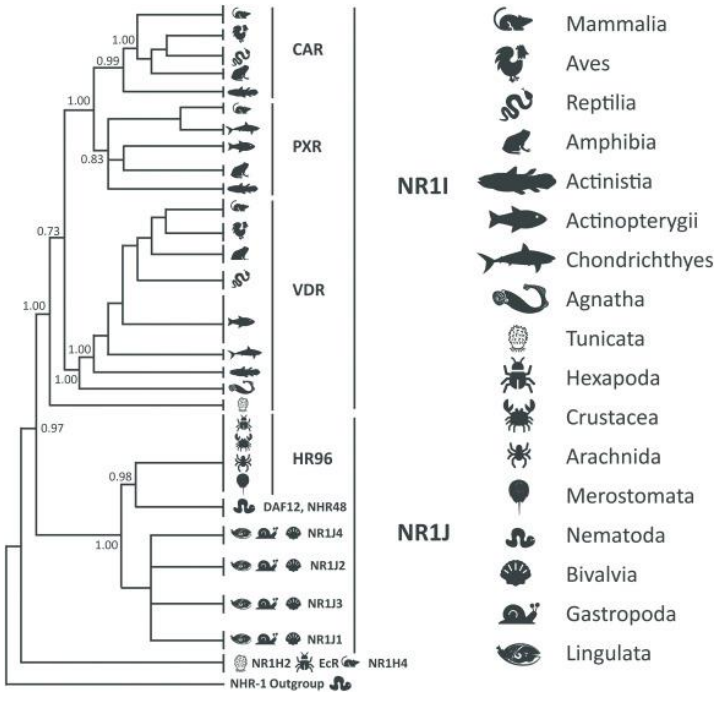


Fig. 5. Phylogenetic tree describing the relationships between NR1I/J in vertebrates and invertebrates [45].

The NR1I group encompasses the VDR (NR1I1), PXR (NR1I2), and CAR (NR1I3), capable of inducing the expression of phase I enzymes (e.g., CYP3A4). PXR and CAR are also involved in the expression regulation of phase II and phase III enzymes [56]. VDR is responsible for regulating calcium and phosphatase homeostasis by binding calcitriol. It is present in fish, amphibians, reptiles, birds, and mammals [56]. PXR is activated by pregnane steroids and is expressed in mammals, with high representation in the liver and intestine in humans and mice [56]. PXR is also present in some teleost fish, such as Pelagic cod (*Melanonus zugmayeri*), but was lost in other species, like Atlantic cod (*Gadus morhua*), and in birds and reptiles [57,58]. Unlike these two, CAR has high constitutive activity, which means that, depending on the compound, its activation can increase or decrease. CAR has only been detected in tetrapods, such as mammals, some birds, and reptiles [56].

PXR has a large and flexible ligand-binding site, thus recognizing many different ligands varying in size and chemical structure and is characteristic of each species [46,56]. It has two main transcriptional targets, the CYP3A subfamily and the P-glycoprotein multidrug resistance pumps, which also have a wide substrate specificity [56]. This broad ligand specificity of PXR suggests that this receptor, and to a lesser extent also CAR, are highly involved in the defense against exogenous and endogenous toxicants.

The role of PXR in defending organisms against toxic compounds is well described in vertebrates and mammals, acting in the distinct phases of xenobiotic metabolism, as mentioned above [14,15,31]. Both NR1I and NR1J have an affinity for lipophilic molecules, acting as sensors of xenobiotic compounds. They bind to a wide variety of ligands, such as endogenous molecules (steroid hormones, secondary bile acids, and fat-soluble dietary vitamins, such as vitamin D) and exogenous compounds (xenobiotics and toxins) [14,59,60]. NR1I and NR1J are activated upon exposure to xenobiotics, presenting similar responses between their members, which could indicate that these subfamilies may play similar roles in the xenobiotic response of vertebrates and invertebrates [14,31,45].

Despite the limited information available about the NR1J1 group in marine invertebrates and in particular in mussels, these transcription factors are thought to be involved, as NR1I members, in the regulation of expression of genes from phases I, II, and III, such as cytochrome P450 (CYP450) and ATP-binding cassette B member 1 gene (ABCB1) [31,45]. CYP450 comprises a family of hemoproteins, widely distributed throughout all living forms, with a role in the biotransformation and detoxification of xenobiotics. In invertebrates, numerous CYPs have been discovered, of which five with sequences similar to the vertebrate CYP1 and CYP3 families and so-called CYP1-like-1, CYP1-like-2, CYP3-like-1,

CYP3-like-2, CYP3-like- 3 were cloned, and their expression assigned in the digestive gland of mussel *M. edulis* [61,62]. On the other hand, ABCB1, an ATP-dependent transmembrane transporter, is mainly involved in the elimination of unmodified exogenous compounds, having been found in the gills of bivalve mollusks, such as the mussels *M. galloprovincialis*, *M. edulis*, *M. californianus* and in the oyster *Saccostrea forskali* [63,64]. In the ark shell *Scapharca subcrenata* and in the blood clam *Tegillarca granosa*, for example, the exposure to DSTs led to the activation of these transporters [65].

Studies involving marine invertebrates and NRs from the NR1J group encompass the discovery that, in mollusks, 3 to 4 genetic lineages from the NR1J group are present (α, β, γ, and δ), from which the SplNR1Jβ receptor was isolated and characterized, from the marine clam *Scrobicularia plana* [14]. In the Pacific oyster, *Crassostrea gigas*, several homologs of the NR1J1 group were discovered, namely CgNR1Jα, CgNR1Jβ, and CgNR1Jδ [66]. Other mollusks, where receptors from this group were found, include the owl limpet *Lottia gigantea* and the freshwater snail *Biomphalaria glabrata*, presenting, respectively, three (LgNR1J1, LgNR1J12 and LgNR1J3) and four (BgNR1J1, BgNR1J12, BgNR1J3 and BgNR1J4) receptors [67]. An expansion of the NR1J1 group, totalizing twenty genes, was discovered in the marine flatworm *Macrostomum lignano* that, together with other receptors, can help this species adapt to different conditions of temperature, salinity, and oxygen concentration, in addition to conferring resistance to a wide range of toxins [43].

The study of the action of these NRs in aquatic organisms contaminated with biotoxins (e.g., DSTs) is gaining increased attention, given their putative role in the biotransformation of these compounds, including OA. The presence of toxicants in the mussel *M. galloprovincialis* has been reported to involve NR1J1 paralogs and the action of detoxification enzymes, such as CYP450 [61,68,69]. In a recent study carried out in CIIMAR (not published, personal communication), four paralogs of the NR1J1 nuclear receptor (NR1J1α, NR1J1β, NR1J1γ, and NR1J1δ) were identified and analyzed in the mussel *M. galloprovincialis*. Their presence may be explained by the great need for adequate detoxification mechanisms to cope with exposure to a great variety of chemical substances present in the aquatic environment.

2. Objectives

The main objective of this project is to study the expression of the NR1J1, CYP3-like, and ABCB1 genes in the marine mussel *Mytilus galloprovincialis* and to hypothesize the molecular and physiological functions of NR1J1 group of genes in filter-feeding marine invertebrates. The specific objectives of the research project consisted of the following:

1. Evaluate the expression of NR1J1 (and the genes under NR1J1 regulation, CYP3-like, and ABCB1 genes) in mussels fed with microalgae *Tetraselmis* sp. and *Isochrysis* sp. to test the hypothesis that diet and food intake affect the expression of these genes;

2. Evaluate the expression of NR1J1 (and the genes under NR1J1 regulation, CYP3-like, and ABCB1 genes) in mussels exposed to okadaic acid (OA) toxin to test the hypothesis that this specific group of marine toxins affects the expression of these genes.

3. Material and methods

3.1. Harvesting marine mussels and maintenance in aquarium

Wild mussels of the species *Mytilus galloprovincialis* were collected in Praia da Memória, located at latitude 41.23041568 and longitude -8.72195363, in Matosinhos (Porto, northern Portugal) (Fig. 6). This place is characterized by water temperatures varying between 13ºC and 17ºC, salinity between 30 and 35‰, and pH between 7.5 and 8.5 [70].

Adult mussels (size approximately 4-6 cm long) were harvested from the rocks (total of 152 animals) and transported in a thermal box containing water from their natural environment to the laboratory, where they were cleaned in order to remove material adhered to the shells and those that did not present external anomalies, were selected.

a) b)

Fig. 6. Praia da Memória a) Geographic location latitude 41.23041568 and longitude -8.72195363 (Source: Google Earth). B) Example of one of the strata from where mussels were collected at Praia da Memória.

3.2. Experimental design

Two distinct experimental trials were conducted to study the influence of the diet with green (non-toxic) algae *Tetraselmis* sp. and *Isochrysis* sp. (trial 1) or the effect of OA (trial 2) on the genes of interest.

In both trials, the animals underwent an acclimatization phase, lasting 2 weeks, which included feeding with a mixture of the microalgae *Tetraselmis* sp. and *Isochrysis* sp. algae at a concentration of 1.0 x 10^7 cells/L twice a day and renewing the seawater once a day. *Isochrysis* sp. (PhytoBloom ref. AADISS003) and *Tetraselmis* sp. (PhytoBloom AADTES003) was provided as freeze-dried material from Necton (Olhão, Portugal).

During this phase and throughout the trials, the temperature to which the animals were exposed varied between 16ºC and 17ºC. The temperature was controlled through a water-cooling system, using a large tank with water, and accommodating transparent glass flasks with the animals inside (Fig. 7). These flasks differed between trials: in the first trial, the total

capacity of the flasks was 4 L of water, while in the second trial, flasks with 600 mL capacity were used. The oxygenation of the systems was ensured throughout through air pumping.

a) b) c)

Fig. 7. Illustrative figure of the cooling and oxygenation systems used in both trials (A), as well as the different clear glass flasks used in each: B) 4 L clear glass flasks used in trial 1; C) 600 mL transparent glass flasks used in trial 2.

The water used was filtered natural seawater, received at CIIMAR, where it is treated to remove sediments and subsequently distributed to the laboratories, passing through sand filters. The detailed conditions of each trial are described below:

3.2.1. Influence of food intake on the expression of NR1J1, CYP and ABCB1 genes

Previous studies with human cell lines conducted at CIIMAR revealed that the cellular contents of the microalgae *Tetraselmis* sp. and *Isochrysis* sp. showed activating properties of *M. galloprovincialis* NR1J1 genes (personal communication). Thus, the first trial aimed to confirm the ability of microalgae content to activate mussel NR1J1 genes *in vivo* by feeding mussels with different densities of the algae *Tetraselmis* sp. and *Isochrysis* sp. for 5 days.

Experimental groups: 1- control without food (C); 2- *Tetraselmis* sp. intake at a concentration of 1 x 10^7 cell/L (T); 3- *Tetraselmis* sp. intake at a concentration of 5 x 10^7 cells/L (T+); and 4- *Isochrysis sp.* at a concentration of 1 x 10^7 cells/L (I). Each experimental group was replicated in three aquaria, each accommodating 6 animals, making a total of 72 mussels used.

Mussels were fed twice a day with the corresponding diets for each group. The gills and digestive glands of 3 mussels from each aquarium were sampled on the 3rd and 5th days of the trial. Tissues from animals from replicate aquaria were pooled, allowing overall 3 pooled biological samples per experimental group. Biological samples were stored in RNA*later* at -20ºC until processing.

3.2.2. Influence of OA on the expression of NR1J1, CYP and ABCB1 genes

Based on two studies on scallops *Argopecten irradians* [71,72], this trial aimed to study the effect of OA on *M. galloprovincialis* and NR1J1 genes expression. For this trial, 300 µg lyophilized OA (98% purity, Alfa Aesar) was previously dissolved in 1 mL of methanol (100%).

Considering the limitations in the amount of toxin available, this trial lasted 2 days, and fewer animals were used. Experimental groups: 1 – Control with food (mixture of microalgae *Tetraselmis* sp. and *Isochrysis* sp., C+); 2 - control without food (C); 3 - control with methanol (M); 4- group exposed to okadaic acid (OA). Each experimental group comprised three glass flasks with 500 mL seawater and 4 mussels per flask, making a total of 48 mussels used.

The group exposed to OA received approximately 148 µL of concentrated OA solution in methanol (100%), making up an OA final concentration of 100nM. The methanol final concentration to which mussels were exposed was 0.00027% (v/v). Okadaic acid was added to the water for two consecutive days. The gills and digestive glands of 2 mussels from each flask were sampled after 24 and 48 h of exposure to the compound and treated independently (no sample pools). Tissues were immersed in RNA*later* at -20ºC until processing. After completing both samplings, one sample from each experimental group was stored for chemical analysis.

3.3. Sample homogenization and RNA extraction

Small pieces of gill and digestive gland were cut to obtain approximately 30 mg of tissue, considering that tissue pools were maintained for the first test. They were then placed in 2 mL tubes containing 350 µL of NR buffer (NZYTech, Portugal) with 3,5µL of β-mercaptoethanol, and a 2 mm diameter sphere, and homogenized using Precellys 24 tissue homogenizer (Bertin Ins., France) with two cycles of 5400 rpm for 30 seconds.

After homogenization, the samples were centrifuged for 3 minutes at maximum speed to eliminate debris. The supernatant was collected in a new 1.5 mL tube, and the same volume of 70% ethanol was added. RNA extraction was performed using NZY Total RNA Isolation KIT (NZYTech, Portugal), following the manufacturer's instructions. The concentration and quality of RNA samples were evaluated with an absorbance method using the DeNovix DS-11 FX (DeNovix Inc. USA) [73].

3.4. cDNA synthesis and RT-qPCR

After equalizing the concentrations of all samples, cDNA was synthesized using the NZY First-Strand cDNA Synthesis Kit (NZYTech, Portugal), following the manufacturer's instructions [73].

Mytilus galloprovincialis-specific primers were designed for the four NR1J1 paralogs, three CYP450 genes, and the ABCB1 ortholog (Table 1). The efficiency and specificity of each primer pair were evaluated using seven 1:5 dilutions of a pool of samples in a CFX384 Touch™ Real-Time PCR Detection System (BioRad) and verifying the existence of a unique melting curve using the next amplification cycle: 10 min at 95º, 40x (15 s at 95º, 1 min at Tm of each primer), 1 min at 95º and melt curve evaluation from 60º to 95º with an increment of 0.5º for 5s. To this end, serial dilutions of samples were produced, and the slope of the Ct (cycle threshold) regression line was calculated with the relative cDNA concentration of this pool [73].

Quantitative PCR was then conducted with the tested primers, using CFX384 Touch™ Real-Time PCR Detection System (BioRad) under the following conditions: denaturation for 10 min at 95 ◦C, amplification by 40 cycles of 15 s at 95 ◦C and 60 s at 60 ◦C, melting curve evaluation 1 min at 95 ◦C, and increase of 0.5 ◦C each 30 s starting in 60 ◦C, end at 95 ◦C for 15 s. With a final volume of 10 µL, each well contained 5 µL of iTaq Universal SYBR

green supermix (Bio-Rad, USA), 0.3 µL of each primer, 2.4 µL of water and 2 µL of the sample. Each sample was present in duplicate on the plates [73].

Table 1. Specific primers used for qPCR.

Name	Primer	Sequence	Size	TmºC
ATP binding cassette subfamily B member 1 FM999809.2	F	CACCATAGCCGAGAACATCC	139	60
	R	CTCCACGCTCTCCAACTAG		
CYP3-like 1 VDI50017.1	F	GGAGGATTGATGAGTTGGGAGGA	141	62
	R	AGACGGCAAAAGAAAGAGTGGTC		
CYP3-like 2 VDI50771.1	F	TCAGTGCAATTGTCGTCGAAAGC	131	62
	R	TCATGGCGAGCGTTTAACATCAG		
CYP3-like 3 VDI64783.1	F	CCCAGGGAACACATTGGAAGTTTCT	137	65
	R	GTCTCCCCGCTGTGAGTGGATG		
NR1J1α VDI41431.1	F	CAAATCAACAAGAAGGAGAGAAACGA	175	62
	R	GGTCGGTCTGATGAAAACAGTTC		
NR1J1β VDI17689.1	F	AAACAGCAGTGGCTTGACCCA	201	61
	R	TGTAACATTTGGCCGATCGGG		
NR1J1γ VDI43945.1	F	CTGTCGGAATTGATCTCTCCAG	230	62
	R	ATCCTGCTGTTGTAAACCTGGTC		
NR1J1δ VDI45893.1	F	GGAACAAGAAGGATGGCGAAC	199	60
	R	ATTCTTTAATCCCACACTTTCAGG		
18S L33451.1	F	TCGATGGTACGTGATATGCCTA	151	60
	R	CGTCACTACCTCCCCGTGC		

3.5. Statistics

Using Bio-Rad CFX Maestro 1.0 Software (BioRad), relative gene expression was estimated based on the Pfaffl method, and the expression of target genes was normalized with the reference gene 18S expression. The level of significance used was $p<0.05$. [73]

With such a small number of samples per treatment, normality was assumed. Using SPSS v.27 (IBM, USA), a one-way ANOVA was performed with fold change values, followed by a Tukey post-hoc test to identify differences between means of different groups. The level of

significance used was $p<0.05$. JASP 0.17.0.0 was used to build violin plots, to observe the differences between all the experimental groups, and the samples from the same experimental group.

4. Results

4.1. Influence of the diet on the expression of NR1J1 genes

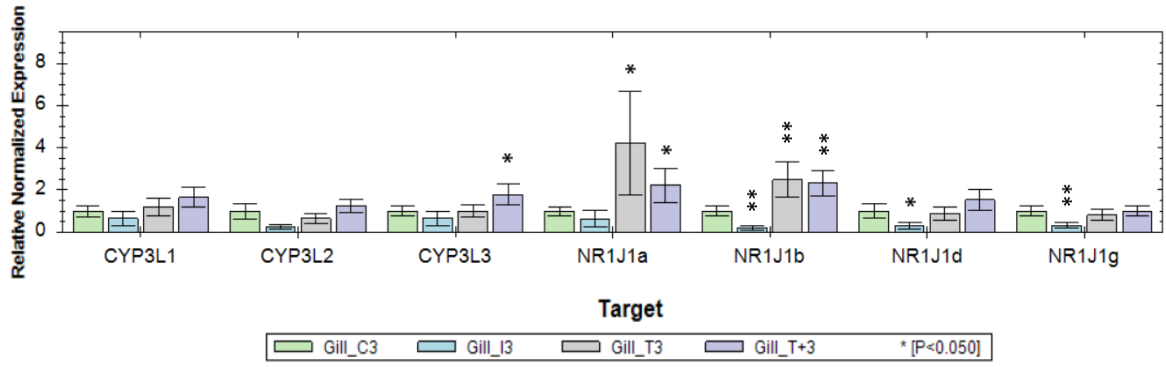
4.1.1. Gene expression in the gills

After 3 days of trial (Fig. 8A), CYP3L1 and CYP3L2 genes showed no significant expression differences in any treatment. Mussels fed with *Tetraselmis* sp. showed a significant increase in the expression of the CYP3L3 ($P = .0468$), NR1J1a ($P = .0237$), and NR1J1b ($P = .00466$) genes. Both concentrations of *Tetraselmis* sp. caused a significant effect on expression of NR1J1a and NR1J1b. However, only at the highest concentration of *Tetraselmis* sp. the CYP3L3 expression was significantly increased. In animals fed with *Isochrysis* sp., all genes were downregulated, namely in NR1J1b ($P = .0013$), NR1J1d ($P = .0334$), and NR1J1g ($P = .0079$).

On the 5th day of the trial (Fig. 8B), *Tetraselmis* sp. led to an increase in the expression of all genes, being, the highest *Tetraselmis* sp. dose responsible for significant differences in all of the genes. On the other hand, contrary to day 3, animals fed with *Isochrysis* sp. showed upregulation of all genes, except NR1J1d, the most significant being NR1J1a ($P = .0069$).

Except for NR1J1a, we can observe a significant increase in gene expression in samples treated with the higher concentration of *Tetraselmis* sp. between day 3 and day 5, and similar to the lowest concentration of *Tetraselmis* sp. at day 5 (Fig. 9). On the other hand, in mussels fed with *Isochrysis* sp., it is also possible to notice an increase in the expression of CYP3L1, CYP3L3, NR1J1b, NR1J1d, and NR1J1g, similar to the higher concentration of *Tetraselmis* sp. at day 5, but not significant between the two different days (Fig. 9).

A)



B)

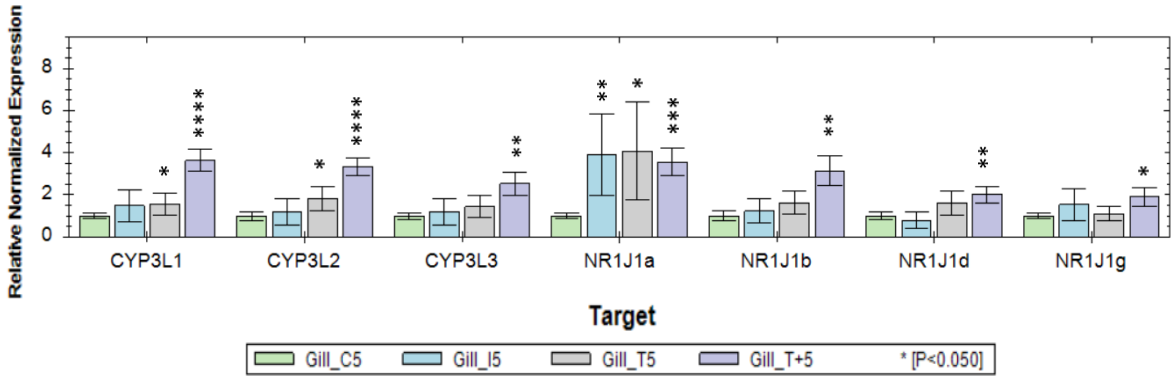


Fig. 8. Relative expression of *M. galloprovincialis* genes in gills, after 3 days (A) and 5 days (B) of trial. Values are reported in relation to control. The group represented by the letter C is relative to the control group (no diet), I is the group fed with *Isochrysis* sp.; and in turn T and T+, correspond to the groups of mussels fed lowest and highest concentrations of *Tetraselmis* sp., respectively. The values represented with the number 3 are the ones from the first sampling (day 3); the number 5 corresponds to the second sampling (day 5). Significant differences with regard to control are labeled with "*" (P<0.05).

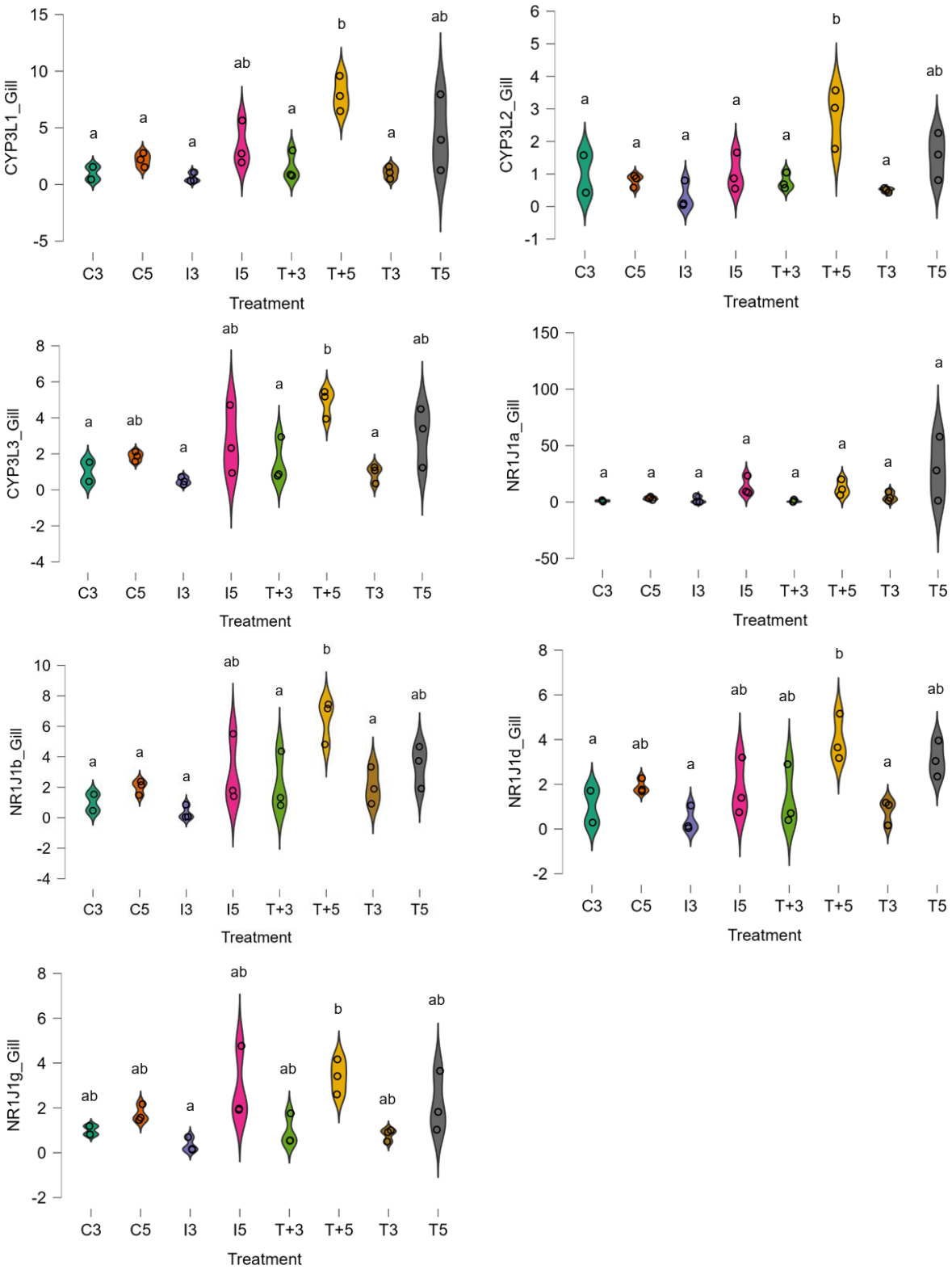


Fig. 9: Relative expression of *M. galloprovincialis* genes in gills, at day 3 and day 5. Values are reported in relation to control of the first sampling, at day 3. The group represented by

the letter C is relative to the control group (no diet); I is the group fed with *Isochrysis* sp.; T and T+, correspond to the mussels fed with the lowest and highest concentrations of *Tetraselmis* sp., respectively. The values represented with the number 3 are the ones from the first sampling (day 3); the number 5 corresponds to the second sampling (day 5). Significant differences with regard to control are labeled with "*" (P<0.05).

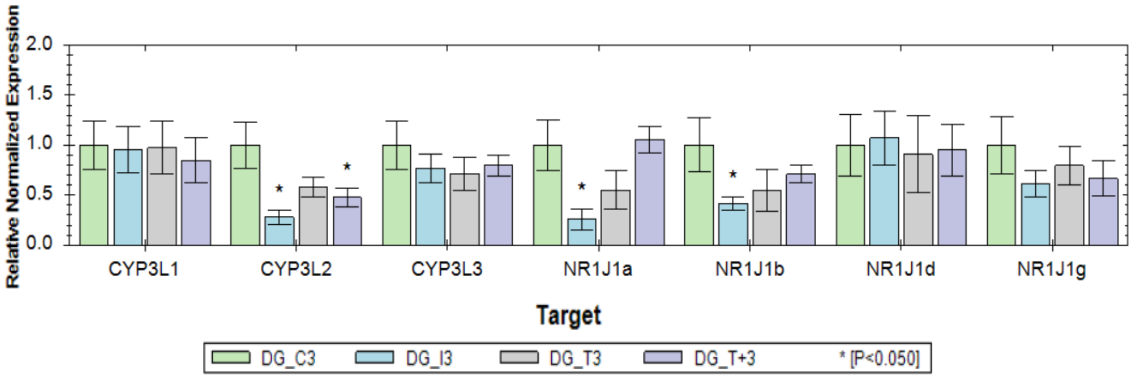
4.1.2. Gene expression in the digestive gland

After 3 days of trial (Fig. 10A), CYP3L1, CYP3L3, NR1J1d, and NR1J1g genes showed no significant differences in expression in any treatment. In mussels fed with *Isochrysis* sp., it is notable a down-regulation of genes CYP3L2 (P = .0057), NR1J1a (P = .0276), and NR1J1b (P = .0488). *Tetraselmis* sp. at the highest concentration only affected CYP3L2 (P = .0306) expression, and the low concentration had no significant effect.

After 5 days of trial (Fig. 10B), downregulation was observed in several genes. *Tetraselmis* sp. treatment decreased the expression of all genes in animals fed with lower concentrations of this alga: CYP3L1 (P = .0359), CYP3L2 (P = .0035), CYP3L3 (P = .0346), NR1J1a (P = .0159), NR1J1b (P = .0161), NR1J1d (P = .0237), and NR1J1g (P = .0219). The highest concentration affected the expression of CYP3L1 (P = .0033), CYP3L2 (P = .0284), CYP3L3 (P = .0405), and NR1J1b (P = .0101). In turn, *Isochrysis* sp. intake altered the expression of NR1J1a (P = .0021) and NR1J1b (P = .0164) genes.

In this tissue, no significant differences were observed between day 3 and day 5 (Fig. 11).

A)



B)

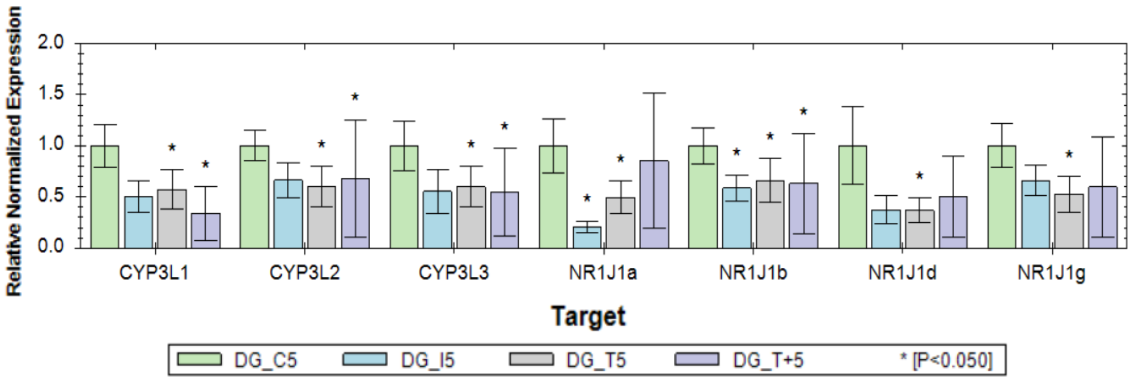
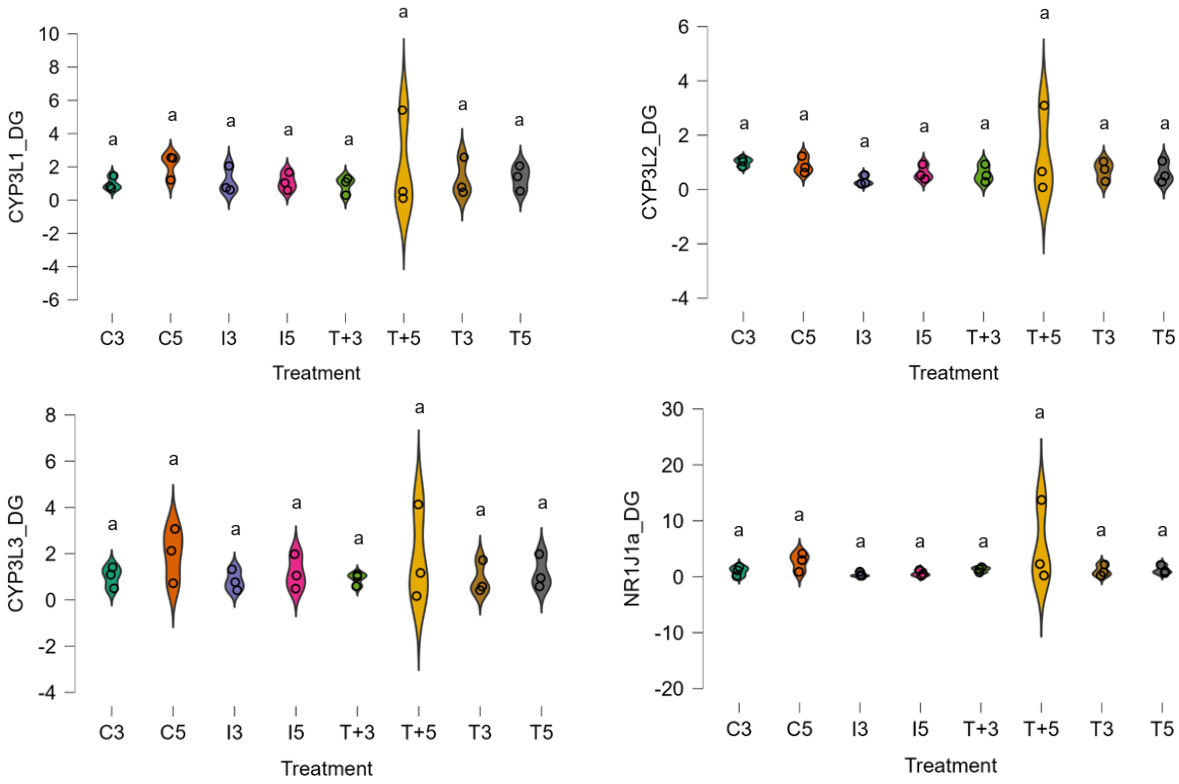


Fig. 10. Relative expression of *M. galloprovincialis* genes in digestive gland, after 3 days of trial (A) and 5 days of trial (B). The group represented by the letter C is relative to the control (no diet); I is the one fed with *Isochrysis* sp.; T and T+ correspond to the mussels fed with the lowest and highest concentrations of *Tetraselmis* sp., respectively. The values represented with the number 3 are the ones from the first sampling (day 3); the number 5 corresponds to the second sampling (day 5). Significant differences with regard to control are labeled with "*" (P<0.05).



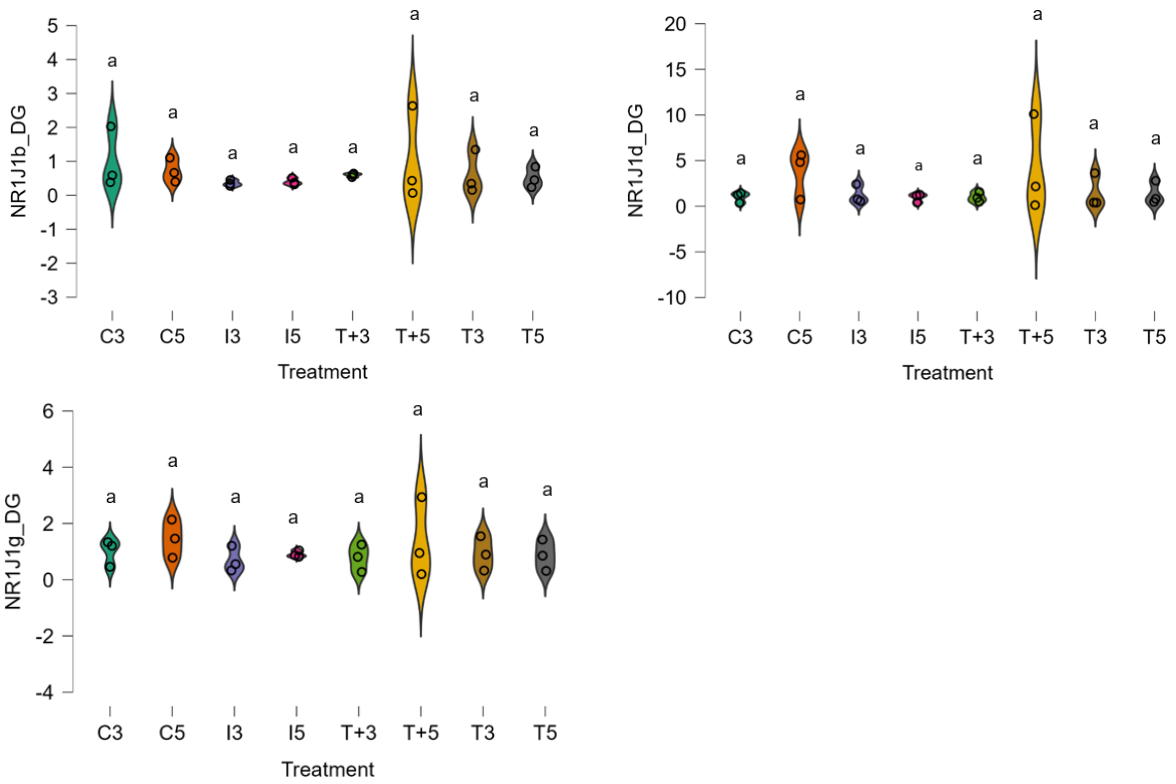


Fig. 11: Relative expression of *M. galloprovincialis* genes in digestive gland at day 3 and day 5. Values are reported in relation to control of the first sampling, at day 3. The group represented by the letter C is relative to the control (no diet); I is the one fed with *Isochrysis* sp.; T and T+ correspond to the mussels fed with the lowest and highest concentrations of *Tetraselmis* sp., respectively. The values represented with the number 3 are the ones from the first sampling (day 3); the number 5 corresponds to the second sampling (day 5). Significant differences with regard to control are labeled with "*" (P<0.05).

4.2. Influence of OA on the expression of NR1J1 genes

4.2.1. Gene expression in the gills

After 24 hours of trial (Fig. 12A), there were significant differences in the expression of all genes when expression levels were compared with the control (with no diet), except CYP3L3. In fact, animals fed with the microalgae mixture (*Tetraselmis* sp. and *Isochrysis* sp.) showed significant increases in the expression of the ABCB1 (P = 0,0362), NR1J1a (P

= .0034), NR1J1b (P = .0014), NR1J1d (P = .00002), and NR1J1g (P = .0095) genes. The presence of OA in the water caused significant effects on the expression of some genes: decreased the expression of CYP3L1 (P = .0009) and CYP3L2 (P = .0021), and increased NR1J1d (P = .0269) and NR1J1g (P = .0487). In turn, the group treated with methanol showed a downregulation of CYP3L2 (P = .0282).

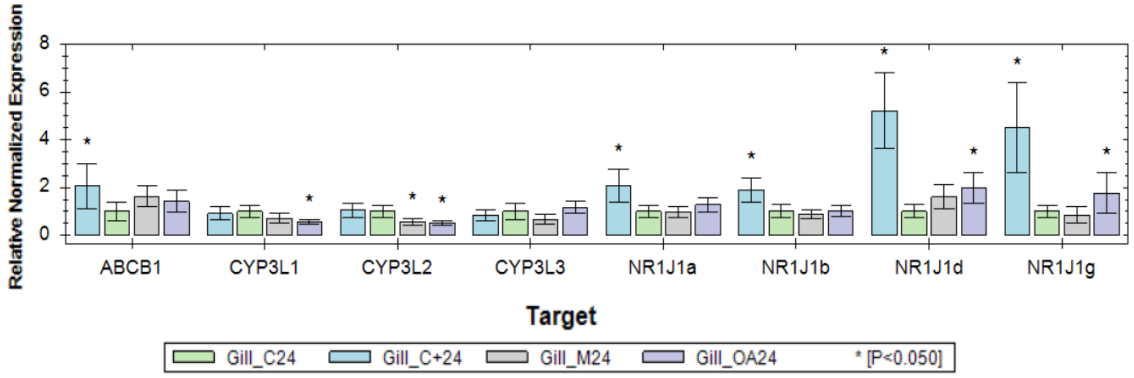
No significant differences in gene expression were found after 48 hours, compared to the control mussels (without diet) (Fig. 12B).

When considering the control and mussels fed with microalgae (C+), results were slightly different, and significant differences in gene expression were found in the group treated with methanol (Annex 1, Figure A1). In this analysis, methanol caused significant downregulation of CYP3L2 (P = .0221), NR1J1a (P = .0130), NR1J1b (P = .0004), NR1J1d (P = .0007), and NR1J1g (P = .0197). On the same note, OA significantly decreased the expression of CYP3L1 (P = .0277), CYP3L2 (P = .0023), NR1J1b (P = .0051), and NR1J1d (P = .0048), and ceased to have a significant effect on NR1J1g.

Another factor to consider is the effect of methanol. When assuming the group treated with methanol as the control (Annex 1, Figure A3), OA had no effect on the expression of any gene studied, neither at 24h nor at 48h. In fact, increased expression of genes could be observed at 24h in the groups fed (C+): CYP3L2 (P = .0220), NR1J1a (P = .0130), NR1J1b (P = .0004), NR1J1d (P = .0007) and NR1J1g (P = .0197). The groups that didn't received food (C) also significantly increased the expression of CYP3L2 (P = .0282). However, after 48h there were no significant differences in any gene.

Between 24h and 48h of treatment, there were no significant effects on gene expression (Fig. 13). Despite not being significant, it is possible to notice a decrease in the expression of NR1J1g and NR1J1d in the groups treated with the mixture of algae, methanol, and OA, between the two times. In these genes, the most significant differences were between the control with food and no food.

A)



B)

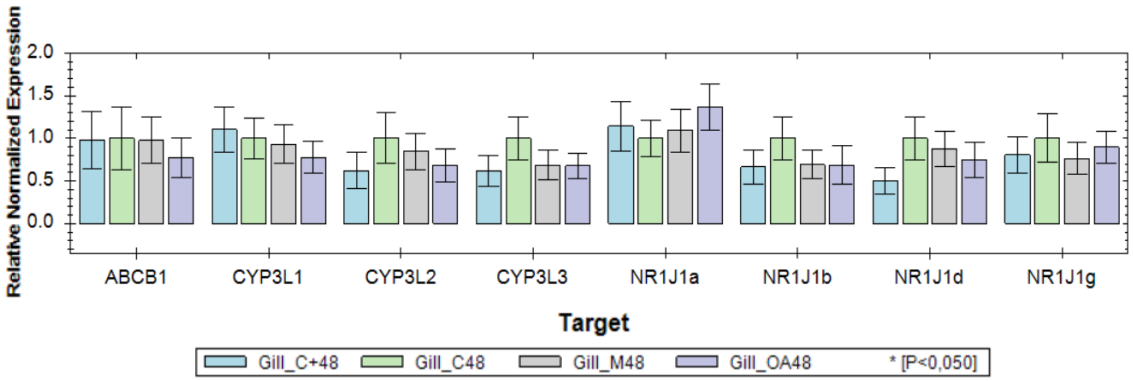


Fig. 12. Relative expression of *M. galloprovincialis* genes in gills, after 24 hours (A) and 48 hours (B). Values are reported in relation to control (C). The group represented by the letter C+ is the one fed with a mixture of *Isochrysis* sp. and *Tetraselmis* sp.; C is relative to control (no diet); M corresponds to the group treated with methanol; OA refers to the group treated with the toxin okadaic acid. The values represented with the number (24h) are the ones from the first sampling; the number (48h) corresponds to the second sampling. Significant differences with regard to control are labeled with "*" (P<0.05).

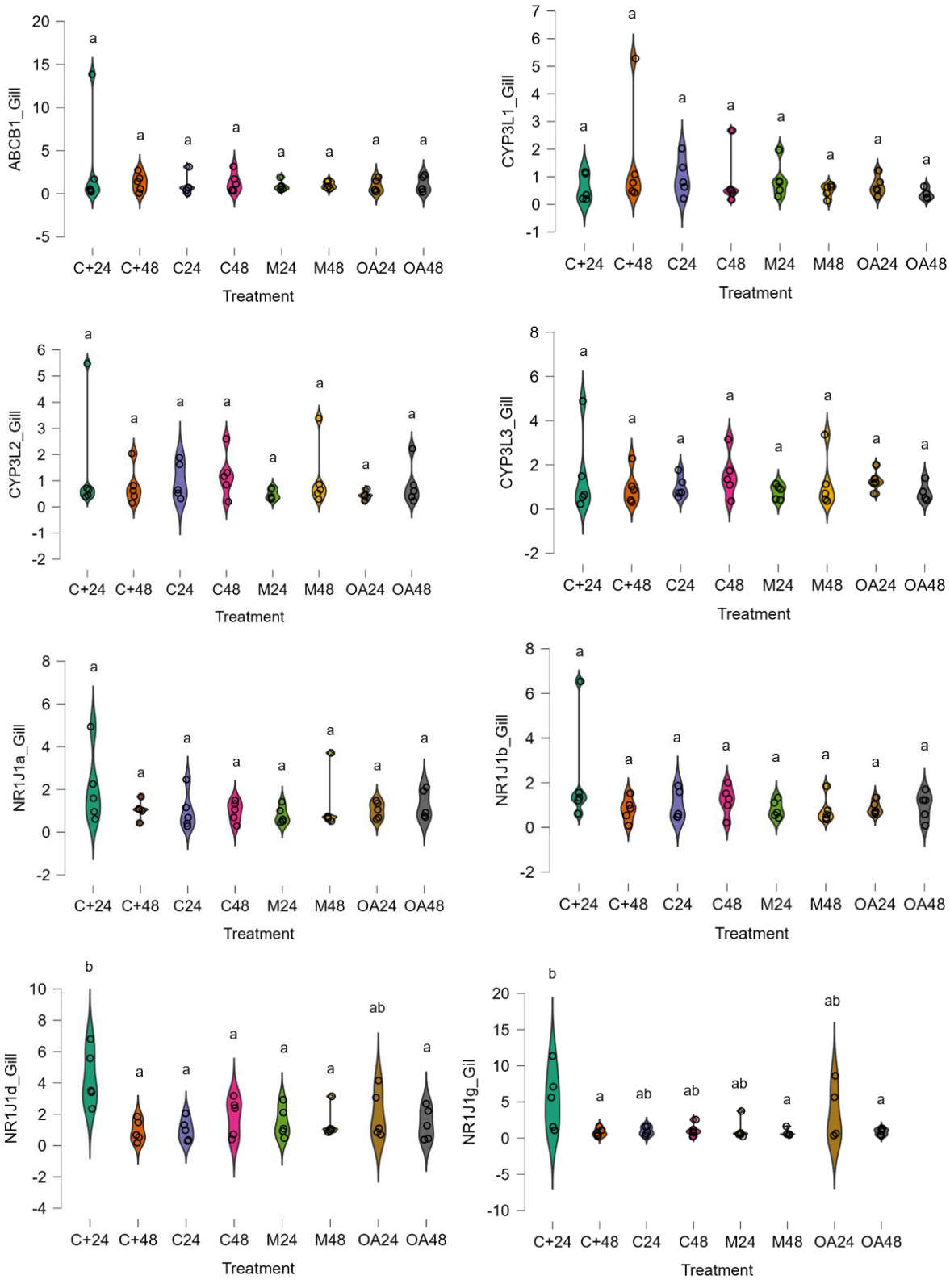


Fig. 13. Relative expression of *M. galloprovincialis* genes in gills, at 24h and 48 h. Values are reported in relation to control of the first sampling, at 24h. The group represented by the letter C+ is the one fed with *Isochrysis* sp*. and Tetraselmis* sp.; C is relative to the control

with no food; M corresponds to the groups treated with methanol; and OA to the group treated with okadaic acid. The values represented with the number 24 are the ones from the first sampling (24h); the number 48 corresponds to the second sampling (48h). Significant differences with regard to control are labeled with "*" (P<0.05).

4.2.2. Gene expression in the digestive gland

After 24 hours of trial (Fig.14A), comparisons with the control without diet (C) revealed significant differences in all genes, with OA and methanol exposure causing over-expression of all genes: OA affected ABCB1 (P = .0001), CYP3L1 (P = .0004), CYP3L2 (P = .0059), CYP3L3 (P = .0009), NR1J1a (P = .000005), NR1J1b (P = .0042), NR1J1d (P = .0018) and NR1J1g (P = .0089); methanol also affected ABCB1 (P = .00008), CYP3L1 (P = .0001), CYP3L2 (P = .0012), CYP3L3 (P = .0008), NR1J1a (P = .000001), NR1J1b (P = .0001), NR1J1d (P = .00007) and NR1J1g (P = .0002). The group fed with algae did not show significant differences in the expression of any of the genes studied.

After 48 hours of trial (Fig. 14B), CYP3L2 did not show significant differences in any of the treatments. Surprisingly, OA did not affect CYP3L3 and NR1J1d expression. In turn, the group fed with algae mixture was responsible for a significant increase in ABCB1 (P = .021) and NR1J1b (P = .0155) expressions.

On the other hand, the number of significant differences at 48h greatly varies when the experimental group fed microalgae is the control (C+) (Annex 1, Figure A2). No significant differences were observed in CYP3L2, CYP3L3, NR1J1d, and NR1J1g expressions. Exposure to methanol seemed to up-regulate CYP3L1 (P = .0209) and NR1J1a (P = .0012), and OA had the same effect on CYP3L1 (P = P = .0119) and NR1J1a (P = .0013), as methanol. In this analysis, the gene expression at 24h was similar to the results when considering the group with no food as the control. However, NR1J1d no longer had any significant differences, and OA did not affect ABCB1 and NR1J1g expressions.

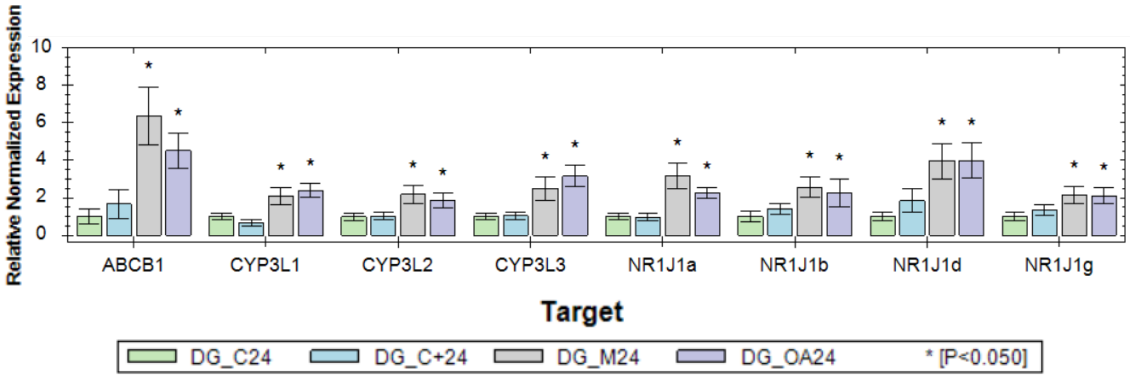
Using the group treated with methanol as the control, differences in gene expression are very few (Annex 1, Figure A4). At 24h, gene expression variations were observed in the groups fed and not fed microalgae (C and C+): C+ affected ABCB1 (P = .0105), CYP3L1 (P = .00002), CYP3L2 (P = .0022), CYP3L3 (P = .0021), NR1J1a (P = .000002), NR1J1b (P = .0041) and NR1J1g (P = .0436); and C affected ABCB1 (P = .00008), CYP3L1 (P = .0001), CYP3L2 (P = .0012), CYP3L3 (P = .0008), NR1J1a (P = .000001), NR1J1b (P =

.000009), NR1J1d (P = .00007) and NR1J1g (P = .000193). OA only affected the expression of NR1J1a (P = .0168) at 24h. At 48h, differences could be observed for genes CYP3L1 (P = .0209) and NR1J1a (P = .0013) in the group fed microalgae (C+). Furthermore, differences in all genes were observed in group (C): ABCB1 (P = .0028), CYP3L1 (P = .0009), CYP3L3 (P = .0004), NR1J1a (P = .0011), NR1J1b (P = .0013), NR1J1d (P = .0385), and NR1J1g (P = .0187) (Annex 1, Fig. A4).

Between the two different sampling times, there were no significant effects in the gene expression (Fig. 15).

Despite not being significant, it is possible to notice a decrease in the expression of NR1J1a in the groups treated with the mixture of algae and methanol at 48h. The groups treated with OA showed an increase in the expression of this gene, although also not significant. In this case, the most significant difference was between the control with no food and methanol, at the same time of sampling (Fig. 15).

A)



B)

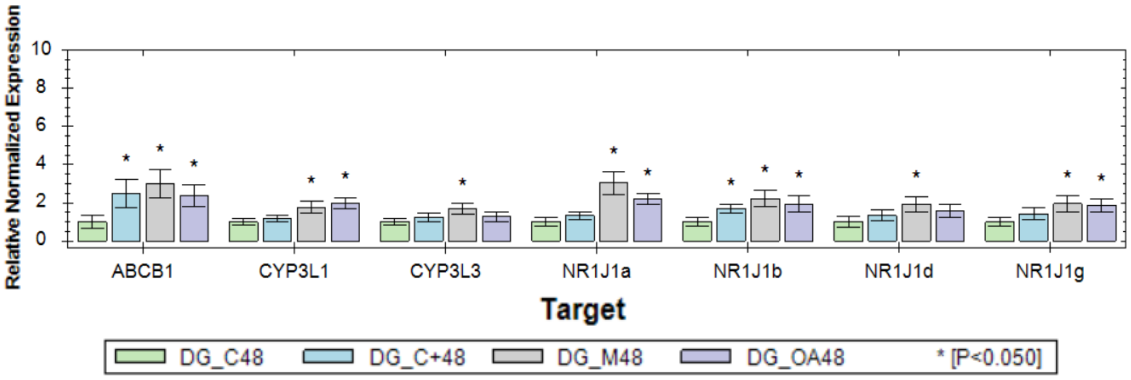
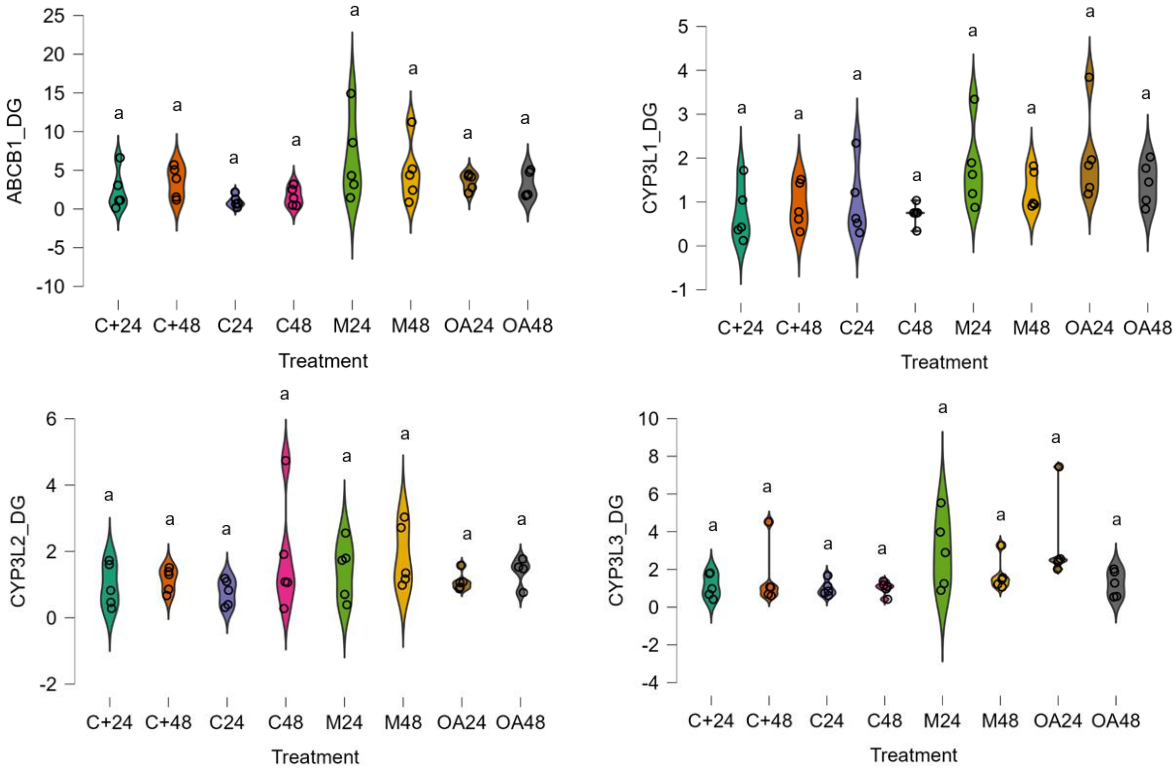


Fig. 14. Relative expression of *M. galloprovincialis* genes in digestive gland, after 24 hours (A) and 48 hours (B). Values are reported in relation to control (C). The group represented by the letter C+ is the one fed with a mixture of *Isochrysis* sp. and *Tetraselmis* sp.; C is relative to control (no diet); M corresponds to the group treated with methanol; OA refers to the group treated with the toxin okadaic acid. The values represented with the number 24 are the ones from the first sampling (24h); the number 48 corresponds to the second sampling (48h). Significant differences with regard to control are labeled with "*" (P<0.05).



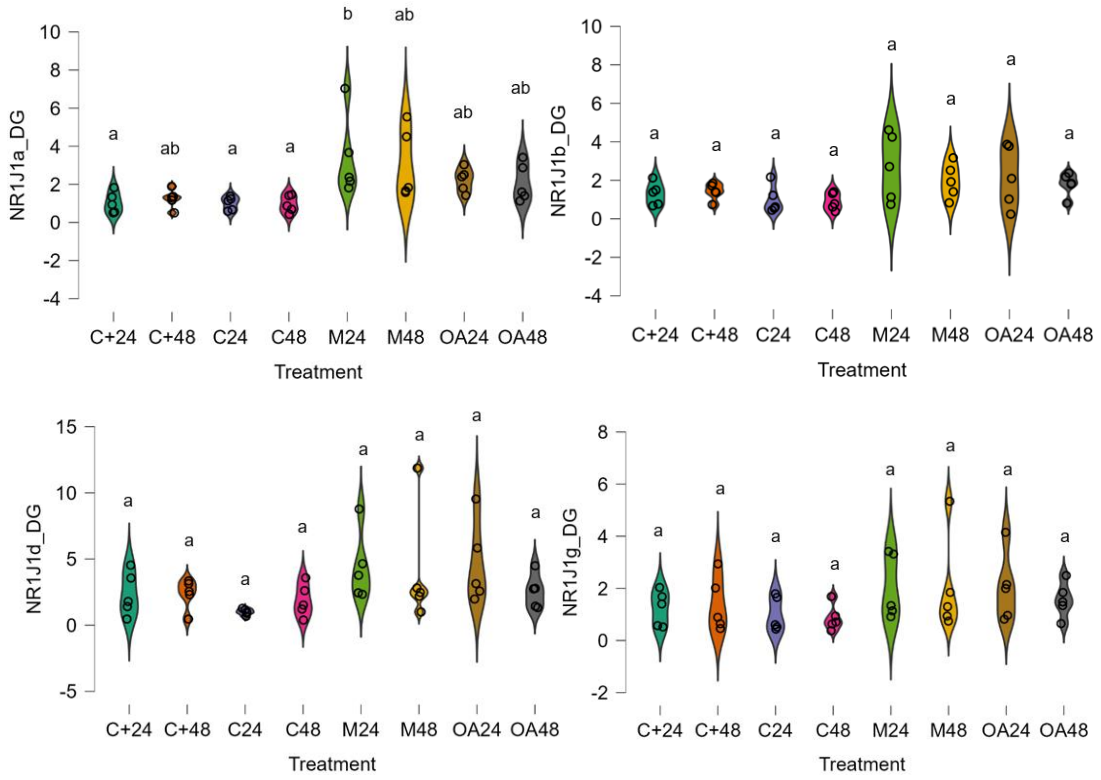


Fig. 15. Relative expression of *M. galloprovincialis* genes in digestive gland, at 24h and 48 h. Values are reported in relation to control of the first sampling, at 24h. The group represented by the letter C+ is the one fed with *Isochrysis* sp. and *Tetraselmis* sp.; C is relative to the control with no food; M corresponds to the groups treated with methanol; and OA to the ones treated with okadaic acid. The values represented with the number 24 are the ones from the first sampling (24 h); the number 48 corresponds to the second sampling (48 h).

5. Discussion

Okadaic acid is a toxin produced by dinoflagellates from the genera *Prorocentrum* and *Dinophysis*. This toxin and its derivatives (dinophysis toxins, DTX) accumulate in shellfish and cause diarrhetic shellfish poisoning in humans. Okadaic acid is a potent inhibitor of serine/threonine phosphatases (PP1 and PP2A), being cytotoxic in a variety of cell lines and causing gastrointestinal diseases in humans [18,22,31]. *M. galloprovincialis* is a highly

consumed shellfish, contributing to overcoming the high demand for fish and seafood for consumption. However, this species can sometimes accumulate toxins like OA, becoming contaminated. The depuration of shellfish to reduce the toxin accumulated has been shown to require long periods and to be not feasible at the industrial scale. In this regard, a better understanding of defensome composition and functions in this group of animals and DST detoxification pathways could lead to innovative depuration strategies, more efficient and suitable for the aquaculture industry [5,8].

Gills and digestive gland are the most commonly studied organs when it comes to detoxification processes because of their functions and characteristics. In fact, the gills are the main interface between the organism and the environment, which increases the probability of being in contact with water pollutants, such as toxins. On the other hand, the digestive gland is the major detoxification site because of the high expression of phase I and phase II xenobiotic metabolizing enzymes [74].

That being said, the objective of this work was to investigate the expression of NR1J1 family genes and genes involved in detoxification, regulated by the first, studying the effect of okadaic acid and mussel feeding *Tetraselmis* sp. and *Isochrysis* sp.

It is important to mention that the effect of the microalgae and okadaic acid in the expression of the referred genes, could have been influenced by the nature of both, since the interaction with organs depends on the size and composition of the substances. In fact, in the first trial, mussels were not exposed to any chemical substance, instead were fed 2 different microalgae cells. Thus, any response caused by these microalgae, must be associated with the processes of ingestion and digestion that occur in the digestive gland. In the second trial, toxin OA was dissolved in water, which can be readily uptaken by the organism, in the gills and digestive gland [75].

In the natural environment, gills are sensory organs to both particles and compounds in suspension or dissolved in water, performing a selective filtration of particles. In this work, gills seemed to be more sensitive to the microalgae particles, than the dissolved OA. This response could be related with the mechanism of particle selection and rejection. [76]

On the other hand, OA could have been assimilated preferentially in the digestive gland since the effect of OA on gene expression increased in this organ.

5.1. Effects of diet on gene expression

Tetraselmis sp. and *Isochrysis* sp. are part of a solution to eliminate toxins by feeding them to contaminated animals, accelerating metabolic mechanisms, and expelling the OA through fecal matter [9,39]. Studies have shown that nutritional regime (microalgae, commercial paste of microalgae) and temperature treatment could improve the elimination of the lipophilic toxin okadaic acid in the naturally contaminated wedge shell *Donax trunculus* [39]. In another study with *M. edulis*, feeding experiments with the diatom *Skeletonema costatum* demonstrated to be important for DSP toxins depuration, with detoxification rates of total OA improving from 60% to 90% [77].

In this study, each alga extract had a different effect on the expression of the studied genes, depending on the time and concentration.

The digestive gland is the organ where food is processed and digested and is also where most of the biotransformation and detoxification reactions occur in bivalves [18,78]. It is thus an organ rich in phase I, II and III xenobiotic metabolizing enzymes. Despite this, in the present work, a downregulation of NR1J1 and phase I enzymes was observed in all the groups treated with *Isochrysis* sp. and the two concentrations of *Tetraselmis* sp.. The decrease of genes involved in toxin biotransformation has already been reported in another study, where the activity of glutathione s-transferase (GST), decreased, in the digestive gland, in the presence of electrophiles [74].

It is also necessary to notice that, in this first trial, the values of gene expression in the digestive gland are much lower than the ones in the gills.

Furthermore, the results reveal the importance of the gills in conducting xenobiotic metabolism reactions. This organ was very sensitive to the intake of microalgae, being able to activate the expression of several NR1J1 members and phase I genes, although the effect was more significant in groups treated with the two concentrations of *Tetraselmis* sp., than the one with *Isochrysis* sp.. The ability of this organ to carry out detoxification reactions was reported in other studies, where gills exhibited rapid upregulation of genes related to biotransformation, preventing the chemical burden on other target tissues, such as, the digestive gland [74,79].

Regarding NR1J1 expression in the gills, our results are consistent with another work addressing gene expression of three NR1J homologous (NR1J1, NR1J3, and NR1J4) in *M. galloprovincialis* against exposure to pharmaceuticals, which concluded that exposure to those drugs strongly and frequently affected the expression of NR1J1 genes [45]. To the

best of our knowledge, this is the only published work confirming the expression *in vivo* of NR1J-like genes in bivalve mollusks.

Although the mechanisms underlying the activation of NR1J1 were not confirmed in this study, it could have occurred during the interaction and recognition of microalgae by gill epithelial cells, triggering molecular signals to activate gene transcription and initiating a defense response to external (potentially toxic) substances.

In general, we can observe that the presence of *Tetraselmis* sp. and *Isochrysis* sp. influence gene expression, i.e., whenever there is an increase or decrease in the expression of NR1J1 genes, there is also the same expression pattern in phase I genes. Thus, considering the function of NR1Is in vertebrates, which regulate the expression of CYPs, and the results of the present work, we can infer that this function can be conserved and the transcriptions factors NR1J1 can also regulate the expression of the phase I genes [31,45]. It is also interesting to notice that, despite all the changes in the expression of NR1J1 genes being accompanied by changes in the expression of phase I genes, NR1J1a and NR1J1b seemed to be the ones with the most significant effect.

The present results bring some clarification on the mechanisms underlying the effects of food supply, demonstrating their importance as drivers for the activation of several xenobiotic detoxification reactions.

5.2. OA effects on gene expression

In this study, the presence of OA in the water caused significant organ-dependent effects on the expression of some genes. However, we must consider that these effects can be influenced by methanol, which was used to dissolve OA.

In general, contrary to what happened in the first trial, it can be noticed that the effect was higher in the digestive gland rather than the gills. These results could be related to the functions of the organs, namely the digestive gland, that is known to perform the biotransformation and elimination of toxicants [18]. Gills seemed to be more sensitive to microalgae, whereas the digestive gland was more sensitive to OA, considering the number of genes that were differentially expressed in the two organs. Furthermore, we can also conclude that methanol, although present in very low amounts (0.00027%, v/v), significantly

affects gene expression. When the effect of methanol is considered, the only effect of OA that stand out is the upregulation of NR1J1a gene.

The expression levels of CYP3 members appeared to be lower in the gills. This is consistent with a study in *M. edulis*, where CYP3-like genes showed higher expression in the digestive gland [62]. Although the CYP3-like genes were upregulated in this tissue, the expression levels dropped after 48h, which may indicate that the increase observed in the early hours was an effective defense response against OA, emphasizing the involvement of this phase I genes in early DSP toxin metabolism [61]. The difference between the expression levels of CYP3-like genes in gills and digestive glands could indicate that the expression of these genes is organ-specific [61].

ABCB1 was upregulated by OA in the digestive gland and in the gills, although in this last tissue, the difference was not significant, which is consistent with studies showing this level of upregulation in both tissues [65,80]. Like the CYP3 members, ABCB1 expression also appears to be tissue-specific, depending on the capacity of absorption, metabolism, and elimination of toxic compounds of each tissue [65]. It is interesting to notice the high levels of ABCB1 expressed at 24h, which could be evidence of the important role that these genes have in OA efflux in the tissues tested in the early stages of contamination since they constitute the first line of defense against toxicants [40,80].

In the gills, the mixture of the two algae also had interesting effects on the expression of the genes: it was responsible for an upregulation of all NR1J1 genes, accompanied by an upregulation of ABCB1. These results could be related to the characteristics of this tissue as the central interface between the organism and the environment and agree with ABCB1 being the first line of defense against toxins, such as OA [40,74].

Shellfish contamination studies with OA or OA-producing alga (*Prorocentrum lima*) have shown that this molecule triggers several molecular responses and alterations in phases I, II, and III xenobiotic-metabolizing enzymes. Variations in the biotransformation phase II enzyme glutathione s-transferase (GST) family members were reported in gills and digestive gland from *Crassostrea ariakensis* [81]. Alterations in the expression patterns of CYP450 have been described in gills and digestive gland from *Perna viridis* exposed to *P. lima* cells to be dependent on the CYP isoform, organ, and *P. lima* cell density [61]. P-glycoprotein (ABCB1) was also over-expressed in the gills of *P. viridis* exposed to the OA-producing *P. lima* cells. The authors advance the hypothesis that this protein could be involved in the resistance mechanisms to DSP toxins [69]. Furthermore, P-glycoprotein showed species and organ-specific expression patterns (*R. philippinarum*, *S. subcrenata,* and *T. granosa*) [65].

In general, the expression of NR1J1 members is upregulated in the gills and digestive gland by OA, being much higher in the digestive gland and consistent with the results obtained for CYP3-like members and ABCB1. Despite the expression NR1J1d and NR1J1g were highly increased in gills, all the NR1J1 genes were significantly altered in the digestive gland. Furthermore, alterations in NR1J1 gene expressions were followed by an alteration in the expression patterns of CYPs and ABCB1 genes, which could be evidence that these NRs are involved in the biotransformation and elimination of toxicants through [31,45]. Hence, considering the role of NR1I members in regulating gene expression of phase I and phase II genes in vertebrates, we can infer that this function can be conserved in mussels, and thus, NR1J1 transcription factors can also regulate the transcription of biotransformation genes.

Although the results of this study corroborate previous studies, it should be borne in mind that the experimental conditions differ considerably from study to study, and animals are not exposed to the same doses of OA toxin.

The main focus of this study was the chemic defensome, centralized in the biotransformation and elimination of toxins and other compounds. However, the effects observed in the expression of the tested genes, lead us to believe that other pathways might have also been influenced. It has been reported, in other studies, that PXR and CAR are involved in modulating energy metabolism. In fact, along with their role in biotransformation and elimination of substances, like toxins, these NR can also crosstalk with other hormone responsive transcription factors, affecting the metabolism of fatty acids, lipids and glucose, and resulting in an influence in energy metabolism, necessary to the good function of the organism. [82,83]

Considering the ortholog relation between NR1J1 and NR1Is, and this function of NR1Is in vertebrates, which are involved in the activation of other pathways, and the results of the present work, we can infer that this function can be conserved and the transcriptions factors NR1J1 may also have a role in the regulation of energy metabolism [45,82,83].

The effects observed in the gene expression in the gills and digestive gland, in this study, could also be related to the mechanisms of respiration and digestion, that happen in these two tissues, respectively, since they are involved in the energy production, and there is the need to anticipate the amount of energy produced, used in the digestion. In fact, because of its functions, the digestive gland shows a complexity in its metabolic pathways, and it has also been reported that feeding and digestion activities in mussels, such as *M. edulis*, consume around 20% of the mussel metabolic energy [84,85].

The limitations in time, amount of toxin available and number of animals affected the second experimental trial. Regarding this trial, it would be interesting to evaluate the uptake of OA and its quantification in the different tissues and also analyse the esterification rate of the toxin. With the present results, and bearing in mind the experimental groups, and the controls used, we can infer that the effects observed were consequence of the dissolved OA, in contact with the mussels, but it is necessary to evaluate the assimilation of this toxin.

6. Conclusions

In the present study, the influence of food intake and OA contamination in the expression of NR1J1 genes, CYP3-like members, and ABCB1 was investigated in mussels. The presence of *Tetraselmis* sp. and *Isochrysis* sp. had a higher influence on the expression of these genes in the gills, which is consistent with other studies. Considering the number of genes that were differentially expressed, gills were more sensitive to microalgae, whereas the digestive gland was more sensitive to OA. The presence of OA and methanol was responsible for more significant alterations in gene expression in the digestive gland than in the gills, possibly due to the functions of this organ in the biotransformation and elimination of toxicants. Both diet and OA influenced the expression of the tested genes, which brings evidence that the regulation of phase I, II, and III genes by the transcription factors NR1Is in vertebrates can be a conserved function of NR1J1 in mussels.

These results demonstrate that food supply and microalgae intake can accelerate the metabolism and elimination of biotoxins, such as OA, in mussels. Nevertheless, considering the complexity of OA metabolism and its toxicity, and the complexity of the defensome system in mussels, further studies are warranted to functionally dissect the mechanistic role of the genes involved in OA metabolism and detoxification in bivalves.

7. References

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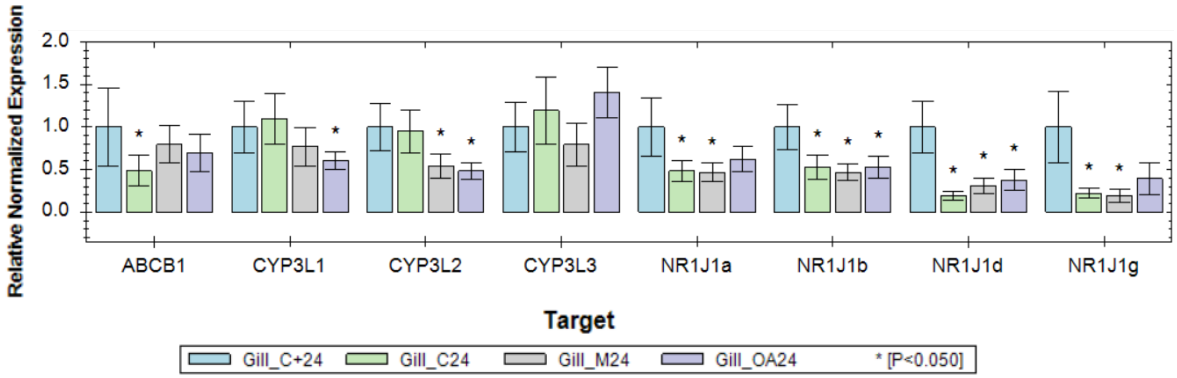
ANNEX 1

1. Influence of OA on the expression of NR1J1 genes (compared to the control with food)

1.1. Gene expression in the gills

A)



B)

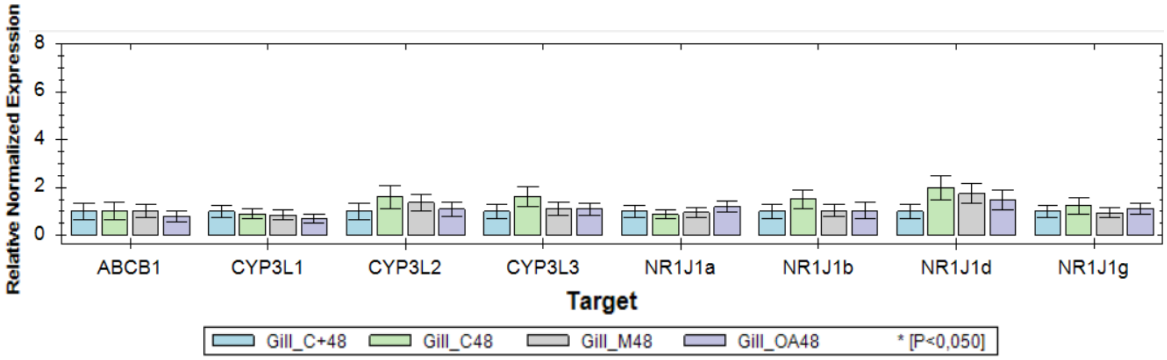
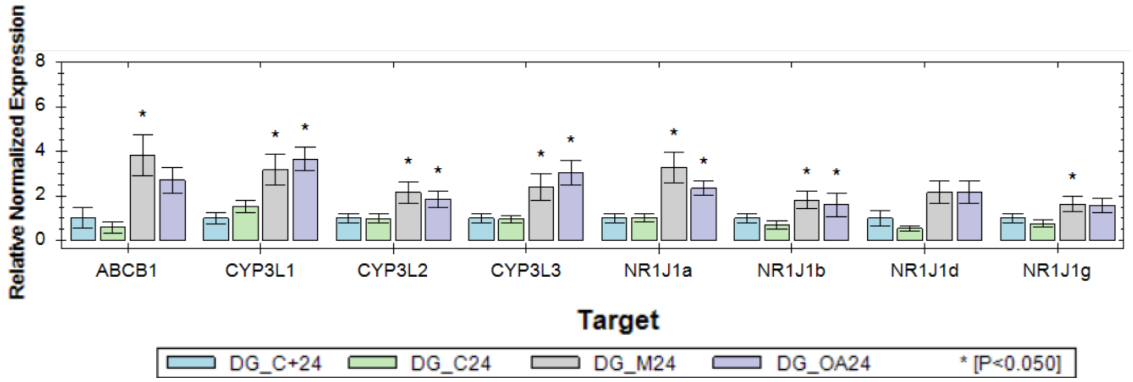


Figure A1. Relative expression of *M. galloprovincialis* genes in gills, after 24h of trial (A) and 48h of trial (B). Values are reported in relation to control with food. The group represented by the letter C+ is the one fed with *Isochrysis* sp. and *Tetraselmis sp.*; C is relative to the group with no diet; M corresponds to the groups treated with methanol; and OA to the one with okadaic acid. The values represented with the number 24 are the ones from the first

sampling (24h); the number 48 corresponds to the second sampling (48h). Significant differences with regard to control are labeled with "*" (P<0.05).

1.2. Gene expression in the digestive gland

A)



B)

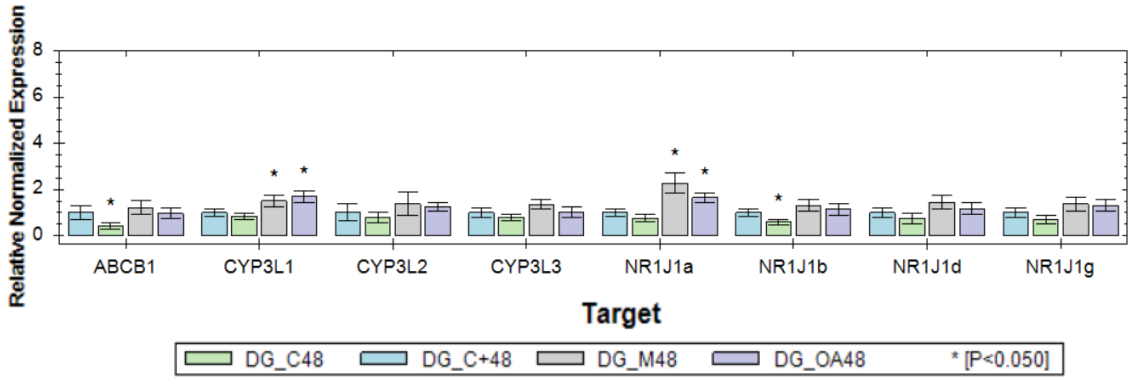
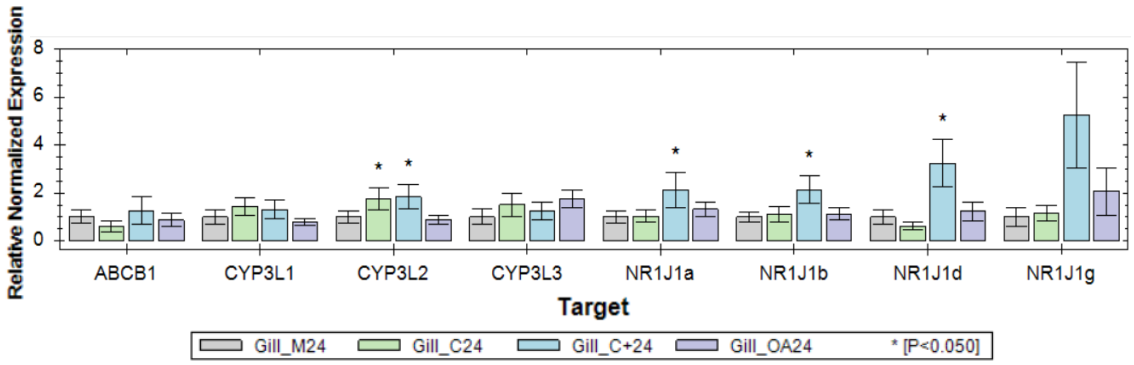


Figure A2. Relative expression of *M. galloprovincialis* genes in digestive gland, after 24h of trial (A) and 48h of trial (B). Values are reported in relation to control with food. The group represented by the letter C+ is the one fed with *Isochrysis* sp. and *Tetraselmis sp.*; C is relative to the group with no diet; M corresponds to the groups treated with methanol; and OA to the one with okadaic acid. The values represented with the number 24 are the ones from the first sampling (24h); the number 48 corresponds to the second sampling (48h). Significant differences with regard to control are labeled with "*" (P<0.05).

2. Influence of OA on the expression of NR1J1 genes (compared to the control with methanol)

2.1 Gene expression in the gills

A)



B)

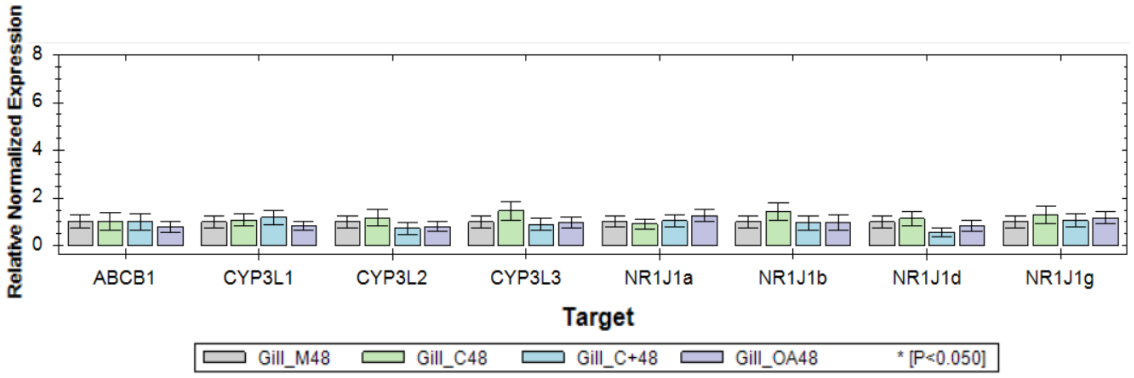
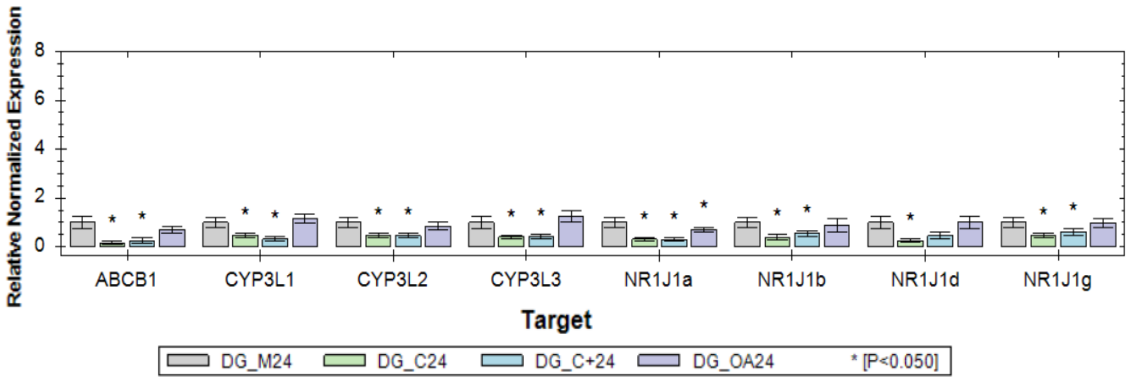


Figure A3. Relative expression of *M. galloprovincialis* genes in gills, after 24h of trial (A) and 48h of trial (B). Values are reported in relation to control with methanol. The group represented by the letter C+ is the one fed with *Isochrysis* sp. and *Tetraselmis sp.*; C is relative to the group with no diet; M corresponds to the groups treated with methanol; and OA to the one with okadaic acid. The values represented with the number 24 are the ones from the first sampling (24h); the number 48 corresponds to the second sampling (48h). Significant differences with regard to control are labeled with "*" (P<0.05).

2.2 Gene expression in the digestive gland

A)



B)

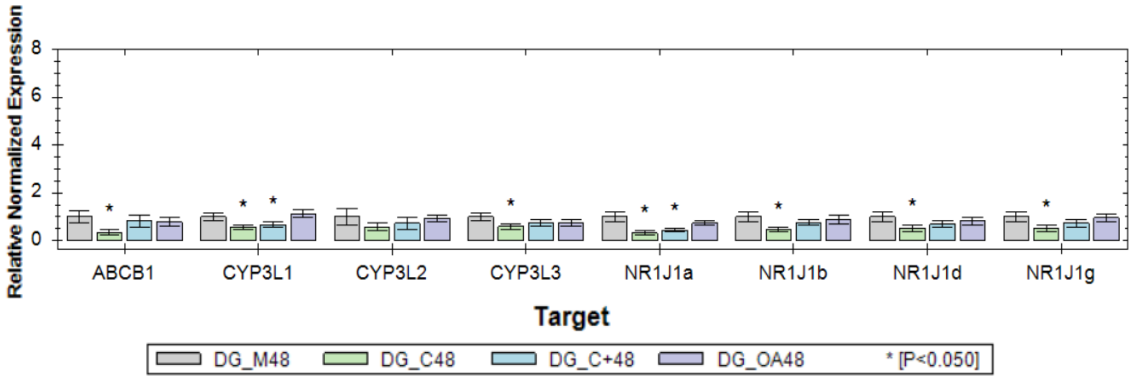


Figure A4. Relative expression of *M. galloprovincialis* genes in gills, after 24h of trial (A) and 48h of trial (B). Values are reported in relation to control with methanol. The group represented by the letter C+ is the one fed with *Isochrysis* sp. and *Tetraselmis sp.*; C is relative to the group with no diet; M corresponds to the groups treated with methanol; and OA to the one with okadaic acid. The values represented with the number 24 are the ones from the first sampling (24h); the number 48 corresponds to the second sampling (48h). Significant differences with regard to control are labeled with "*" (P<0.05).