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FACULTAD DE CIENCIAS DEL MAR Y AMBIENTALES. DEPARTAMENTO DE QUÍMICA FÍSICA
INSTITUTO DE INVESTIGACIÓN MARINA (INMAR)

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Ecotoxicological assessment of galaxolide and tonalide as contaminants of emerging concern in marine ecosystems

Evaluación ecotoxicológica de la galaxolida y la tonalida como contaminantes de interés emergente en ecosistemas marinos

TESIS DOCTORAL

Programa de Doctorado en Gestión y Conservación del Mar

Friday Ojie Ehiguese

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HACEN CONSTAR:

Que el trabajo recogido en la presente Memoria titulada "Evaluación ecotoxicológica de la galaxolida y la tonalida como contaminantes de interés emergente en ecosistemas marinos", presentada por D. Friday Ojie Ehiguese ha sido realizado bajo su dirección y autorizan su presentación y defensa para optar al Grado de Doctor por la Universidad de Cádiz.

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Abstract

The polycyclic musk compounds Galaxolide (HHCB) and Tonalide (AHTN) are the most frequently consumed fragrance materials applied in many household and personal care products. These substances have been detected in all environmental compartments and, due to their inherent lipophilicity, they easily bioaccumulate in aquatic organism. The aim of this PhD thesis was to assess the risk of environmental concentrations of HHCB and AHTN in the marine environments. In order to provide a more complete assessment on marine biota, empirical approach based on data of ecotoxicological assays was explored based on environmental risk assessment (ERA) scheme. In this case, it was intended to employ organisms from different trophic levels like microalgae, echinoderms, bivalves, shrimps, and fish. This diversification in the species allows assessing how organisms with different biological complexity can be affected. In addition, different endpoints were used for each species, taking as basis the sensitivity and relevance of the endpoints. Another important aspect considered to select the endpoints was their level of biological organization, so that the responses measured were based on acute and chronic exposure, focusing on growth, survival, development, behaviour, and biomarkers at the biochemical and molecular levels.

The results obtained in this thesis show that environmental concentrations of both compounds are detrimental to microalgae growth posing significant risk to *Phaeodactylum tricornutum* and *Isochrysis galbana*. It was also observed that environmental concentrations of HHCB and AHTN significantly altered the early life stages endpoints such as fertilization, larval development, and survival in tested species. Furthermore, this thesis has proven that in a scenario of heterogenous pollution where lethality is not expected to occur, HHCB and AHTN may trigger spatial avoidance, which might reduce the local biodiversity of ecosystems due to emigration to safer environments. Sublethal effects assessed in bivalves after chronic exposure to HHCB and AHTN showed that these substances are bioavailable to marine organisms and they have the potential to induce oxidative stress, genotoxicity, neurotoxicity and alter the health status of marine organisms. It was also observed that both compounds modulated endocrine disruption biomarkers in small fish. Despite the significant alterations in endocrine disruption biomarkers induced by both compounds, AHTN appeared to be a more potent inhibitor of endocrine activity in the marine environments.

This PhD Thesis has demonstrated the adverse effects of environmental concentrations of HHCB and AHTN in marine ecosystems. Therefore, the data presented in this study should be integrated with other available data required for policy actions that will aid the conservation and management of the sea.

Resumen

Los compuestos de almizcle policíclico galaxolida (HHCB) y tonalida (AHTN) son los componentes de azmicles que se aplican con más frecuencia en muchos productos domésticos y de cuidado personal. Estas sustancias se han detectado en todos los compartimentos ambientales y, debido a su lipofilicidad inherente, se bioacumulan fácilmente en organismos acuáticos. El objetivo de esta tesis doctoral fue evaluar el riesgo de concentraciones ambientales de HHCB y AHTN en los ambientes marinos. Con el fin de proporcionar una evaluación más completa de la biota marina, se exploró un enfoque empírico basado en datos de ensayos ecotoxicológicos siguiendo el esquema de evaluación de riesgos ambientales (ERA). Se emplearon organismos de diferentes niveles tróficos como microalgas, equinodermos, bivalvos, camarones y peces, para evaluar cómo organismos con diferente complejidad biológica podrían verse afectados. Otro aspecto importante considerado a la hora de determinar las respuestas a tiempo final fue su nivel de organización biológica, por lo que las respuestas medidas se basaron en la exposición aguda y crónica, enfocándose en el crecimiento, supervivencia, desarrollo, comportamiento, biomarcadores a nivel bioquímico y molecular.

Los resultados obtenidos muestran que la concentración ambiental de ambos compuestos es perjudicial para el crecimiento de microalgas, lo que representa un riesgo significativo para *Phaeodactylum tricornutum* e *Isochrysis galbana*. También se observó que las concentraciones ambientales de HHCB y AHTN alteraron significativamente los puntos finales de las primeras etapas de la vida, como la fertilización, el desarrollo larvario y la supervivencia en las especies probadas. Además, se ha demostrado que en un escenario de contaminación heterogénea donde la letalidad no es esperada, HHCB y AHTN pueden desencadenar una evasión espacial que podría empobrecer los ecosistemas locales debido a la emigración a ambientes menos impactados. Los efectos subletales evaluados en bivalvos después de la exposición crónica a HHCB y AHTN mostraron que estas sustancias están biodisponibles para los organismos marinos y tienen el potencial de inducir estrés oxidativo, genotoxicidad, neurotoxicidad y alterar el estado de salud de los organismos marinos. También se observó que ambos compuestos modulaban biomarcadores de disrupción endocrina en peces. A pesar de las alteraciones significativas en los biomarcadores de disrupción endocrina inducidas por ambos compuestos, la AHTN pareció ser un inhibidor más potente de la actividad endocrina en los ambientes marinos.

Esta tesis doctoral ha demostrado los efectos adversos de concentración ambiental de HHCB y AHTN en los entornos marinos. Por lo tanto, los datos presentados en este estudio deben integrarse con otros datos disponibles necesarios para las acciones políticas que ayudarán a la conservación y gestión del mar.

List of Abbreviations

AC50	Median avoidance concentration
AChE	Acetylcholinesterase
ADBI	Celestolide
AHMI	Phantolide
AHTN	Tonalide
ASTM	America Society for Testing and Materials
ATII	Traseolide
CAS	Chemical Abstract Services
CDNB	1-chloro-2,4-dinitobenzene
CEC	Contaminants of Emerging Concern
CI	Chemical Ionization
COX	Cyclooxygenase
DDT	Dichlorodiphenyltrichloroethane
DEET	N,N-diethyl-m-toluamide
DNA	Deoxyribonucleic Acids
DPMI	Cashmeran
DTNB	5,5-Dithiobis (2-nitrobenzoic acid)
EDC	Endocrine Disrupting Chemical
EHMC	2-ethyl-hexyl-4-trimethoxycinnamate
ERA	Environmental Risk Assessment
EROD	Ethoxyresorufin-*O*-deethylase
EUAR	European Union Risk Assessment Report
FS	Forced Exposure System
GC	Gas Chromatography
GPx	Glutathione Peroxidase

GR	Glutathione Reductase
GSH	Reduced Glutathione
GSSH	Glutathione Disulphide
GST	Glutathione-*S*-transferase
HHCB	Galaxolide
IPPC	Integrated Pollution Prevention and Control
LC50	Median Lethal Concentration
LPO	Lipid Peroxidation
MA	Musk Ambrette
MDA	Malondialdehyde
MEC	Measured Environmental Concentratrion
MFO	Mixed-Function Oxidase
MK	Musk Ketone
MO	Monooxygenase
mRNA	messenger Ribonucleic Acid
MS	Mass Spectrometry
MT	Musk Tibetene
MX	Musk Xylene
NADPH	Nicotinamide adenine dinucleotide phosphate
NFS	Non-Forced Exposure System
NMC	Nitro Musk Compound
NOEC	No Observed Effect Concentration
NSAID	Non-Steroidal Anti-Inflammatory Drugs
OECD	Organization for Economic Co-operation and Development
PAH	Polycyclic Aromatic Hydrocarbon
PCB	Polychlorinated Biphenyl

PEC	Predicted Environmental Concentration
PID	Population Immediate Decline
PMC	Polycyclic Musk Compound
PNEC	Predicted No Effect Concentration
PPCP	Pharmaceutical and Personal Care Products
PUFA	Polyunsaturated Fatty Acids
PVC	Polyvinyl Chloride
qPCR	Quantitative Polymerase Chain Reaction
REACH	Registration Evaluation Authorization and Restriction of Chemicals
RIFM	Research Institute for Fragrance Materials
ROS	Reactive Oxygen Species
RQ	Risk Quotient
SMC	Synthetic Musk Compounds
STP	Sewage Treatment Plants
TBARS	Thiobarbituric Acid Reactive Substance
TL	Total Lipids
TMP	Tetramethoxypropane
USEPA	United States Environmental Protection Agency
WFD	Water Framework Directives
WWTP	Wastewater Treatment Plant

Chapter 1

General Introduction

1.1. General Overview

The marine environment covers about 71% of the earth's surface and contains more biodiversity than the terrestrial and freshwater ecosystems altogether. Marine biodiversity is supported by the unique complex network of interactions given energy and material transfers between the biotic and abiotic components, which foster stability amongst marine communities. The biological networks, where the success of species is directly or indirectly connected through biological interactions, constitutes ecosystem function, through which ocean and coastal ecosystems provide natural benefits (fisheries, recreations, industrial raw materials, etc) upon which humans depend (Sun *et al*., 2016). Since the last industrial revolution era, anthropogenic activities have become the nexus of threats to the stability of marine ecosystems and its capacity to support life due to transport of agricultural chemicals from farm sites, domestic and industrial effluents as well as atmospheric deposition of contaminants.

The impacts of legacy contaminants on the marine ecosystems from industrial and agricultural chemicals such as polyvinyl chlorides (PVCs), polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), pesticides and insecticides have been documented as pollutants in the marine environment (Carls *et al*., 2008; Coteur *et al*., 2001; Donkin *et al*., 1997; Filipak Neto *et al*., 2008; Hylland, 2006; Jansson *et al*., 1993; Karacik *et al*., 2009; Mayer *et al*., 1977; Wang and Wang, 2005; Zhang *et al*., 2016). They have been found to bioaccumulate in marine organisms and several studies have shown that they cause various effects such as growth inhibition, behavioural changes, oxidative stress, hepatotoxicity, embryotoxicity, carcinogenicity, genotoxicity, neurotoxicity and mortality in marine microalgae, invertebrates and fish (Carls *et al*., 2008; Coteur *et al*., 2001; Donkin *et al*., 1997; Filipak Neto *et al*., 2008; Hylland, 2006; Karacik *et al*., 2009; Mayer *et al*., 1977; Wang and Wang, 2005; Zhang *et al*., 2016). In the last two decades, evidence has emerged regarding presence of chemicals that were previously not on priority list for environmental monitoring consequent to improved technology in analytical chemistry that allows quantification of environmental chemicals at nanoscale. These groups of chemicals are part of everyday life because they are consumed as personal care products, pharmaceutical active substances, antimicrobial agents/disinfectants, and preservatives, otherwise known as contaminants of emerging concern (CECs).

1.2. Contaminants of Emerging Concern (CECs)

CECs have been defined by the United States Environmental Protection Agency (USEPA) as new substances not captured within regulatory framework for monitoring and whose impacts on human and the environment is poorly understood (Montes-Grajales *et al*., 2017). They are used for various household and

personal care purposes as part of our everyday life and therefore increasing their concentrations in environmental matrices. For most CECs, the route of entrance is mainly via wastewater treatment plants (WWTPs) effluents (Bueno *et al*., 2012; Díaz-Garduño *et al*., 2017; Picot Groz *et al*., 2014), where they follow water gradient to wide scale presence in the marine environment. These contaminants are unique environmental pollutants because many of them have the capacity to elicit biological effects at low concentration. Amongst other CECs, pharmaceutical and personal care products (PPCPs) are the most frequently detected in environmental matrices.

Pharmaceutical products (PPs) have attracted attention in different regions across the globe (Barnes *et al*., 2008; Focazio *et al*., 2008; Kleywegt *et al*., 2019) and in the year 2000, the European Union Water Framework Directives identified 33 priority substances of concern including PPs and specifically added diclofenac, iopamidol and carbamazepine in 2007 to the list while ibuprofen, clofibric acid, triclosan, phthalates and bisphenol A were proposed to be added to the list (Ebele *et al*., 2017). They have been measured in different aquatic ecosystems, confirming their ubiquity and reported concentrations are consistently higher in hospital, domestic and industrial sewage treatment plants (STP) followed by freshwater and marine environments (Alidina *et al*., 2014; Bendz *et al*., 2005; Brausch and Rand, 2011; Diaz-Garduño *et al*., 2016; Ebele *et al*., 2017; González-Alonso *et al*., 2017; Gonzalez-Rey *et al*., 2015; Kay *et al*., 2016; Lei *et al*., 2018; Loos *et al*., 2013; McEneff *et al*., 2014; Mezzelani *et al*., 2018; Pereira *et al*., 2015; Weigel *et al*., 2004). They have been detected in groundwater (Sui *et al*., 2015), drinking water (Yang *et al*., 2017) and in adipose tissues of many aquatic organisms (Klosterhaus *et al*., 2013; McEneff *et al*., 2014; Mezzelani *et al*., 2016a, 2016b; Picot Groz *et al*., 2014; Xie *et al*., 2019). Recent studies have demonstrated an increase in the number of PPs measured in the aquatic ecosystems (Garduño *et al*., 2016 and others).

Varieties of personal care products (PCPs) have also been detected in the aquatic environment, including disinfectants, insect repellants, preservatives, UV-filters, and fragrances. Unlike PPs that are used internally at a prescribed dosage, PCPs are externally applied, and the quantity used is at personal discretion and need, thus accounting for their high concentrations in WWTPs effluents and receiving water body (Brausch and Rand, 2011). Most frequently detected PCPs and their metabolites include triclosan, triclocarban, N,N-diethyl-m-toluamide (DEET), paraben, 2-ethyl-hexyl-4-trimethoxycinnamate (EHMC), to mention a few and their environmental occurrence and toxicity has been reviewed by Brausch and Rand (2011). The current research focuses on the toxicity of polycyclic musk compounds in the marine environment. Below, a brief description of the various classes of synthetic musk compounds (SMCs) is discussed and their occurrence, bioaccumulation, and toxicity in the aquatic ecosystems in general is reviewed.

1.3. Fragrances

Fragrances are low molecular weight (<300) chemical compounds which are applied in a wide range of personal care products such as body lotions, deodorants, washing soap and detergents, perfumes, toothpastes, various cosmetics, etc (Reiner and Kannan, 2011) to give a characteristic and acceptable scent. Fragrances are derived from musk – a wide range of natural and synthetic products. Natural musks are extracted from fatty tissues of plants and animals which are sacrificed to extract musky organs. This practice has contributed to reduction in musk deer abundance and has therefore been banned. SMCs, on the other hand, are chemically produced to reflect the same physical characteristics as natural musk. They are classified based on the functional group used in the formulation and consists of nitro musk compounds, polycyclic musk compounds, macrocyclic musk compounds and alicyclic musk compounds. Brief literature reviews are provided for nitro musk compounds and extensive review of polycyclic musk compounds with analysis of their physiochemical parameters, usage, environmental occurrence, and effects are discussed below.

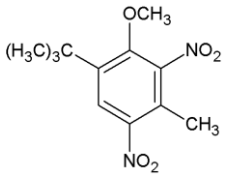
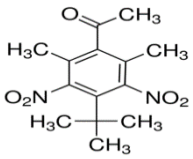
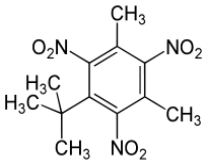
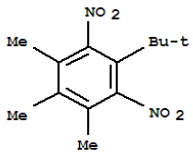
1.3.1. Synthetic Musk Compounds (SMCs)

The evolution of SMCs dated back to the 19th century when Baur stumbled on a nitro compound that possessed a good scent while working on explosives. He further improved on the synthesis to produce the first known synthetic musk named after him – *musc Baur* (Sommer, 2004). Other SMCs synthesized with alkylated nitrobenzene derivatives were grouped together as nitro-musk compounds (NMCs), they are composed of methylated nitrates and acetylated benzene rings and include musk ketone (4-tert-butyl-2,6-dimethyl-3,5-dinitroacetophenone), musk xylene (1-tert-butyl-,5-dimethyl-2,4,6-trinitrobenzene), musk ambrette (2,6-dinitro-3-methoxy-4-tert-butyltoluene), musk moskene (1,1,3,3,5-pentamethyl-4,6-dinitro-2H-indene) and musk tibetene (1-tert-butyl-3,4,5-trimethyl-2,6-dinitrobenzene). Their structural formular and physiochemical properties are presented in Table 1.1.

NMCs are highly lipophilic and possess high n-octanol/water partition coefficient ($LogK_{ow}$) and, consequently, they are persistent in the environment and can bioaccumulate in human and aquatic organisms. However, musk xylene and musk ketone are the most frequently detected in different environmental matrices at high concentrations. The environmental performance of this class, including bioaccumulation and toxicity revealed they are persistent, bio-accumulative and toxic. Wet weight bioconcentration potential (BCF_w) of up to 5,820 and 6,740 were measured in Japanese carp after 10 weeks laboratory exposure, respectively, to 10 µg/L and 1 µg/L of musk xylene (Chemical Inspection and Testing

Institute, Japan, 1992). Different nitro musk compounds have been measured in several aquatic organisms from freshwater and marine environment as well as human adipose tissues (Gatermann *et al*., 1999; Gatermann *et al*., 2002; Müller *et al*., 1996; Suter-eichenberger *et al*., 1998). Primary demyelination, central and peripheral nervous systems damage characterized by degeneration of myelin and axon in rat fed with musk ambrette have been reported (Spencer *et al*., 1984). NMCs were found to exert toxic effects related to carcinogenicity, mutagenicity, neurotoxicity, and genotoxicity (Luckenbach and Epel, 2005; Mersch-Sundermann *et al*., 1996; P. S. Spencer *et al*., 1984) and have therefore been restricted as fragrance materials in the perfumery industry.

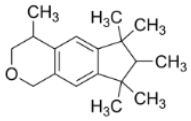
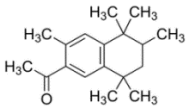
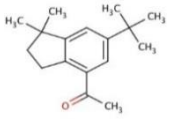
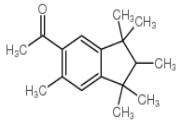
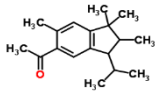
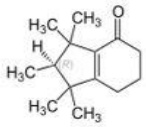
Table 1.1: Chemical structures and characteristics of the nitro musks category.

Common Name	Chemical Structure	CAS No	Log K_{OW}	Molecular Weight (g/Mol)	Boiling Point (^{0}C)	Vapour Pressure (Pa)
Musk Ambrette (MA)		83-66-9	5.7	268.269	185	0.00333
Musk Ketone (MK)		81-14-1	5.8	294.307	395.00	0.00004
Musk Xylene (MX)		81-15-2	4.9	297.267	200-202	0.00003
Musk Tibetene (MT)		145-39-1	5.9	266.297	391.01	0.00076

1.3.2. Polycyclic musk Compounds

The polycyclic musk compounds (PMCs) were synthesized to replace the nitro functional group due to their instability in alkaline medium and photochemical reactivity. PMCs are bi-cyclic aromatic compounds which comprised of acetylated and highly methylated pyran, tetralin and indane skeletons (Sumner *et al*., 2010). Although the industrial synthesis of this group are moderately difficult, they are, however, considered very important fragrance materials in perfumery due to binding capacity to fabrics and their characteristic musky scent (Swidish Society for Nature Conservation, 2000). Galaxolide (1,3,4,6,7,8-Hexahydro-4,6,6,7,8,8-hexa-methylcyclopenta-g-2-benzopyran-HHCB) and tonalide (7-acetyl-1,1,3,4,4,6-hexa- methyl-1,2,3,4-tetrahydronaphthalene-AHTN) are the most consumed polycyclic musk globally and others include phantolide (6-acetyl-1,1,2,3,3,5-hexamethyl-indane-AHMI), celestolide (4-acetyl-1,1-dimethyl-6-tert.-butylindane-ADBI), traseolide (5-acetyl-1,1,2,6-tetramethyl-3-iso-propyldihydroindane-ATII) and cashmeran (6,7-dihydro-1,1,2,3,3-pentamethyl-4(5H)-indanone-DPMI). Their chemical structures and some physical characteristics are presented below (Table 1.2). Other SMCs are macrocyclic and alicyclic musk compounds. At the moment, their usage in the perfumery industry is as best experimental and therefore have not been classified as CECs.

Table 1.2: Chemical structures and characteristics of the polycyclic musk category.

Common Name	Chemical Structure	CAS No	$LogK_{OW}$	Molecular Weight (g/Mol)	Water Solubility (mg/L)	Vapour Pressure (Pa)
Galaxolide (HHCB)		1222-05-5	5.9[a]	258.404	1.75[a]	0.0727[a]
Tonalide (AHTN)		1506-02-1	5.7[a]	258.405	1.25[a]	0.0608[a]
Celestolide (ADBI)		13171-00-1	5.4	244.378	0.22	0.019
Phantolide (AHMI)		15323-35-0	5.9	244.378	0.9	0.132
Traseolide (ATII)		68140-48-7	6.3	258.405	0.3	0.009
Cashmeran (DPMI)		33704-61-9	5.9	206.329		5.20

a. Balk and Ford (1999a)

1.3.3. Occurrence and concentrations of synthetic musk compounds

Source network for SMCs entrance into the aquatic environment is described in Figure 1. SMCs are released into the environment during production, compounding, application in consumer products and

most importantly from household uses. Although the quantity released during production, compounding and industrial application into the aquatic environment is minimal (European Commission, 2008b, 2008a), it is expected that release from end users into the aquatic environment from wastewater water treatment plants effluents, direct input and via atmospheric deposition will be higher (Figure 1).

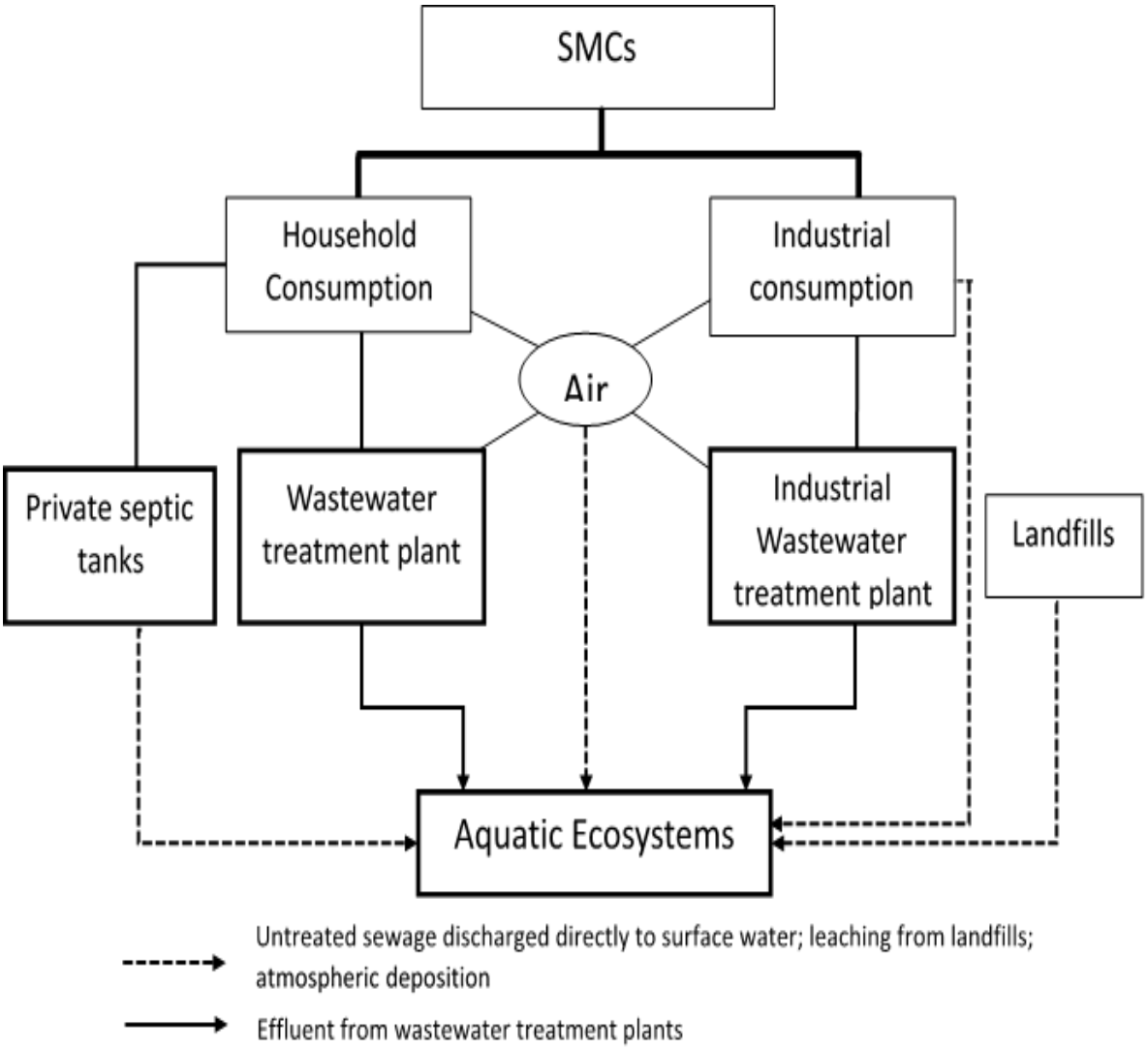


Figure 1: Diagram showing source network for Synthetic musk compounds (SMCs) entrance into the aquatic ecosystems

Environmental concentration of synthetic musk belonging to the nitro group was first found in the Tama river, near Tokyo Japan in 1981 (Peck *et al*., 2006). Further investigations revealed that the nitro group is highly persistent and possesses high bioaccumulation potential (Zhang *et al*., n 2013; Zhang *et al*. 2013b).

This group has been demonstrated to be genotoxic and carcinogenic (Nair *et al*., 1986) and thus, placed under restricted use by European Chemical Agency.

The most widely consumed PMCs are HHCB and AHTN. According to European Union Risk Assessment Report (EUAR) for HHCB and AHTN (European Commission, 2008a, 2008b), each of HHCB and AHTN has one production site in Europe. As of 2000, between 1000 and 5000 ton/y of undiluted HHCB and AHTN was produced in Europe. The estimated quantity of these substances used in 1996 was approximately 8000 ton (Chen *et al*., 2010). Data from Research Institute for Fragrance Materials (RIFM) revealed that between 1993 and 2006 average quantity of HHCB and AHTN used in Europe was 1648 ton/y and 454 ton/y, respectively, and 25% percent of each substance being applied in production of detergent for industrial and domestic cleaning purposes (European Commission, 2008b, 2008a). In the United States, all fragrances consumed has been doubled since 1990 (Roosens *et al*., 2007) and increased by 25% between 1996 and 2000, from estimated quantity of 5200 to 6500 ton (Peck *et al*., 2006). Recent data from EPA showed that the USA consumed approximately 1600 and 1700 metric tons per year of HHCB in 2008 and 2011, respectively, and the rate is estimated to keep increasing, due to increase in market demand for fragrance materials (USEPA, 2014). The quantity of HHCB and AHTN is equal to 95% of total market volume of fragrance materials (Balk and Ford, 1999a; Pedersen *et al*., 2009). Europe consumption of HHCB and AHTN in 1995 alone is approximately 15.5 mg/day (Rimkus, 1999). In Italy, the consumption of HHCB and AHTN has been estimated at 7.23 and 1.81 g per year per capita, respectively (European Commission, 2008b).

These compounds have been found in various environmental compartments (Table 1.3): air, water and sediment (Fromme *et al*., 2001; Peck *et al*., 2006; Peck and Hornbuckle, 2006). Owing to their external applications, they do not undergo metabolic transformation and as such are released untransformed into the aquatic ecosystem via sewage treatment plants, direct atmospheric deposition, leaching from landfills and untreated sewage disposal from private properties (Figure 1) (Fromme *et al*., 1999; Ramskov *et al*., 2009). Furthermore, most sewage treatment plants are not adapted to completely eliminate SMCs from municipal and industrial wastewater. Investigations have shown that only about 50% of the total SMCs are eliminated from sewage treatment plants, while the rest enter the receiving rivers and oceans via sewage outfall (Heberer, 2002; Lee *et al*., 2010) and are diluted along the river gradient downstream (Ricking *et al*., 2003). Their occurrence in seawater, which in some cases distant from source (WWTP), is an evidence of their persistence, although atmospheric deposition could be a vital source of SMCs in marine environments. Also, for many cities on the coasts, treated sewage materials are discharged over a short distance into coastal ecosystems and owing to the partial removal of SMCs from WWTPs, these areas could be inundated with these contaminants.

Table 1.3: Measured environmental concentrations of galaxolide (HHCB) and tonalide (AHTN) in the environmental compartments.

Country	Environmental compartments	HHCB	AHTN	Reference
Canada	STP Effluent (ng/L)	205 - 1300	110 - 520	Ricking *et al*., 2003
England	Tamar Estuarine (ng/L)	6.00 - 30	3.00 - 15	Sumner *et al*., 2010
England	STP Effluent (ng/L)	987 - 2089	55 - 159	Sumner *et al*., 2010
England	SPM (dry wgt) (ng/g)	12.00 - 29	1.00 - 11.00	Sumner *et al*., 2010
England	Sediment (ng/g dry wt)	11.00 - 17.00	2.00 - 10.00	Sumner *et al*., 2010
Germany	Surface water (ng/L)	70 - 1590	20 - 530	Frommme *et al*., 2001
Germany	STP Effluent (ng/L)	LOD - 13330	LOD - 4360	Frommme *et al*., 2001
Germany	Sediment (ng/g)	15 - 3150	20 - 1100	Frommme *et al*., 2001
Germany	Surface water (ng/L)	20 - 12500	30 - 6800	Hebere, 2002
Germany	Surface water (ng/L)	10 - 180	10.00 - 70	Dsikowitzky *et al*., 2002
Germany	SPM (ng/g)	5000 - 191000	2 - 1399000	Dsikowitzky *et al*., 2002
Germany	North Sea (ng/L)	0.09 - 4.8	0.08 - 2.6	Bester *et al*., 1998
Germany	Elbe Estuary (ng/L)	95 - 136	65 - 200	Bester *et al*., 1998
Italy	Molgora River (ng/L)	0.05 - 1141	<0.25 - 364.42	Villa *et al*., 2012
Italy	suspended sediments (ng/g)	<0.0007 - 17993	0.00035 - 4321	Villa *et al*., 2012
Netherlands	Rhine river (ng/L)	10 - 220	10 - 130	Breukel & Balk, 1996
Netherlands	suspended sediments (ng/g)	50 - 160	100 - 540	Breukel & Balk, 1996
Spain	STP Effluent (ng/L)	LOD - 1225	LOD - 146	Rosal *et al* 2010
Spain	STP Effluent (ng/L)	1800 - 9000	100 - 900	Bueno *et al* 2012
Spain	STP Effluent (ng/L)	4750	780	Pintado-Herrera *et al*, 2014
Spain	River Water (ng/L)	1890	190	Pintado-Herrera *et al*, 2014
Spain	Effluent (ng/L)	5603	1583	Diaz-Garduno *et al*., 2017
Sweden	STP Effluent (ng/L)	157 - 423	42 - 104	Ricking *et al*., 2003

The measured environmental concentrations of PMCs recorded in various parts of Europe and America are presented in Table 1.3. These concentrations are approximations of the exact concentrations of these compounds in various environmental compartments due to the sensitivity and selectivity of the instrumental and analytical approaches used in their determination. The chromatographic (GC) approach has been widely embraced owing to its compatibility with sample preparation techniques that necessitate subsequent liquid desorption or thermal desorption of the analytes and thus can be used with various sample preparation techniques (Vallecillos *et al*., 2015). However, this approach has limitations related to identification and quantification of targeted musk fragrances due to the complexity of environmental matrices. Again, because chromatographic peaks of musk fragrances sometimes co-elute with matrix components, it be difficult to separate them with one-dimensional GC. GC in combination with chemical ionization (CI) is quite sensitive for nitro musk fragrances and their amino metabolites (Peck and Hornbuckle, 2006). Ion trap (IT) in MS or tandem MS/MS modes is the second most selected analyzer to determine PCMs, NMs and MCMs in different kinds of samples because of its ability to achieve higher selectivity and sensitivity (Vallecillos *et al*., 2015). In most of the environmental concentrations presented in Table 1.3, GC-MS with a quadrupole as the analyzer technique in terms of instrumental analysis of PCM fragrances was used in environmental samples. However, microextraction techniques have recently been developed as environment-friendly methodologies in analyzing PCMs in environmental water and solid samples.

1.3.4. Bioaccumulation of synthetic musk compounds

Most emerging contaminants are difficult to monitor directly because of their low concentrations in the environment, ranging from nano- to micro- units and, therefore, not well graded in accordance with their potential to elicit toxic effects in organisms. However, available data though insufficient suggest that these substances do exist, and they could represent a threat to the marine ecosystems. Chemical monitoring alone seems not sufficient to be relied upon in deriving good quality standards for the marine environment. In addition, biological monitoring may provide a good complement to bridging this gap by studying contaminants accumulation in organisms.

Environmental contaminants are distributed in organisms via the skin, buccal cavity, nostrils, and other permeable membranes into the system. Their fate in organisms depends, amongst others, on certain physical and chemical characteristics such as lipophilicity, polarity, ionization, chemical structure, molecular mass, as well as the lifecycle stage of the organism during exposure. Most polycyclic compounds are lipophilic

and thus can penetrate the phospholipid bilayer of cell membranes and bind with other cellular molecules. Depending on other factors, xenobiotics can be metabolized or accumulated in various tissues within an organism: liver, adipose tissues, kidneys, and gills.

The term bioaccumulation is used to describe the uptake of xenobiotics directly from the environment and/or via food intake and it is an indicator of potential persistence and toxicity of contaminants (Wang, 2016). Bioaccumulation of PMCs from different aquatic organisms such as mussels, crustaceans, and fish from freshwater and marine environments sampled from the wild have been documented (Fromme *et al*., 1999; Fromme *et al*., 2001; Rimkus *et al*., 1997; Rüedel *et al*., 2006) and, in most of the studies, HHCB and AHTN remained the predominant PMCs detected. Concentrations of HHCB and AHTN as high as 6400 ng/L and 2130 ng/L lipid weight, respectively, have been measured in bream fish from the Elbe River in Germany (Rüedel *et al*., 2006). Similarly, Fromme *et al*. (2001) found HHCB in eels at concentrations with a value range of 445 – 6470 µg/kg lipid weight from Rivers Spree, Dahme and Havel, Germany. Under laboratory condition, the bioaccumulation factor based on wet weight (BAF_w) for various SMCs in fish were 4200 – 5100 for musk xylene (Rimkus *et al*., 1997), 1584 for HHCB and 597 for AHTN (Balk and Ford, 1999b). BAFw measured in eels from surface waters in Berlin with a high proportion of municipal sewage effluents were 995 and 1421 for HHCB and AHTN, respectively (Heberer, 2002). Gatermann *et al*. (2002) reported the values SMCs in four fish species from a pond receiving water from sewage treatment plants and detected a concentration of 40000 ng/L for musk xylene, while HHCB and AHTN were lower than previous values. The authors concluded that the wide variation in values of SMCs detected in different species could be due to species dependent accumulation and metabolism of contaminants in fish (Gatermann *et al*., 2002).

There are few reports on the environmental concentrations of PMCs in marine environments and the only compounds reported are HHCB and AHTN (Bester *et al*., 1998). It is doubtful that the dearth of information regarding the presence of PMCs in the marine environment reflect the actual status of these substances for the marine ecosystems because of the increasing consumption globally and Europe in particular; and secondly because many cities are located on the coast and have their sewage treatment plants directly emptying into the marine environment. Notwithstanding, reports have shown they bioaccumulate in marine organisms (Kannan *et al*., 2005; Moon *et al*., 2011, 2012). Moon *et al*. (2012) reported bioaccumulation profiles of polycyclic aromatic hydrocarbons and SMCs in liver tissues and bubbler from minke whales and common dolphins from Korean coastal waters. They noted that, in all the samples of liver tissues and bubbler from minke whales and dolphins from the coastal waters, HHCB were found to be predominant with no exception. The concentrations in ng/g lipid weight found in the liver tissues and bubbler of both organisms ranged from <2.3 to 169 and <2.3 to 50; and 24 to 187 and 19 to 72 of HHCB and AHTN,

respectively. The highest concentrations of both compounds were found in the liver and bubbler of minke whales.

To further our understanding of the biomagnifications potential of PMC and to identify those species that accumulate higher concentrations through their diet and habitat, Nakata (2005) measured SMCs in lugworm, mussel, crustacean, fish, marine birds and mammals from tidal flat and shallow water areas of Ariaka Sea, Japan. In all the samples analyzed, HHCB and AHTN were the dominant compounds while NMCs were undetected. An earlier investigation of oysters in 61 locations around Japanese coastal waters reported the predominance of HHCB and AHTN (Nakata *et al*., 2007). HHCB concentrations in organisms from the Ariaka Sea were 3 to 10-fold higher than those of AHTN and the highest concentrations were found in clams, ranging from 258 ng/g (lipid wt.) to 2730 ng/g (lipid wt.), followed by crustaceans and fish in the tidal flat. However, the results could not establish any correlation between HHCB concentrations in clams and the organisms in the successive trophic levels in the food chain (Nakata *et al*., 2007), suggesting that the concentrations in the organisms in higher trophic levels where more of bioconcentration rather than biomagnifications, which further corroborated the ubiquity of these compounds in marine environment.

1.3.5. Toxicity of synthetic musk compounds

Environmental chemicals interact with biotic components to produce biological effects. Naturally, organisms are equipped with internal mechanisms to mediate the effects and protect themselves from irreversible damage. This process releases signals used in ecotoxicological studies as early warning mechanisms to protect the organism at individual level before population level effects occur. Like other environmental contaminants, the potential toxicity of SMCs was evaluated even before they were first detected in environmental samples. In 1964, Davis, Taylor, Jones and Brouwer (cited by Nair *et al*., 1985) found that rat feed with musk ambrette in a sub-chronic assay had retarded growth, testicular atrophy and gradual paralysis of the hind limb. Subsequent investigation by Spencer *et al*. (1984) detected the neurotic effect of musk ambrette in rats. Furthermore, musk ambrette and musk xylene were tested for mutagenicity in *Salmonella typhimurium* strains TA100 and TA98 strains with and without rat liver S-9 activation system. Musk ambrette was found to cause a concentration-dependent increase in mutagenicity in *S. typhimurium* TA100 requiring the activation with rat-liver S-9 fraction (Nair *et al*., 1986). Female zebrafish dietary exposure to musk ketone for 8 weeks to two different dose levels before spawning were found to reduce body weight and length, coupled with reduced liver and gonad somatic index (Carlsson and Norrgren, 2004). Again, dose-dependent reduction in fecundity, increased early life-stage mortality and reduced median survival time was also reported in zebrafish (Carlsson and Norrgren, 2004). Consequently,

NMCs widely returned genotoxic and mutagenic effects and acute toxicity in early life stages (Api *et al*., 1995; Api *et al*., 1996).

Toxicity data of PMCs, especially HHCB and AHTN in marine environments are incipient and previous studies demonstrated that both substances are potentially toxic to aquatic organisms, including early stages of some organisms. Toxicity of xenobiotics on early life stages has serious ecological consequences because inhibition of embryo development, growth and survival could limit the recruitment capacity of the individual species, contributing to population decline. Population decline at any trophic level, for example primary consumers, will facilitate alteration in the community structure.

Exposure of algae, crustaceans, mollusks, earthworm and fish to HHCB and AHTN showed that both substances might inhibit growth of algae (Balk and Ford, 1999b; Ding *et al*., 2020). The microalga, *Pseudokirchneriella subcapitata* exposed to HHCB (0.0625 - 1.0 mg/L) and AHTN (0.042 - 0.85 mg/L) inhibited microalgae growth up to 56%, producing NOEC values of 0.201 mg/L and 0.374 mg/L, respectively (Balk and Ford, 1999b). Recently, Ding *et al*. (2020) demonstrated that HHCB inhibited the growth of the microalgae *Navicula* sp. and *Scendesmus quadricauda*, producing IC_{50} values of 0.050 mg/L and 0.336 mg/L, respectively. Similarly, acute toxicity reported for other organisms like crustaceans, mollusks, earthworm and fish, returned LC_{50} values higher than environmental relevant concentrations (Artola-Garicano *et al*., 2003; Balk and Ford, 1999b; Gooding *et al*., 2006). Lethal and sublethal toxicity of HHCB and AHTN to glochidial (larval) and juvenile life stages of the freshwater mussel *Lampsilis cardium* showed that at 24 h dose-response mortality was detected, producing LC_{50} values ranging from 454 to 850 µg/L for AHTN and from 1000 to >1750 µg/L for HHCB (Gooding *et al*., 2006). In another study, HHCB and AHTN were found to inhibit larval development after 5 days exposure of marine copepod (*A. tonsa*) at median effect concentration (EC_{50}) of 0.026 mg/L for AHTN and 0.056mg/L for HHCB (Wollenberger *et al*., 2003).

Although the above literatures suggested that PMCs are not acutely toxic to organisms at environmental relevant concentrations, there are, however, sublethal effects of ecological relevance already detected. For example, 0.02 mg/L of HHCB impaired larval development of the copedop *Nitocra spinipes* (Breitholtz *et al*., 2003) and Gooding *et al*. (2006) reported life stage dependent effects.

Aquatic sediments are often reservoirs of chemical contaminants that can potentially elicit detrimental effects to aquatic organisms, both pelagic and benthic, and consequently have toxic impacts across aquatic ecosystems. The concentrations of chemicals in sediments may be several orders higher than in water layers because contaminants that may not dissolve in water, can adsorb to particulate matter and may cause

significant effects in benthic organisms. Therefore, investigating sediment toxicity of PMCs is essential to understanding the impact these substances may impose on benthic and pelagic aquatic biota. Results of sediment toxicity test with HHCB on the freshwater mud snail *Potamopyrgus antipodarum* revealed that on exposure to 30 and 100 μg/g, HHCB reduced juvenile survival by 10% and 20%, respectively, and other endpoints such as low reproductive potential, reduced feeding in adults and juveniles as well as reduced growth rate were identified as potential effects triggered by HHCB (Pedersen *et al*., 2009). Similarly, the earthworm *Eisenia fetida* exposed to AHTN (>8.2 μg/cm^2) and HHCB (>3.2 μg/cm^2) showed morphological symptom of sick earthworm at 24 h, and 100% mortality after 48 h of exposure to AHTN (>85.9 μg/cm^2) and HHCB (> 53.7 μg/cm^2) (Chen *et al*., 2011).

1.4. Hypothesis and Objectives

The widespread applicability of PMCs as fragrance materials in household and personal care products together with external application and disperse use contribute to their ubiquity in environmental matrices. The PMCs, such as HHCB and AHTN, have been detected in marine environments in different parts of the world and the main route of entrance being sewage treatment plants and atmospheric deposition. They are lipophilic, have high octanol-water partition coefficient and could be persistent or pseudo-persistent in marine environments. The interaction and potential toxicity they may exert on marine biota, particularly sedentary species that are continuously exposed to these compounds and impacts on early life stages of marine species and marine algae growth is elusive.

1.4.1. Hypothesis

The present research work is focused on the following hypothesis:

- On a short-term exposure, environmental concentrations of HHCB and AHTN may inhibit growth of microalgae such as *Phaeodactylum tricornutum*, *Tetraselmis chuii*, *Isochrysis galbana* and *Raphidocelis subcapitata*. HHCB and AHTN may significantly affect the fertilization success, larvae development of sea urchin (*Paracentrotus lividus*), motility and survival of artemia (*Artemia franciscana*), larvae development of mussels (*Mytilus galloprovincialis*), increase mortality of yolk sac larvae of fish (*Sparus aurata*) and may also interfere in the spatial displacement of organisms (namely the shrimp *Palaemon varians*) from their habitat and trigger population immediate decline of marine organisms.

- Under chronic exposure, HHCB and AHTN may alter biochemical changes in the clams *Ruditapes philippinarum* that may cause significant physiological impacts such as oxidase stress, genetic damage and neurotoxicity. Exposure to these substances may also modulate gene expression of endocrine biomarkers in yolk sac larvae of the fish *Cyprinodon variegatus*.

1.4.2. Objectives

The main objectives of the present research work are:

- To determine the short-term effects of environmental concentrations of HHCB and AHTN on marine organisms such as *Paracentrotus lividus, Artemia franciscana, Mytilus galloprovincialis, Sparus aurata* early life stages, growth inhibition of the marine microalgae, *Phaeodactylum tricornutum, Tetraselmis chuii, Isochrysis galbana*, following EU guideline to characterize the risk of both compounds in the marine ecosystems.

- To determine the spatial avoidance of the shrimps *P. varians* in response to the repellence caused by the exposure to a contaminant gradient of HHCB and AHTN in a non-forced, multi-compacted exposure systems as a complementary approach to risk assessment and estimate the population immediate decline of the shrimps.

- To determine the sub-lethal effects of the exposure to environmental concentrations of HHCB and AHTN using the bioindicator species *Ruditapes philippinarum.* An integrated biomarker approach is applied to determine biochemical changes in biomarkers related with biotransformation, oxidative stress, genotoxicity and neurotoxicity.

- To assess the effects of HHCB and AHTN at the molecular level on yolk sac larvae of *Cyprinodon variegatus* after 96 h exposure, using qPCR to quantify gene expression levels of endocrine biomarkers.

- To provide an overview about the potential risk of HHCB and AHTN on the structure and functioning of the marine aquatic ecosystems.

The above objectives were set to provide understanding of the potential toxicity of low concentrations of HHCB and ANTN in the marine environments.

1.5. Structure of the thesis

The present PhD thesis entitled "Ecotoxicological assessment of galaxolide and tonalide as contaminants of emerging concern in marine ecosystems" has been divided into eight chapters which are explained below.

Chapter 1: This chapter includes general introduction of the research area, highlighting the significance of the marine ecosystems and the species richness it holds, followed by diversity of contaminants of emerging concern and their threat to marine biota. The types of synthetic musk compounds and their physiochemical properties were also explained. The occurrence of synthetic musk compounds in the marine environments was elucidated, identifying all possible routes of entrance, detection and concentration in the media and tissue accumulation in organisms. Thereafter, toxicity data of synthetic musk compounds in different environmental compartments were presented with emphasis on the effect assessment data of the polycyclic musk compounds, galaxolide and tonalide in the aquatic environment. The hypothesis and objectives of the thesis were highlighted followed by the structure of the thesis.

Chapter 2: This chapter focuses on the methodological approach used for the development of this thesis. Here, the theoretical framework of environmental risk assessment was explained followed by the array of test organisms and the endpoints used. This chapter also deals with the different bioassays that were performed, the variety of biomarkers used and the analytical methods for each biomarker that was assessed.

Chapter 3: This chapter entitled presented all the research work that was performed during the PhD thesis development.

Section 3.1 is entitled "Acute toxicity and overview of the potential risks of galaxolide and tonalide on structure and functioning of marine ecosystems" addressed the impacts of short-term exposure of marine microalgae, invertebrates, and fish to environmental concentrations of galaxolide and tonalide by measuring endpoints related to microalgae growth and early life stages of invertebrates and fish to quantitatively estimate the risk to marine biota. This chapter includes the article published by Ehiguese *et al*. (2021, Processes 9, 371).

Section 3.2: This section entitled "Spatial avoidance as a complementary tool for environmental risk assessment of galaxolide and tonalide in marine environment" deals with the avoidance response of the shrimp *Palaemon varians* exposed to a contaminant gradient of galaxolide and tonalide under a multi-compartmented non-forced exposure system that allows the shrimps to escape from a more contaminated compartment to less contaminated area. This chapter includes the article published by Ehiguese *et al*. (2019, Chemosphere 232, 113 – 120)

Section 3.3: This section entitled "The sub-lethal effects of environmental concentrations of galaxolide and tonalide on the manila clams *Ruditapes philippinarum*" highlighted the sub-lethal effects induced by environmental concentrations of galaxolide and tonalide on the adult manila clams *R. philippinarum* by measuring a battery of biomarkers after chronic exposure. This chapter includes the article published by Ehiguese *et al*. (2020, Marine Environmental Research 160).

Section 3.4: This Chapter entitled "The use of molecular biomarkers to assess the endocrine disrupting potential of galaxolide and tonalide in marine organisms" presents results of the study on the effects of galaxolide and tonalide on biosynthesis of steroidogenic hormones in yolk sac larvae of the sheepshead minnow *Cyprinodon variegatus*. This chapter includes the article published by Ehiguese *et al*. (2021, Environmental Research 196).

Chapter 4: This chapter includes the general discussion, highlighting the relevance of this PhD thesis and presents future perspectives.

Chapter 5: This chapter highlighted the major conclusions derived from this PhD thesis.

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Chapter 2

Methodological Approach

2. Methodological Approach

For the development of the current PhD Thesis, several methodologies widely used in environmental risk assessments (ERA) were employed. In order to provide a more complete assessment about the potential risks of the polycyclic musk compounds galaxolide (HHCB) and tonalide (AHTN) on marine biota, empirical approach based on data of ecotoxicological assays was explored based on ERA scheme. In this case, it was intended to employ organisms from different trophic levels like microalgae, echinoderms, bivalves, shrimps, and fish. This diversification in the species allows assessing how organisms with different biological complexity can be affected. In addition, different endpoints were used for each species, taking as basis the sensitivity and relevance of the endpoints. Another important aspect considered to select the endpoints was their level of biological organization, so that the responses measured were based on acute and chronic exposure, focusing on growth, survival, development, behavior, and biomarkers at the biochemical and molecular levels. Some details about the ecotoxicological endpoints described in the following chapters of the current thesis have been discussed below.

2.1. Environmental Risk Assessment

The emergence of new chemicals in addition to existing substances used for industrial, agricultural, and domestic purposes are at an alarming rate and are continuously increasing. In fact, according to Chemical Abstract Service (CAS), approximately 4000 new substances were added to the chemical register each day in addition to more than 33 million chemicals and over 59 million sequences registered from 1907 to January 2008 (Binetti *et al*., 2008). Considering possible chemical contaminations, this implies serious consequences for human and environmental safety and, as such, information regarding potential chemical hazards is important for human and environmental protection. Therefore, protocols for environmental risk assessment (ERA) of chemicals aiming at regulating and managing the potential impacts that their production and usage might impose on different environmental compartments are in force in different geography. As the aquatic ecosystems is the ultimate sink for contaminants pollutants either by direct discharge or through hydrologic and atmospheric processes (van der Oost *et al*., 2003), important regulatory documents as the Registration Evaluation Authorization and Restriction of Chemicals (REACH) (European Commission, 2006), the Integrated Pollution Prevention and Control (IPPC) (EC, 2008) and the Water Framework Directives (WFD) (EC, 2000) aim to safeguard the environments from chemical perturbations. Specifically, WFD is a specific legal document enacted for the protection of inland surface waters, transitional waters, coastal waters, and groundwater in its member states. The document provided a general framework for ecological protection and a minimum chemical standard for all surface waters (ECD, 2000).

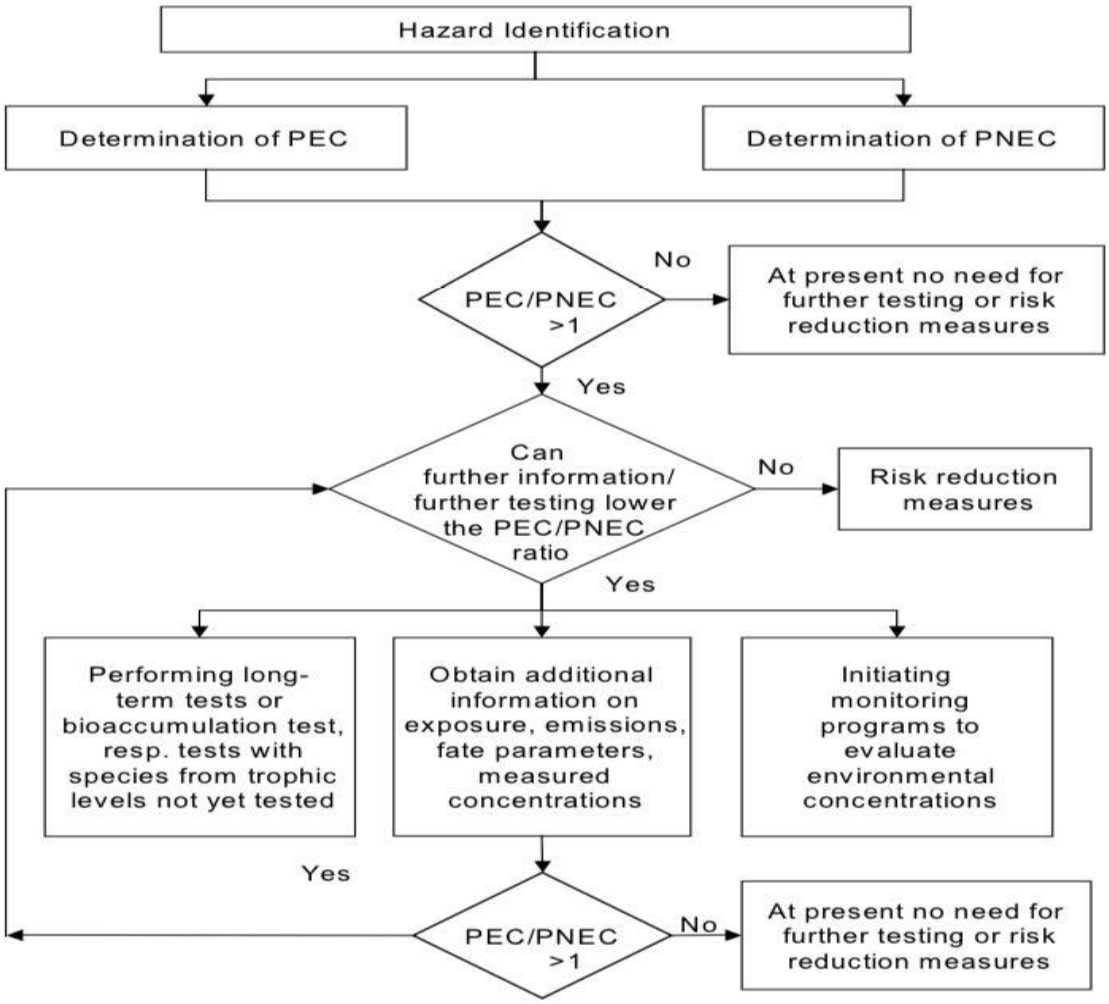


Figure 2.1: Environmental risk assessment procedure. Source: EC, 2003

ERA is the process of quantifying the likelihood that contaminants or anthropogenic activities will cause adverse effects on ecosystems and their components with a degree of certainty using scientific methodologies (van der Oost *et al*., 2003). The ERA process involves a scientifically driven risk analysis and a more politically oriented risk management; and could be performed on retrospective or introspective basis. Although most ERA is concerned with predictive assessment of environmental concentrations and effects, attention is presently more focused on retrospective assessment, evaluating sources of contaminants (e.g., effluent, spill), exposure and effects. Risk analysis of contaminants in the aquatic environment as described by European Union Technical Guidance Document on Risk Assessment (EC, 2003) involves hazard identification, exposure assessment, risk characterization and risk classification (Figure 2.1). In retrospective ERA, risk characterization is computed using *measured environmental concentrations* (MEC)

instead of predicted environmental concentration (PEC). Toxicity data obtained from single species or several species exposure representing different trophic levels, divided by an appropriate assessment factor are used to calculate *predicted no effect concentration* (PNEC). The ratio of MEC and PNEC gives a quantitative estimate (risk quotient) upon which risk can be classified.

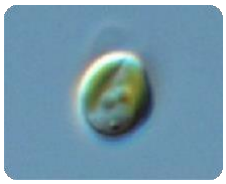
In this research, extensive literature survey of MEC of the polycyclic musk compound HHCB and AHTN was undertaken. Most data found were performed in Europe and one in Canada. Details are presented in Table 1.3 (Chapter 1) and have been discussed above. PNEC data used in this study were derived from laboratory toxicity tests. The ratio of MEC and PNEC were computed to derive the risk quotient and risk classification of HHCB and AHTN in marine ecosystems.

2.2. Acute toxicity tests

Contaminants in the marine environments affect life in several ways, depending on the concentration and the duration of exposure. Exposure to high concentration on a short-term tends to lethality, mostly to sensitive species and even resistant organisms may become sensitive at some stages in their life cycle. Therefore, short-term exposure to contaminants for effect should not only be concern with endpoints related to lethality but sub-lethal effects at a stage in the life cycle of the test organism. Sub-lethal effects related to the ability to survive, growth and reproduce will affect population size and reduction in sensitive population may lead to changes in the community structure, that can alter ecosystem functioning (Fleeger *et al*., 2003).

ERA for the aquatic ecosystems combines assessment of exposure and effect data generated from acute and chronic toxicity tests. As a result, acute toxicity tests were conducted in static bioassay with microalgae, invertebrates, and fish to generate effective concentrations (EC) for HHCB and AHTN. Following the procedure described by Garrido- Perez *et al*. (2008), 72 h growth inhibition tests were performed with the microalgae *Phaeodactylum tricornutum*, *Tretraselmis chuii* and *Isochrysis galbana* (Table 2.1), change in biomass was measured indirectly through absorbance and data were expressed as percentage of growth inhibition. These microalgae have been used as sensitive tools for ERA of effluents (Díaz-Garduño *et al*., 2017), pesticides (Magnusson *et al*., 2010), pharmaceutical and personal care products (Aguirre-Martínez *et al*., 2015; Ferreira *et al*., 2007).

Table 2.1: Description of bioindicator species, toxicity tests and endpoints.

Organism	Species	Trophic level	Toxicity test (duration)	Image
Algae	*Phaeodactylum tricornutum*	Primary producer	Growth inhibition (72-h)	
Algae	*Tetraselmis chuii*	Primary producer	Growth inhibition (72-h)	
Algae	*Isochrysis galbana*	Primary producer	Growth inhibition (72-h)	
Algae	*Raphidocelis subcapitata*	Primary producer	Growth inhibition (72-h)	
Artemia	*Artemia franciscana*	Primary consumer	Motility and survival	
Sea urchin	*Paracentrotus lividus*	Primary consumer	Larval development (48-h)	

Organism	Species	Trophic level	Toxicity test (duration)	Image
Mussels	*Mytilus galloprovincialis*	Filter feeder Primary consumer	Larval development (48-h)	
Clams	*Ruditapes philippinarum*	Filter feeder Primary consumer	Sub-lethal effects: xenobiotics biotransformation (EROD & GST), Oxidative stress (GPx, GR, LPO), DNA damage, neuroendocrine toxicity (AChE, COX) and TL (21-days)	
Shrimp	*Palaemon varians*	Secondary consumer	Spatial avoidance	
Fish	*Sparus aurata*	Carnivores Secondary consumer	Survival test (96-h)	
Fish	*Cyprinodon variegatus*	Omnivore Secondary consumer	Sub-lethal effect: endocrine disruption (*cyp19* & *vtg1*) (3-days)	

A battery of acute toxicity tests with invertebrates were performed with the brine shrimp *Artemia franciscana*, the sea urchin *Paracentrotus lividus*, and the mussel *Mytilus galloprovincialis*, beside the tests with yolk sac larvae of the fish *Sparus aurata* (Table 2.1). For each test, early life stages of the organisms were exposed to environmental concentrations of HHCB and AHTN. The acute toxicity tests were performed following standard protocols and endpoints related to fertilization success, larvae development, motility and mortality were evaluated.

For each test, IC_x/EC_x (concentrations that cause growth inhibition or any effect) values were calculated using the ICPIN statistical program, provided by USEPA on its website, for the analysis of toxicity data of the chemicals.

2.3. Spatial avoidance test

Traditional toxicity testing involves exposure of organisms to environmental stressors in a closed condition, known as forced exposure, where the organisms are continually exposed to the test substance. Under this condition, the endpoints recorded may be totally linked to the toxic effects of the stressor. On the other hand, spatial avoidance is an endpoint measured in a non-forced exposure system that permits emigration of organisms from a noxious environmental condition to a safer area, thus indicating the aversive character of the samples (Jutfelt *et al*., 2017; Tierney, 2016). This approach has been used as a behavioral response to simulate how organisms will potentially respond to a scenario of heterogeneous pollution in the marine environments, detecting and avoiding potentially dangerous contaminant concentrations.

Determination of active avoidance was first performed by Shelford and Allee (1913), but spatial avoidance to assess organisms' behavioral response to contaminants was later performed with fish in tubes containing contaminants and clean water at both ends, thus allowing the fish to detect differences in contamination levels and to move to less contaminated environment (Jones, 1947). Different exposure systems including two compartments, steep gradients, laminar flow chambers, avoidance/preference chambers, fluvarium systems and dilution gradients involving several compartments have been developed and employed to assess contamination-driven avoidance (Folmar, 1976; Gunn and Noakes, 1986; Hartwell *et al*., 1989; Moreira-Santos *et al*., 2008; Richardson *et al*., 2001; Smith and Bailey, 1990; see also review by Jutleft *et al*., 2017).

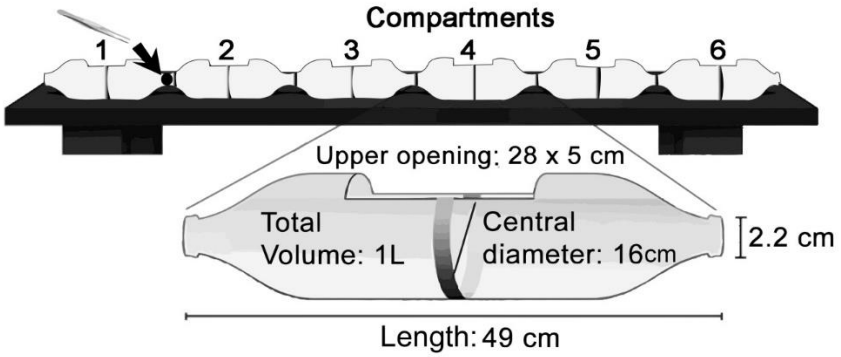


Figure 2.2: Non-forced multi-compartmented static exposure system used in the avoidance tests (Islam *et al*., 2019).

The use of the free-choice, non-forced, multi-compartmented exposure system (Figure 2.2) developed by Lopes *et al*. (2004), in which contamination gradients or patches are simulated, have also proven to be a suitable approach to assess how contaminants can interfere in the spatial distribution of organisms (see review by Araújo *et al*., 2016b, Araújo and Blasco, 2019). Laboratory exposure of fish, amphibians, decapods, mollusks, dipterans, copepods and annelids to fungicides, effluents, metals and organic compounds to determine avoidance responses at sublethal concentrations in a free-choice multi-compartmented exposure system have been reported and indicated that contaminants potentially can, at some extent, drive the spatial distribution and habitat selection processes by organisms (Araújo *et al*., 2016a, 2014b, 2014c, 2014a; Dornfeld *et al*., 2009; Moreira-Santos *et al*., 2008; Rosa *et al*., 2012; Silva *et al*., 2017). This non-forced, multi-compartmented approach simulates a realistic heterogeneous contamination scenario where organisms are not restricted to continuous exposure like in the forced exposure, thus providing a complementary approach to environmental risk assessment based, not exclusively on toxicity, but on contamination-driven displacement patterns of organisms.

2.4. Chronic toxicity test and the use of biomarkers in ERA

Occurrence and concentrations of contaminants in environmental matrices is determined by chemical monitoring, yet the presence of chemical substances in a compartment of an aquatic ecosystem does not necessarily mean toxic effects. Therefore, it must be confirmed that there is a connection between external levels of exposure, internal levels of tissue contamination and early adverse effects (van der Oost *et al*., 2003). Early warning signs (biomarkers) for detecting adverse effects are important to protecting ecosystem health because risk management based on population effects in response to contamination mitigation may

be irreversible. Therefore, simple and rapid tools capable of giving relevant information regarding toxicity of a contaminant in any environmental compartment is required. Biomarkers are quantitative measures in body fluids, cells or tissues indicating biochemical change that potentially alter the physiology of organisms consequent to exposure to contaminants or other environmental stressors (Hook *et al*., 2014; Lionetto *et al*., 2019; van der Oost *et al*., 2003). They are good indicators that organisms have been exposed to xenobiotics and have been distributed within tissues, causing toxic effects at sensitive sites.

Biomarkers used in both laboratory and field experiments can supply essential relationships between laboratory toxicity and field assessment and it offers many advantages for comparing the relative toxicity of specific chemicals or complex effluents (van der Oost *et al*., 2003). Advantageously, they serve as intermediates between contaminant sources and higher level effects and, very importantly, give information on the potential toxicity of pollutants rather than mere quantification of their presence (Hook *et al*., 2014).

Biomarkers have been used in ERA of many legacy contaminants in aquatic ecosystems. Luna-Acosta *et al*. (2015) used biomarkers to establish the potential relationships between organic contaminants accumulated in the soft tissues of juvenile oysters, defense response and physiological condition by transplanting oysters in different sites of the Marennes–Oléron Bay. The authors determined correlation between PAHs and DDT body burdens, antioxidant, and immune-defense responses in the oysters. Furthermore, many studies on potential toxicity of organic contaminants such as endosulfan, phenanthrene, aroclor 1254, PAHs have been linked with oxidative stress and DNA damage in aquatic organisms under laboratory conditions (Griffitt *et al*., 2007; Park *et al*., 2009; Rodgers *et al*., 2020; Tao *et al*., 2013; Zhang *et al*., 2014). Biomarkers have also become very useful in detecting the potential toxicity of contaminants of emerging concern, domestic and industrial effluents in aquatic ecosystems. Recently, activities of pharmaceutical and personal care products in the aquatic ecosystems were studied by examining different biomarkers in echinoderm, bivalves, mollusks, and fish; effects related to oxidative stress, DNA damage, neurotoxicity, endocrine disruptions were linked with exposure to these contaminants (Aguirre-Martìnez *et al*., 2015; Aguirre-Martínez *et al*., 2016, 2015b; Desbiolles *et al*., 2018; Yu *et al*., 2013). Despite the usefulness of biomarkers in ERA, limitations related to species representativeness, cascade effects of one biomarker on other biomarkers responses and data interpretation are challenges confronting the usefulness of biomarkers (Hook *et al*., 2014; van der Oost *et al*., 2003). Therefore, caution must be exercised when interpreting results of laboratory toxicity tests (Hook *et al*., 2014).

Biomarkers are broadly categorized into biomarker of exposure, which indicate the degree an organism is exposed to environmental contaminant or its metabolites, or the product of an interaction between a chemical agent and target molecule or cell that can be measured in a compartment within an organism

(Hook *et al*., 2014; van der Oost *et al*., 2003). Biomarker of effect indicates quantifiable biochemical, physiological alterations or other changes in an organism exposed to environmental contaminants or its metabolites producing effects linked to adverse health status or disease (Hook *et al*., 2014; van der Oost *et al*., 2003). On the other hand, biomarkers of susceptibility reflect the changes in an organism that makes it susceptible to environmental contaminants. Biomarkers of exposure and effect have been widely incorporated in field and laboratory studies and have provided useful information for environmental risk management of many contaminants.

2.4.1. The use of clams as model organism for biomarker-based evaluation of marine contamination

Coastal ecosystems and transitional waters are the most known biologically reproductive ecosystems and consist of rich biodiversity due to high food supply, nutrients, and sunlight availability, and they are home to many marine plants and animals. Yet, they are the most contaminated ecosystems due to anthropogenic activity, constantly being inundated with contaminants from agricultural runoffs, industrial and municipal discharges. Therefore, in a point source pollution scenario, the ability of an organism to survive depends on its capacity to fight (from a physiological point of view) against or fly (from a behavioral point of view) from contamination. While pelagic organisms might rapidly avoid such areas by emigrating, sedentary species like bivalves are only partially able to drift in part and are constantly exposed to contaminants, making them good bioindicator species for biomonitoring of coastal ecosystems.

The clams *Ruditapes philippinarum* (Table 2.1) are euryhaline marine benthos that originated from the Indo-Pacific region but have been introduced into coastal waters along the Atlantic and Mediterranean corridors for aquaculture purposes. They are highly successful and have been found to dominate native species (*R. decussatus*) in Europe (Moschino *et al*., 2010). As infauna suspension filter-feeders inhabiting sandy-mud bottoms, they burrow into sediments and thrust their inhalant siphon at or below the sediment surface to feed (Kanaya *et al*., 2005; Moschino *et al*., 2012). They feed mainly on particulate organic matters, microphytobenthos and sediment organic matters (Dias *et al*., 2019; Komorita *et al*., 2014). Because of their feeding behavior and ecology, inhabiting water, and sediment interface, they have been recommended as good bioindicator species for investigating sediment and water contamination (Li *et al*., 2006; Moschino *et al*., 2012).

The use of *R. philippinarum* as a model organism for biomarker-based assessment of marine contamination gained traction in the last decades and has widely been used to investigate the potential toxicity of pesticides

(Tao *et al*., 2013), metals (Aouini *et al*., 2018; Ji *et al*., 2019; Santana *et al*., 2017; Wang *et al*., 2011), wastewater effluent (Díaz-Garduño *et al*., 2018, 2016; Maranho *et al*., 2015a; 2015b), pharmaceutical products (Aguirre-Martinez *et al*., 2013; 2016; Almeida *et al*., 2015; Matozzo *et al*., 2012; Milan *et al*., 2013; Trombini *et al*., 2019) and nanoparticles (De Marchi *et al*., 2017; Marisa *et al*., 2018, 2016; Volland *et al*., 2015). Therefore, this research explores the possibility of the clams to provide useful information required for the ERA of HHCB and AHTN in marine environments after chronic exposure by evaluating biochemical alterations in digestive gland and gonad tissues.

2.4.2. Biomarkers used in this study

Biomarkers for assessing the impacts of contaminants or environmental stressors on organisms can be measured at different levels of biological organization and includes biochemical, molecular, physiological, histopathological, and behavioral changes. This study determined biochemical, molecular and behavioral changes in marine organisms exposed to environmental concentrations of HHCB and AHTN. Hook *et al*. (2014) recommended a combination of suites of biomarkers measured across multiple levels of function to allow better estimation of the contaminant eliciting changes in fitness with concomitant changes in cellular function and fitness. Additionally, redundancy in measures would likely reduce the probability of a false positive that could occur owed to a spurious result in a single measure. The suite of biomarkers used in this study are discussed below.

2.4.2.1. Ethoxyresorufin-O-deethylase (EROD)

The marine ecosystems are constantly inundated with contaminants emanating from anthropogenic activities, consequently subjecting the inhabiting organisms to stress of combating the effects of contaminants. An important step involved in coping with the stress of environmental contamination is to metabolize xenobiotics to excretable and harmless form. This toxicants biotransformation mechanism is mediated by the cytochrome P450-dependent monooxygenase (MO) or mixed-function oxidase (MFO) system (van der Oost *et al*., 2003). The cytochrome P450 isoforms are a large and versatile assemblage of enzymes that possess membrane bound heme-protoporphyrin (Rewitz *et al*., 2006; Sharifian *et al*., 2020). Depending on their location in the cell, they can be classified into two including microsomal P450s which is resident in the membrane of endoplasmic reticulum and function in phase I biotransformation of exogenous lipophilic substances, notably PAHs, pesticides, drugs, and other substances with similar chemical structure. The second class of cytochrome P450 are the mitochondrial P450s which are presumed

to metabolize endogenous substances like steroid hormones. However, reports have demonstrated that some mitochondrial P450s are actively involved in biotransformation of exogenous substances (Rewitz *et al*., 2006). Cytochrome P450 enzymes catalyze different reactions, including oxidation, reduction, and hydrolysis. Phase I xenobiotic biotransformation is an oxidative reaction catalyzed by cytochrome P450-dependent MFO system in a reaction cycle initiated by substrate binding with the prosthetic heme ferric ion in the enzyme leading to reduction via electron transfer from NADPH reductase. The process is catalyzed by the addition of an atom of dioxygen to the substrates (van der Oost *et al*., 2003; Rewitz *et al*., 2006; Sharifian *et al*., 2020).

Among the enzymes that catalyze phase I xenobiotics biotransformation, EROD seems to be the most sensitive probe for measuring the response of the cytochrome P4501A in aquatic organisms (van der Oost *et al*., 2003). Biochemical measurement of EROD activity is assayed fluorometrically, initiated by NADPH to detect changes in 7-hydroxyresorufin, determined as the quantity of resorufin produced per mg protein per minute (Gagné, 2014). This cost-effective method is rapid and produces valuable information on xenobiotics bioavailability. EROD activity has been employed in numerous fields and laboratory assessment of a variety of aromatic polycyclic compounds and other chemicals with structural similarity, because these compounds can activate the Aryl hydrocarbon receptor. Notably, PAHs have been reported to induce EROD activity in fish (Fuentes-Rios *et al*., 2005; Joachim *et al*., 2017; Vieweg *et al*., 2017), shrimps (da Silva Rocha *et al*., 2012), clams (Zhang *et al*., 2014) and scallops (Guo *et al*., 2017a; Jin *et al*., 2014; Xiu *et al*., 2016). Similarly, pesticides and other organic micropollutants have been reported to induce EROD activity in marine organisms (Almeida *et al*., 2010; Larguinho *et al*., 2014; Tao *et al*., 2013).

The applicability of EROD activity in ERA of contaminants span beyond legacy contaminants. In the last decades, increasing evidence has shown the relevance of EROD activity as a biomarker of exposure to emerging contaminants including pharmaceutically active and personal care products. Exposure of the polychaeta *Hediste diversicolor* to several pharmaceutical products for 14-day, fluoxetine, 17α-ethynyl estradiol and propranolol showed significant increase EROD activity (Maranho *et al*., 2014). Similarly, other pharmaceutical and personal care products have been reported to have increased EROD activity in bivalves and fish (Aguirre-Martinez *et al*., 2013; Grabicova *et al*., 2013; Li *et al*., 2012; Martín-Díaz *et al*., 2009; Zhang *et al*., 2020). Therefore, application of this biomarker will help us understand the bioavailability and biotransformation of HHCB and AHTN in the marine environments.

2.4.2.2. Glutathione-S-transferase (GST)

The byproducts of phase I metabolism are not in all cases excretable and in some instances could be more toxic than the parent compounds and, therefore, need to go through phase II metabolism. This phase involves conjugation reactions where nonpolar compounds are conjugated with a polar functional group resulting in a non-bioactive, excretable form (Omiecinski *et al*., 2011). However, xenobiotics possessing carboxyl, hydroxyl and amine functional groups are directly detoxified via phase II conjugation reaction without initial phase 1 detoxification (Sharifian *et al*., 2020; van der Oost *et al*., 2003). The conjugation of xenobiotics is catalyzed by the enzymes "transferases", GST being the most widely studied biomarker of phase II metabolism. GST catalyzes the conjugation of electrophilic substances and metabolites of phase I metabolism with glutathione for subsequent detoxification. In addition to phase II detoxification, GST plays a significant antioxidant function, mopping up reactive oxygen species (ROS) that could attack important proteins and nucleic acids molecules due to chemical and environmental stress. Varieties of GST isoenzymes (alpha, mu, pi, theta, zeta and sigma-like GST) have been identified in aquatic organisms associated with intracellular detoxification and defense mechanism (Bathige *et al*., 2014; Omiecinski *et al*., 2011; Umasuthan *et al*., 2012; van der Oost *et al*., 2003).

For biochemical analysis of GST as biomarker of environmental contamination, total GST is mostly assayed using 1-chloro-2,4-dinitobenzene (CDNB) as substrate. Other substrates have also proven to be useful in assessing the induction of GST activity in aquatic organisms exposed to xenobiotics. While selecting biomarkers for assessing environmental contamination, the molecular biology of the model organism must be known to understand the capacity of the organism to give relevant information due to exposure to environmental chemicals. Out of 15 GST isoforms found in plants and animals, 7 (μ, Ω, δ, θ, ρ, π and GST microsomal) have been characterized and isolated in gills, hemolymph, digestive gland and gonads of *R. philippinarum* (Bathige *et al*., 2014; Umasuthan *et al*., 2012), hence the choice of this species in this study.

The induction and/or inhibition of GST catalytic activity in aquatic organisms after exposure to contaminants have been widely reported and van der Oost *et al*. (2003) reviewed the significant and suitability of GST activity in fish for ERA. GST catalytic activity in *R. philippinarum* have been used to study the environmental effects of metals (Aouini *et al*., 2018; Wang *et al*., 2011), pharmaceutical products (Aguirre-Martínez *et al*., 2016; Maranho *et al*., 2014; Matozzo *et al*., 2012; Trombini *et al*., 2019) and nanoparticles (De Marchi *et al*., 2017; Marisa *et al*., 2018, 2016; Volland *et al*., 2015).

2.4.2.3. Glutathione Peroxidase (GPx) and Glutathione Reductase (GR)

ROS are endogenous byproducts of metabolism, generated in the cell of aerobic organisms during normal cellular activities related to phosphorylation, mitochondria and microsomal transport chains and oxido-reductase enzymes activities (Regoli and Giuliani, 2014). ROS are radical and non-radical oxygen species such as superoxide anion, hydrogen peroxide and hydroxyl radical and are essential in cell-signaling and homeostasis, balanced by antioxidant enzymes to guarantee cellular integrity. However, environmental change such as hypoxia condition, elevated water temperature, water acidification, increased pH and contaminants exposure can strongly potentiate the intracellular production of oxyradicals via the induction of CYP 450 pathway (Regoli and Giuliani, 2014). Therefore, exposure to PMCs is suspected to produce similar effects, being highly lipophilic (Balk and Ford, 1999).

Elevated oxyradicals in the cell might overwhelm the coping capacity of antioxidants, consequently, leading to cytotoxic adverse effects including oxidative stress (Regoli and Giuliani, 2014; Sureda *et al*., 2011). Furthermore, antioxidants scavenge the interaction of oxyradicals with important biomolecules in a complex network involving scavengers and antioxidant enzymes including GPx and GR. GPx is one of the most important antioxidant enzymes responsible for breaking the product of superoxide dismutase (H_2O_2) into water, giving off oxygen in the process. GPx catalytic activity requires reduced glutathione (GSH) as electron donor, and GSH is oxidized in the process to glutathione disulfide (GSSH) (Regoli and Giuliani, 2014). In the reaction loop, GSSH is catalytically converted back to GSH by GR in the presence of NADPH as a cofactor. GR maintains the balance between GSH and GSSH ratio, the criticality of any alteration in GR enzymatic activity impinges this equilibrium, reducing glutathione scavenging potential with concomitant oxidative stress.

Biochemical analysis of GPx and GR follows the measurement of fluorescein in a highly sensitive fluorescence. Consequently, the induction or inhibition of GPx and GR in marine organisms exposed to environmental chemicals is a useful biomarker in ERA and have been widely employed in laboratory and field studies.

2.4.2.4. Lipid Peroxidation (LPO)

LPO is the oxidative degradation of lipid molecules initiated by oxyradicals in the cells of organisms. As earlier discussed, exposure to contaminants potentiates ROS in the cell, which are converted to harmless products by antioxidants. However, antioxidants enzymes can be overwhelmed by excess ROS, limiting their coping capacity and consequently degrading the cell membrane phospholipid bilayer that protects the

cell by its selective permeability. Lipid peroxidation occurs when ROS attack lipids such as polyunsaturated fatty acids (PUFAs) by replacing hydrogen atoms from its methylene bridge with oxygen, to form fatty acid radicals and water. The fatty acid radicals react with oxygen to produce peroxyl fatty acid radicals and these unstable radicals continue to propagate by reacting with free fatty acid radical and lipid peroxidation. Peroxidation of lipid bilayer in the cell membrane is significant to organism's physiology, interfering with cellular integrity by reducing the cell's selective permeability to substances that might pose danger to nuclear materials in the cell.

The endpoints of lipid peroxidation are production of reactive aldehydes such as malondialdehyde (MDA) and 4-hydroxynonenal (4HNE) (Ayala *et al*., 2014). MDA is widely used as a potent marker of LPO and can be measured biochemically following the thiobarbituric acid reactive substance (TBARS) procedure (Wills, 1987). The method involves heating biological samples with reagents consisting of trichloroacetic acid and thiobarbituric acid, using tetramethoxypropane (TMP) as a standard solution. MDA in the biological sample reacts with the reagent to form a pink chromogen, quantified spectrophotometrically at 516 nm (excitation) and 600 nm (emission) filter (Aguirre-Martínez *et al*., 2016).

LPO has been extensively used as indices of oxidative damage in aquatic organisms exposed to contaminants in both field (Rodríguez-Ortega *et al*., 2002; Sureda *et al*., 2011) and laboratory (Aguirre-Martínez *et al*., 2016; Maranho *et al*., 2014; Silva *et al*., 2012) studies.

2.4.2.5. DNA Damage

Oxidative stress due to chemical exposure has been known to effectuate DNA damage. DNA is a highly reactive molecule, and it is susceptible to endogenous and exogenous chemical modifications. Most endogenous DNA damage occur during hydrolysis and oxidation of the chemically reactive DNA with water and ROS, respectively. This process has been demonstrated to contribute to hereditary diseases and cancer predisposition in humans (Ayala *et al*., 2014; Perrone *et al*., 2016). On the other hand, exogenous DNA damage occurs consequent to exposure to physical, chemical and environmental agents leading to excess ROS production in the cells. Overabundance of ROS causes approximately 100 different oxidative base lesions and 2-deoxyribose modifications and has been linked with human diseases (Ayala *et al*., 2014). Furthermore, chronic exposure to chemical agents can compromise DNA backbone potentiating single strand breaks.

For environmental monitoring purposes, DNA strand breaks or damages are measured in organisms as a biomarker of exposure and effect to genotoxic substances and provide opportunity to evaluate genotoxic

chemicals in aquatic ecosystems, providing information regarding environmental health and also serve as early warning signals of environmental contamination. Although DNA damage as a biomarker of environmental contamination does not provide any specific mechanism of effect, its applicability to many cell types makes it an important biomarker of exposure to genotoxicants.

A number of approaches have been used to quantify DNA damage in the laboratory. This study employed the DNA precipitation assay described by Olive (1988) based on 2% SDS-KCl precipitation of DNA-Protein crosslink, which uses fluorescence to measure DNA strands (Gagnè *et al*., 1995). When DNA breaks because of exposure to contaminants, the strands are released from cellular protein into the supernatant when centrifuged at low speed (Aguirre-Martínez *et al*., 2016). It is then possible to quantify the amount of double or single stranded DNA at the end of the assay (Gagnè *et al*., 1995). This method is reliable own to its rapidity and cost-effectiveness and have been widely used in field and laboratory studies to assesses genotoxicity of effluents, PAHs, pesticides, pharmaceuticals, and personal care products in the aquatic environments (Gagné *et al*., 2017; Guo *et al*., 2017b; Mamaca *et al*., 2005; Xiu *et al*., 2016).

2.4.2.6. Acetylcholinesterase (AChE)

AChE is a cholinergic enzyme present at the postsynaptic cleft of neuromuscular junction and catalyzes the hydrolytic metabolism of the neurotransmitter acetylcholine (Gaitonde *et al*., 2006). In environmental biomonitoring, AChE has widely been used as a biomarker to diagnose the exposure of organisms to neurotoxic substances that can bind with AChE active site, preventing its catalytic deactivation of acetylcholine. The mechanism of action follows that when action potential develops, acetylcholine is released from the presynaptic cleft into the synaptic cleft and is catalytically degraded by AChE into choline and acetate, therefore transmitting neural messages across the cells. However, AChE inhibitors interfere with this process by binding to AChE catalytic sites, leading to build up of acetylcholine (Gaitonde *et al*., 2006; Sturm *et al*., 2000; van der Oost *et al*., 2003).

AChE inhibition has been reported in bioindicator species exposed to organic compounds, metals, pharmaceutical and personal care products in field and laboratory studies (Aguirre-Martínez *et al*., 2016; Maranho *et al*., 2015; Matozzo *et al*., 2005; Stefano *et al*., 2008). Chronic AChE inhibition has the propensity for continuous nerve firing, alteration of postsynaptic cell function, and cholinergic toxicity (Nallapaneni *et al*., 2008; Pope *et al*., 2005; Song *et al*., 2004; Waseem *et al*., 2010). Therefore, early detection of exposure to AChE inhibitors in marine environments measured in bioindicator species could facilitate protection of ecosystems health.

AChE catalytic activity can be measured in post-mitochondrial fraction using 5,5-Dithiobis (2-nitrobenzoic acid) (DTNB) solution to determine the absorbance of AChE (Guilhermino *et al*., 1996). Although this method could be prone to interference, yet, it is sensitive, rapid and cheap, making it suitable as a biomarker of neurotoxicity in the marine ecosystems.

2.4.2.7. Cyclooxygenase (COX)

Cox is a bifunctional enzyme of the fatty acid oxygenases belonging to the myeloperoxidase superfamily, responsible for oxidation of arachidonic acid to prostaglandins (Chandrasekharan and Simmons, 2001; Sales and Jabbour, 2003). Prostaglandin biosynthesis is thought to be catalyzed by two COX-isoenzymes (COX-1 and COX-2); however, COX-3 has been isolated in mammals, regarded as spliced variant of COX-1 and play a different role in eicosanoid biosynthesis (Sales and Jabbour, 2003; Sharma *et al*., 2019). COX-1 is constitutively expressed and plays an active role as analgesic of inflammation by non-steroidal anti-inflammatory drugs (NSAID), while COX-2 is inductively expressed.

COX is a major target of anti-inflammatory drugs; its inhibition is analgesic to symptoms of inflammation and has therefore been used as a biomarker of inflammation in environmental monitoring (Díaz-Garduño *et al*., 2018). Various pharmaceutical products have been reported as inducer of COX in *Corbicula fluminea* (Aguirre-Martínez *et al*., 2018), *M. edulis* (Gagné *et al*., 2017), *H. diversicolor* (Maranho *et al*., 2015) and *Oryza latipes* (Flippin *et al*., 2007). As well, wastewater effluent induced COX activity in *R. philippinarum* (Díaz-Garduño *et al*., 2018) and *Elliptio complanata* (Gagné *et al*., 2007), suggesting that other contaminants apart from pharmaceuticals may alter COX activity in marine organisms. In addition, COX plays a vital role in reproductive physiology and pathology. Its synthesis of prostaglandins is involved in the control of oogenesis and spermatogenesis in aquatic invertebrates (Di Costanzo *et al*., 2019) and controls the effect of serotonin in the spawning process in bivalves (Matsutani and Nomura, 1987). COX catalytic activity is assayed in the laboratory following the oxidation of 2,7-dichlorofluoresceine in the presence of arachidonate (Fujimoto *et al*., 2002) and formation of fluorescein is quantified fluorometrically at 485/530 nm. The methodology provides a rapid and cost-effective means to assess alteration of COX activity in organisms exposed to environmental contaminants.

2.5. Molecular biomarkers of endocrine disruption

A wide variety of contaminants in the marine environments are endocrine disruptors because they interfere with the biosynthesis, transport, metabolism, or removal of natural hormones responsible for maintaining

homeostasis and regulation of developmental processes in marine organisms even at low concentrations (Goksøyr, 2006; Scholz and Mayer, 2008). The impacts of endocrine disrupting chemicals (EDCs) in fish physiology and reproductive health are enormous as these chemicals can alter sex determination, interfere with growth, lower immunity, and reduced survivability. Most research on the effects of EDCs in fish are focused on the steroid reproductive hormones simply because these hormones control important endpoints in environmental risk assessment of potential EDCs and, again, sex different in fish is very labile from embryonic stage as these chemicals tends to interfere and result in skewed sex ratio. However, because hormones are secreted by internal glands and transported as chemical messengers in blood to target cells, using plasma concentrations of hormones as endocrine disruption endpoints are challenging and laborious. Research has shown that endocrine disruption is vividly connected with molecular interactions and consequently expression of appropriate biomarkers can be used to predict reproductive effects of EDCs. Using small fish provides a huge advantage in this regard because they are easy to handle in the laboratory, they are cost effective, easy to breed with many young and short life-cycles (Scholz and Mayer, 2008).

Like all vertebrates, reproduction in fish is controlled by the brain-pituitary-gonadal axis where signals from brain control the release of luteinizing hormone and follicle stimulating hormone from the pituitary gland. These gonadotropin hormones control the synthesis and release of steroid hormones from the gonads that are responsible for the control of sexual behavior, development of secondary sexual characteristics and gametes development and maturation. Therefore, biomarkers directly involved in the steroidogenesis and steroid biosynthesis are important molecular endpoints of endocrine disruption in fish. As a result, this PhD thesis studied two important steroidogenic molecular biomarkers that play key roles in fish reproduction to elucidate the endocrine disrupting potential of HHCB and AHTN in marine environments.

Relative expressions of *cyp19* and *vtg1* are molecular biomarkers of endocrine disruption. *Cyp19* gene codes for the cytochrome P450 enzyme aromatase, an important biomarker of endocrine disruption in teleost (Cheshenko *et al*., 2008). Aromatase is the enzyme involved in the conversion of androgen to estrogen which play a key role in the control of sexual differentiation, maturation and reproduction (Cheshenko *et al*., 2008). *Cyp19* is regarded as a major target for endocrine disrupting chemicals because modulation of its expression and function may potentially disrupt the level of estrogen production (Cheshenko *et al*., 2008; Kazeto *et al*., 2004) and it is frequently assessed in fish because its susceptibility to endocrine disruptors is conserved throughout life (Le Page *et al*., 2011). On the other hand, *vtg1* is an estrogen receptor element gene that regulates vitellogenin synthesis in its promoter region and it is largely expressed in the liver of adult fish (Tingaud-Sequeira *et al*., 2012; Tran *et al*., 2019) but studies have shown that this gene is active in 24 hour post fertilized embryos (Hao *et al*., 2013; Muth-Köhne *et al*., 2016). Therefore, alteration in *vtg1* expression will reduce vitellogenin production which could lead to significant

reduction in oocyte quality and maturation in female teleost (Muth-Köhne *et al*., 2016). Also, expression of both genes provides understanding of mechanism of action and represent molecular initiating events in the adverse outcome pathways of endocrine disrupting chemicals.

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Chapter 3

Studies

3.1. Acute toxicity and overview of the potential risks of galaxolide and tonalide on structure and functioning of marine ecosystems

Summary

This section addressed the short-term toxicity of environmental concentrations of the polycyclic musk compounds (PMCs) Galaxolide (HHCB) and tonalide (AHTN) in the marine environments. HHCB and AHTN have been detected in various environmental compartments and in biological tissues. Yet, there are limited data regarding the ecotoxicity of these compounds in the marine environmental, making difficult to fully assess the risk they might pose to marine biota. Therefore, the aim of this study was to assess the effects of these substances on *Paracentrotus lividus, Artemia franciscana, Mytilus galloprovincialis, Sparus aurata* early life stages, and growth inhibition of the marine microalgae, *Phaeodactylum tricornutum, Tetraselmis chuii, Isochrysis galbana* when exposed to a range of concentrations of 0.005 – 5 µg/L of each compound. Then, to use the endpoints data to characterize their environmental risk to marine biota following EU guidelines. Therefore, endpoint related to growth inhibition of the exposed microalgae were measured after 72-h exposure. For the early life stages tests, endpoints related to *A. franciscana* motility and survival, *P. lividus* fertilization and larval development, *M. galloprovincialis* larval development and *S. aurata* survival were measured after exposure to HHCB and AHTN using static bioassays.

The results obtained in this study showed that environmental concentrations of HHCB and AHTN have minimal to moderate effects on microalgae growth, and in some cases, the growth was confounded by the presence of the organic solvent used in the dissolution of the tested chemicals. Also, the growth inhibition results showed that microalgae are differentially sensitive to both compound because only *P. tricornutum* and *I. galbana* were found to be at risk due to the presence of these substances in the marine environments. For the early life stages tested, it was clear that both compounds have no effects on artemia motility and survival because all the tested concentrations returned over 90% motility and survival of the young Artemia. Furthermore, *P. lividus* larval development proved to be the most sensitive endpoints and both substances significantly reduced *M. galloprovincialis* and *P. lividus* larval development. Even though fish survival test is mostly used for regulatory purpose to prioritize chemicals, the impact of HHCB and AHTN on the survival of *S. aurata* was moderately sensitive making it impossible to calculate the median effective concentrations. Because the goal of environmental protection is to protect the most sensitive species, *P. tricornutum* and *I. galbana* growth inhibition tests, and *P. lividus* and *M. galloprovincialis* larval development were recommended for screening of these substances and other contaminants of emerging concern in the marine environments.

The results of this study have been published in a peer reviewed journal as an open access publication entitled “The effects and risk assessment of the polycyclic musk compounds Galaxolide® and Tonalide® on

marine microalgae, invertebrates and fish (**Ehiguese *et al***., 2021. **Processes** 9, 371. https://doi.org/10.3390/pr9020371). The accepted manuscript formated to the journal recommended reference style is presented below.

The author's personal contribution included conceptualization, methodology, samples analyses, data curation, project management, original draft preparation, writing and editing of manuscript as well as first and corresponding authorship of the article.

The experimental set up were carried out at the Laboratorio 8 - INMAR, Physical Chemistry Department, University of Cadiz, Puerto Real Campus, Spain.

Introduction

Polycyclic musk compounds (PMCs) have been included in the priority lists of the European Commission existing substances regulation [1]. PMCs are synthetic chemicals, produced in large quantities as a replacement for nitro musks, which have been banned because of their environmental persistence and adverse effects on humans and the environment [2]. They have wide applicability in household and personal care products such as detergents, shampoos, lotions, perfumes, as well as additives in cigarettes and fish baits [2], [3]. Due to their high water solubility, inherent lipophilicity and biological stability, coupled with external application and the fact that they do not undergo biotransformation, it is not surprising to find them as contaminants in aquatic ecosystems at concentrations ranging from ng/L to µg/L [4].

In particular, the PMCs marketed as Galaxolide® (HHCB) and Tonalide® (AHTN) represent about 95% of total fragrance materials in the perfume industry [5] and are the most commonly detected PMCs in environmental compartments and biological tissues [6]–[12]. Fromme *et al*. [8] detected HHCB and AHTN in surface water in Berlin (Germany) at concentration values ranging from 70 – 1590 ng/L and 20 – 530 ng/L, respectively. Similarly, Heberer [3] also reported high levels of HHCB and AHTN in surface water in Berlin at concentrations ranging from 20 – 12500 ng/L and 30 -1100 ng/L, respectively. Moreover, these substances have been detected in surface waters in almost every country in Europe [8], [12], [13]. Although most studies were for the freshwater environment, there are data confirming their presence in the marine environment [6], [7], [11], [14], and reported values over 5 µg/L depending on the proximity to a sewage treatment plants (STP). Sumner *et al*. [11] studied the transport of PMCs from an STP effluent into coastal waters and reported that the concentrations of HHCB and AHTN in the open sea depends on the distance from STP and the dilution power towards the open sea [11]. For instance, the authors measured the concentration of HHCB in STP effluent ranging from 987 – 2098 ng/L, being diluted towards the sea over 2 km, to 6 – 28 ng/L. However, this level could be higher near urban coasts where STP effluents are directly discharged into coastal waters, which could represent a potential threat to the survival of nearshore organisms, an important part of the marine ecosystems.

Bioaccumulation of PMCs, particularly HHCB and AHTN, have also been reported in marine organisms including crustaceans, bivalves, fish, marine birds and mammals [9], [15]–[17] at levels of environmental concern. The concern over the potential effects is not only related to the environment, but also the impacts on food safety and, consequently, public health. Therefore, Vandermeersch *et al*. [18] reviewed emerging contaminants in seafood, acknowledging that HHCB and AHTN were the most commonly detected PMCs, with concentrations reaching 160 and 45 µg/g lipid weight in mollusks and fish, respectively [19]. Similarly, in the framework of the FP7 ECsafefood project, where 62 commercial seafood samples (mackerel, tuna,

salmon, seabream, cod, monkfish, crab, shrimp, octopus, perch and plaice) in the European Union were analysed for residues from personal care products, HHCB was detected at concentrations ranging from 2.5 – 414.4 µg/kg dry weight, with the highest concentration measured in fish (sole) and AHTN found at concentrations ranging from 2.5 – 12.2 µg/kg dry weight [20]. Furthermore, HHCB and AHTN have the potential to elicit adverse effects in marine organisms due to the bio-concentration factors (based on lipid content) of 3504 and 5017 [8] as consequence of high octanol water partition coefficients (LogKow) of 5.9 and 5.7 [21], respectively.

Data on the acute, sub-chronic and chronic aquatic toxicity of HHCB and AHTN are available for algae, crustaceans, mollusks, bivalves and various fish [22]–[31]. Although the majority of the studies were focused on freshwater ecosystems, Breitholtz *et al*. [22] and Wollenberger *et al*. [30] investigated the acute, sub-lethal and lethal effects of these substances on the marine copepods, *Nitocra spinipes* and *Acartia tonsa*, respectively. While Wollenberger *et al*. [30] concluded that HHCB and AHTN inhibited larval development in *A. tonsa* at low concentration and should be considered very toxic, Breitholtz *et al*. [22] reported low adverse effects in *N. spinipes*.

Several attempts have been made to assess the environmental risk of HHCB and AHTN in the aquatic environment. Balk and Ford [5] provided an insight by using acute and chronic toxicity data from freshwater and terrestrial organisms to estimate the environmental risk, but with no reference to the marine environment. Other environmental risk assessments (ERAs) performed so far [1], [32]–[34] revealed a paucity of ecotoxicity data for the marine environment needed to effectively assess the risk of these substances.

The aim of this research was to assess the potential environmental risk of HHCB and AHTN in the marine environment by exposing organisms from different trophic levels at early life stages to environmental concentrations of HHCB and AHTN. Therefore, acute toxicity tests were performed using marine organisms such as microalgae (*Phaeodactylum tricornutum*, *Tretraselmis chuii* and *Isochrysis galbana*), crustaceans (*Artemia franciscana*), echinoderms (*Paracentrotus lividus*), bivalves (*Mytilus galloprovincialis*) and fish (*Sparus aurata*), and a candidate freshwater alga – *Raphidocelis subcapitata*. Secondly, the potential risks these substances might exert on the marine ecosystems were estimated following European Chemical Agency (ECHA) guidelines [35]. Furthermore, understanding the toxicity of HHCB and AHTN in lower trophic organisms will help to predict possible bottom-up and top-down effects, which could lead to a functional and structural disruption of the ecosystems [36]–[39]. The species used in the current study have been used to examine the effects of effluents from sewage treatment plants [40], [41], metals [42], [43], organic solvents [44] and inorganic chemicals [45], [46] in marine environments.

Also, these species have been endorsed by international organisations for ERA of contaminants due to their sensitivity[35] [47]–[50].

2. Materials and Methods

2.1. Chemical selections

Analytical grade HHCB and AHTN were purchased from Sigma Aldrich, Spain. The physiochemical properties, preparations in organic solvent and determination of exposure concentrations followed details found in Ehiguese *et al*. [51]. In brief, HHCB and AHTN were dissolved in dimethyl sulfoxide (DMSO) (0.001% v/v) in glass vials to form a stock solution. Concentrations (0.005, 0.05, 0.5 and 5.0 µg/L) of each substance were prepared by diluting the stock solutions in 18.2 MΩ-cm Nanopure water.

2.2. Acute Toxicity Test

2.2.1. Microalgae growth inhibition test

Microalgae growth inhibition tests were performed using three marine and one freshwater species, following the procedure reported by Garrido-Perez *et al*., [52]. Inocula of *P. tricornutum*, *T. chuii* and *I. galbana* (marine species) were provided by the Laboratory of Marine Culture of the University of Cádiz, Spain, and *R. subcapitata* (strain 61.81) was provided by the Culture Collection of Algae at Goettingen University, Germany. Seawater microalgae culture media was prepared by adding nutrients (macro- and micronutrients and vitamins) according to f/2 medium [53] to synthetic seawater according to the formula from USEPA [50]. For the freshwater species, the culture media were prepared according to the concentrations of COMBO Media [54] in Nanopure water. Before exposure to HHCB and AHTN, an inoculum from each species of microalgae was cultured in fresh media and in the same chamber of assay (19±1 ºC; 11000 lux; photoperiod 14/10 light/day). Inocula were maintained for three days to reach the exponential growth phase, in order to provide acclimatised and healthy cells for the tests.

An aliquot of 0.25 mL of each inoculum was added to 3.75 mL of the culture media spiked with different concentrations of contaminants. The exposure was performed in triplicates including two controls: (1) culture media without contaminants (normal growing of the microalga in a fresh medium), and (2) culture media without contaminants but including DMSO (to test the toxic effects of the organic solvent). The addition time of the microalgae was as short as possible, to ensure that they had similar biomass at the initial

time. After 30 min inoculation, the initial biomass (B_0) was measured, representing time zero (T_0). The biomass was measured indirectly through absorbance at 680 nm (maximum chlorophyll peak in a fresh culture of microalga), with a TECAN 2000 micro-well plate reader. This measurement was repeated at 24, 48 and 72 h. The endpoint observed in this test was the inhibition of biomass growth at 72 h compared to the control. The minimal growth acceptable for the control was 16 times higher than the initial density.

2.2.2. Artemia toxicity test

The cysts of *A. franciscana* are certified biological material (AF450, INVE) and were acquired from Acuazul, S.C. (Spain). Before starting the trial, dehydrated artemia cysts were induced to hatch. In a one-litre Erlenmeyer flask containing 300 mL of natural seawater (35 g/L salinity), approximately 100 mg of cysts were resuspended and left with aeration and continuous illumination throughout the hatching time, which occurs within 24-36 h. Once the artemia hatched, the aeration was removed. The hatched nauplii were placed in clean seawater to avoid moving empty or unhatched cysts. Therefore, all nauplii used in the test belong to the same cohort. The test was carried out in triplicates using Petri dishes made of glass, and 60 mm diameter. On each plate 10 nauplii were placed using a glass Pasteur pipette to avoid stress to the nauplii. Subsequently, seawater spiked with each contaminant was added to a total volume of 10 mL. The test was conducted at 20±0.4 ºC and in the dark to minimize the swimming of the nauplii and the consumption of energy. The test lasted for 72 h and records were taken at 24, 48 and 72 h. The *Artemia* were counted using a stereomicroscope at a magnification of 2x and 4x, identifying dead specimens (mortality was recorded when they showed no sign of any movement of their limbs for 30 seconds) and those that presented motility problems such as movements in circles, asynchronous, among others. The test was considered acceptable when survival of over 80% was recorded in the control group after 72 h.

2.2.3. Sea urchin toxicity test

The sea urchin *P. lividus* fertilization and larval development tests were performed following procedures described by Fernandez and Beiras [55], and Environment Canada [48]. Individuals were collected from the uncontaminated rocky subtidal environment off the Bay of Cádiz (Spain) at 1.5 – 2 m depth. They were immediately transported to the laboratory in a cooler box. Matured individuals were dissected, and eggs and sperm were collected using a micropipette. For the fertilization test, 10 µL of sperm were added to the aliquots containing 10 mL of the test concentrations arranged in sequence for 10 min; then, 1 mL of eggs was added to each test tube, swirled gently, and allowed to proceed for 10 min. The endpoint for fertilization

success was the presence of a fertilization membrane. A larval development test was performed by adding 1 mL of fertilized eggs to beakers containing test solutions in triplicate, including the controls (seawater and DMSO), in dark conditions for 48 h. The test was considered valid when development in the control was ≥ 80% and the result expressed as percentage of normal pluteus stage, normalized to the corresponding seawater control. Both fertilization and larval development tests were conducted in a controlled chamber at a temperature of 20 ºC.

2.2.4. Mussel larvae development test

The mussels *M. galloprovincialis* were purchased from an aquaculture farm in north-western Spain. The depurated mussels were transported to the laboratory at 8 ºC. Animals with matured gonads were held at 9 ºC for two weeks to acclimate to the laboratory condition. The toxicity test was performed following the ASTM protocol for acute toxicity of saltwater bivalves [56]. Mussels were placed each in a beaker containing filtered seawater and induced to spawn by thermal stimulation at 19 ºC. Eggs and sperm were filtered to remove debris using 75 µm and 37 µm screens, respectively. Before fertilization, the egg and sperm quality and density were evaluated under a microscope. Aliquots of 10 µL of sperm were added to the eggs for fertilization (10^6/egg) and fertilization success was assessed under the microscope. Fertilized eggs were added to test solutions at 50 eggs/ml and incubated for 48 h at 16±0.2 ºC. The test was performed in triplicate including seawater and solvent controls (DMSO). Samples were fixed with 40% formalin at the end of the test and 100 larvae were counted under the microscope (X 40 magnification) per replicate distinguishing between normal developed larvae (D-shaped) and malformed larvae. Test results were accepted when normal developed larvae in control was ≥80%.

2.2.5. Fish larva mortality test

The effects of environmental concentrations of HHCB and AHTN on the survival of seabream (*S. aurata*) larvae was tested following OECD guidelines for fish early-life stage toxicity test [47]. Yolk-sac larvae (3 – 5 h post hatch) of *S. aurata* were obtained from the laboratory of Marine Culture, Faculty of Marine and Environmental Sciences, University of Cádiz, Spain. Individuals (n = 70) of the yolk-sac larvae were added to each beaker (Pyrex©) containing 600 ml of seawater spiked with the concentrations of contaminants, and each treatment was run in triplicate. Seawater and solvent (DMSO) controls were also tested. The exposure proceeded for 96 h, physiochemical parameters were monitored using the CRISON CM35+ and 40MM+

multiparameter probes (Crison-Hachs Lange S.L.U., Spain) and values recorded during the exposure were: Temperature (16.01±0.2 ºC), salinity (34.2±0.3‰), pH (7.7±0.2) and oxygen (>5 mg/L). The test was considered valid if mortality did not exceed 10% in the control group. Mortality in each treatment group was recorded and data were expressed as the percentage of survived larvae at each experimental condition.

2.3. Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics, version 24.0. Significant differences between organisms exposed to the different concentrations of PMCs or to the organisms from control were checked using a one-way ANOVA followed by multiple comparisons of Turkey's or Dunnett's test. Statistically significant differences were set at $p < 0.05$. Spearman's rank order of correlation was calculated between the measured effects and the exposure concentrations for pairwise comparison, setting significance levels at $p < 0.05$ and $p < 0.01$. In addition, SPSS Probit response model and PriProbit 1.63 softwares [57] were used to calculate the IC_x/EC_x (concentrations that cause growth inhibition or any effect, respectively, to *x*% of the population).

The Risk Quotient (RQ) was derived from the ratio of the measured environmental concentrations (MECs) listed in Table 1 to the PNEC (predicted no effect concentration). PNEC is the effect concentrations (EC) or inhibition concentration (IC) obtained from the tests divided by an assessment factor (European Comission Joint Research Centre, 2003).

$$\mathrm{RQ} = \frac{\mathrm{MEC}}{\mathrm{PNEC}} = \frac{\mathrm{MEC}}{\frac{\mathrm{EC}}{1000}} \text{ or } \frac{\mathrm{MEC}}{\mathrm{IC}/10} \qquad (1)$$

Where:

If RQ < 1 = no risk expected, and no further evaluation required

If RQ > 1 = potential risk and further evaluation is required

3. Results

3.1. Microalgae growth inhibition tests

Data of growth inhibition for microalgae are presented in Figure 1. There was no critical difference between microalgae exposed to seawater control and solvent control, although microalgal growth response was observed in the group treated with DMSO. In brief, it is possible to observe that toxicity of both compounds was minimal for the four microalgae, although AHTN seemed to be slightly more toxic. For *P. tricornutum*, growth inhibition for both compounds were observed in some concentrations. However, significant differences ($p < 0.05$) in growth in relation to the control only occurred with exposure to AHTN at 0.005 µg/L by 16.2% (Figure 1A). On the one hand, *T. chuii* growth was inhibited by AHTN and the decrease in biomass was significantly ($p < 0.01$) concentration dependent (Table S1 – Supplementary Materials). On the other hand, the exposure to HHCB produced a stimulation (hormesis) in the growth (Figure 1B) and similar biphasic responses was seen in *I. galbana* biomass after exposure to both musk compounds (Figure 1C). The freshwater microalgae, *R. subcapitata* growth decreased after exposure to HHCB and AHTN. In the case of AHTN, significant ($p < 0.05$) concentration dependent growth inhibition of *R. subcapitata* was observed (Table S1 – Supplementary Materials), and the highest inhibition occurred at 5 µg/L by 23.5%. In contrast, *R. subcapitata* growth inhibition by HHCB was more severe at 0.05 µg/L by 14.5% in relation to the control (Figure 1D).

3.2. Artemia, sea urch, mussels and fish early life stage toxicity tests

For the organisms tested for fertilization, larvae development, motility and mortality, the validity criteria for the control experiments (seawater control and solvent control – DMSO) were not exceeded. Moreover, there was no significant difference ($p < 0.05$) in responses detected in organisms exposed to seawater control and DMSO.

The effects of HHCB and AHTN on survival and motility of *A. franciscana* are shown in Figure 2A and 2B, respectively. Both responses showed no significant difference ($p<0.05$) compared to the control. For the *A. franscana* mortality test, percentage mortalities in the controls were 3.33% and 6.67% for seawater and DMSO, respectively. The effect of HHCB on the survival of *A. franciscana* was very minimal with the highest mortality of 10% recorded at a 0.5 µg/L HHCB. The effect of AHTN on *A. franciscana* mortality was also very low, with only 3.33% mortality at 0.05 and 0.5 µg/L. Other concentrations of AHTN tested had no effect on *A. franciscana* survival. Artemia motility test was not sensitive to either compound (Figure 2B).

Table 1: Measured Environmental Concentrations (MEC) of Galaxolide (HHCB) and Tonalide (AHTN) in seawater from different locations. Measured values are in ng/L. Maximum values (bold) were used in calculating risk quotient (RQ).

Country/Location	**HHCB**	**AHTN**	**Reference**
Germany (North Sea)	0.09 - **4.8**	0.08 - **2.6**	[6]
Germany (Elbe Estuary)	95 - **136**	65 - **200**	[6]
United Kingdom (Tamar Estuarine - Plym Sound)	6.00 - **30**	3.00 - **15**	[11]
Spain (Bay of Cadiz)	**230±0.1**	NA	[14]
Singapore (Coastal water)	1.66 - **21.8**	0.244 - **1.85**	[58]

NA = Data not available.

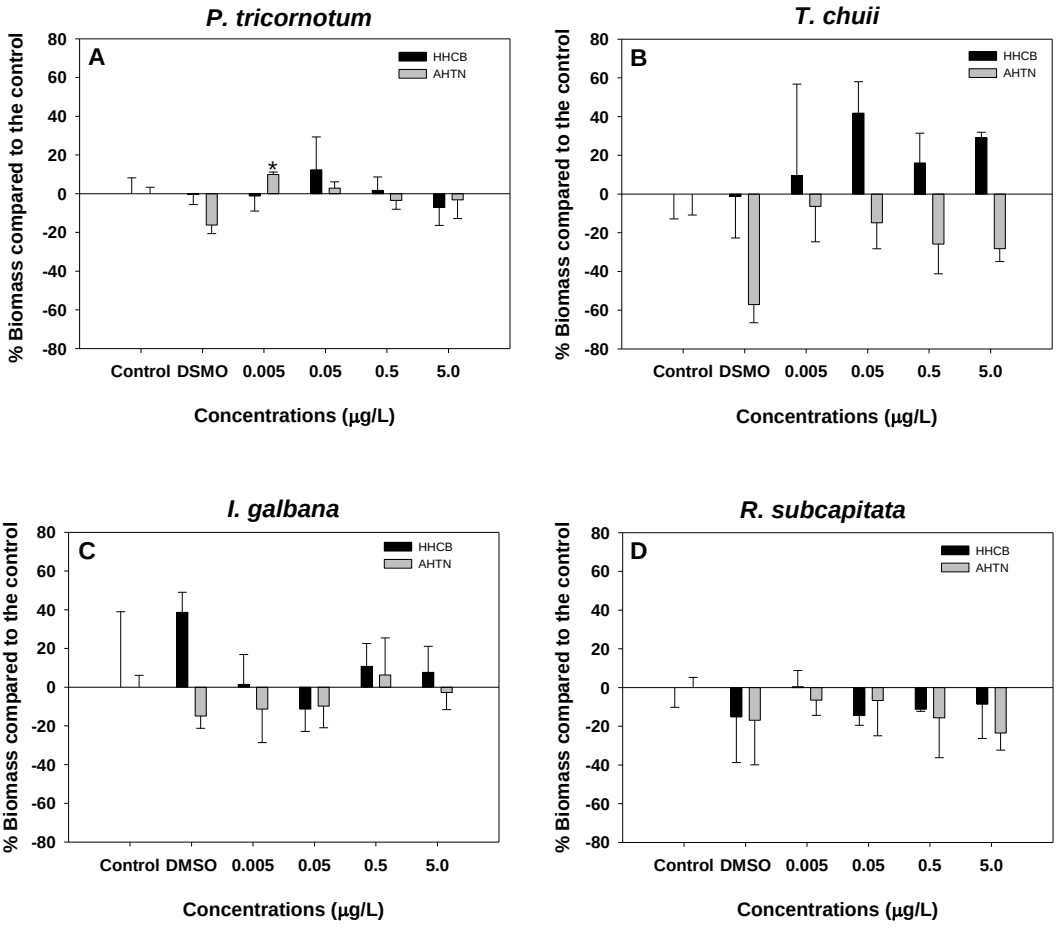

Figure 1, A - D: Microalgae exposed to galaxolide and tonalide for growth inhibition tests after 72 hours. Biomass (%) compared to the control of (A) *P. tricornotum*, (B) *T. chuii*, (C) *I. galbana* and (D) *R. subcapitata* (negative values corresponding to % growth inhibition). Asterisks (*) indicate significant difference ($p<0.05$) in relation to control.

The effects of HHCB and AHTN on *P. lividus* fertilization and larval development tests are presented in Figure 2C and 2D, respectively. The percentage of sea urchin that were able to fertilize in the controls were 89.50% and 90.00% for seawater and DMSO, respectively. Percentage fertilization of sea urchin tested with 0.005 µg/L, 0.05 µg/L, 0.5 µg/L and 5 µg/L HHCB were 86.33%, 82.33%, 81.00% and 82.33%, respectively. Under exposure to AHTN, sea urchin fertilization success recorded at 0.005 µg/L, 0.05 µg/L, 0.5 µg/L and 5 µg/L were 89.00%, 83.33%, 86.33% and 86.33%, respectively. Although the effect is minimal, *P. lividus* fertilization failure induced by both substances was more pronounced for HHCB with significant effects at 0.05 – 5 µg/L, while a significant difference ($p < 0.05$) was only observed at 0.05 µg/L AHTN compared to control (Figure 2C). The results for the sea urchin larval development tested with

HHCB and AHTN are presented in Figure 2D. The percentages of larval development in the seawater and solvent controls were 83.50% and 81.50%, respectively. The number of oocytes that were able to develop to pluteus stage was significantly ($p < 0.01$) concentration dependent (Table S2 – Supplementary Materials) and a significant ($p < 0.05$) decrease in larvae development was recorded at 5 µg/L HHCB compared to the control (Figure 2D). The percentage of larval that were able to develop from morula stage to normal pluteus stage for 0.005 µg/L, 0.05 µg/L, 0.5 µg/L and 5 µg/L HHCB were 85.00%, 85.00%, 68.00% and 44.33%, respectively, while similar concentrations of AHTN reduced larvae development by 63.00%, 75.33%, 54.67% and 62.33%, respectively.

The results for the larval development toxicity test for *M. galloprovincialis* exposed to HHCB and AHTN are presented in Figure 2E. The number of fertilized eggs that developed after 48 h to D-veliger stage in the control was 98%. However, the embryotoxicity of HHCB to mussels was significantly ($p < 0.01$) concentration dependent (Table S2 – Supplementary Materials). From 0.05 – 5 µg/L HHCB, the percentage of abnormal larvae development was significantly ($p<0.05$) different from the control (Figure 2E) and the percentage effect increased to 19.88% in the highest concentration tested (5 µg/L). Similarly, significant toxicity of AHTN to embryos of *M. galloprovincialis* was observed in 0.5 µg/L and 5.0 µg/L with percentages of abnormal larvae of 8.36% and 11.63%, respectively (Figure 2E).

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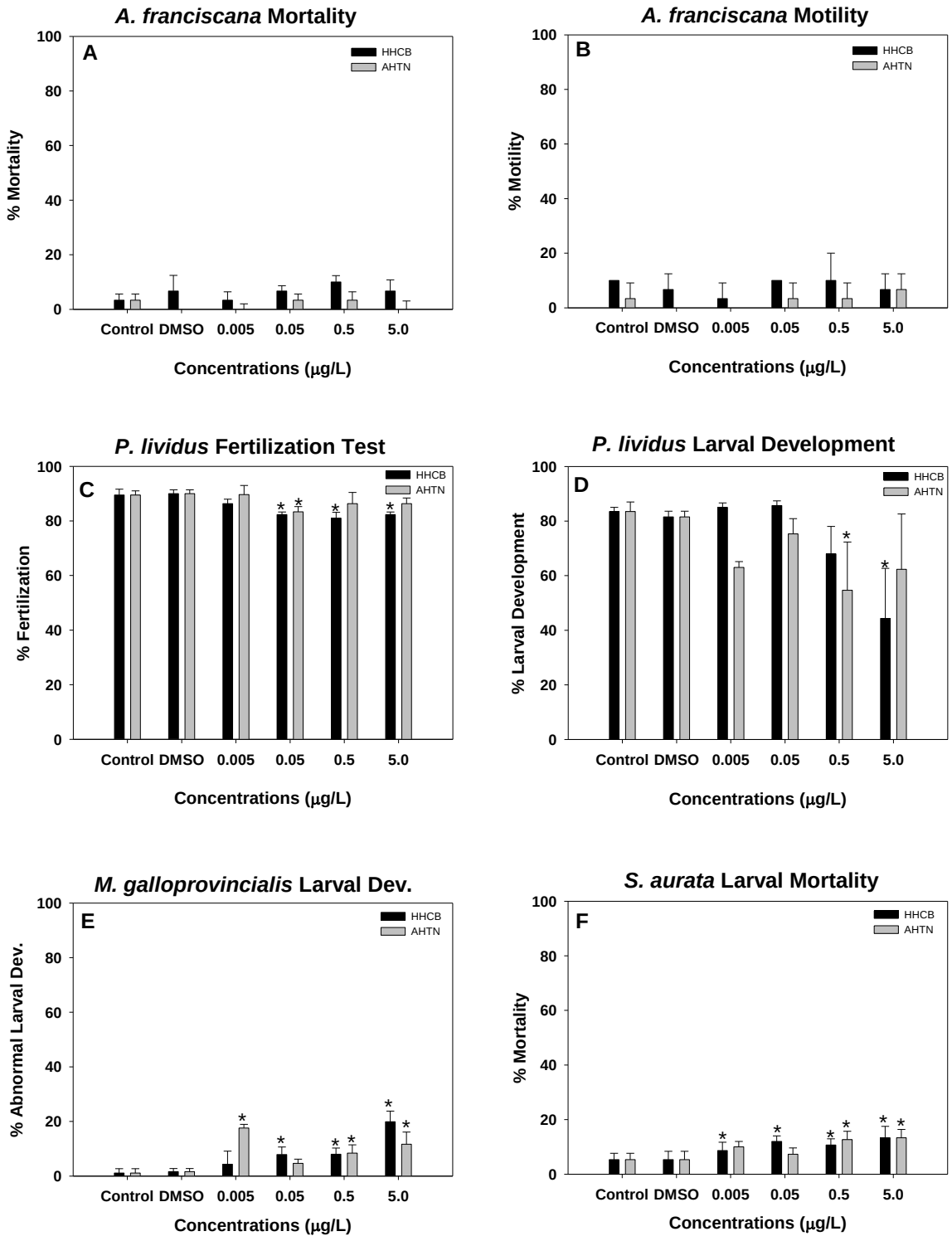

Figure 2: *A. franciscana* 72 h mortality and motility, *P. lividus* fertilization and 48 h larval development, *M. galloprovincialis* 48 h larval development and *S. aurata* 96 h larval mortality tests exposed to galaxolide and tonalide. Asterisks (*) indicate significant difference ($p<0.05$) in relation to control.

The results of the effect of HHCB and AHTN on the survival of yolk-sac larvae of *S. aurata* larval after 96 h exposure was significantly ($p < 0.01$) concentration dependent (Table S2 – Supplementary Materials) and the measured effect is presented in Figure 2F. After 96 h, the percentage mortality of yolk-sac larvae of *S. aurata* in seawater and DMSO controls was 5.33% each. Percentages of mortality in fish exposed to 0.005 µg/L, 0.05 µg/L, 0.5 µg/L and 5 µg/L of HHCB were 8.67%, 12.00%, 10.67% and 13.33%, respectively. On the other hand, the percentage mortality of fish tested with the same range of concentrations of AHTN were 10.00%, 7.33%, 12.67% and 13.33%, respectively.

3.3. Risk Quotient (RQ)

Quantitative risk estimation of chemicals in the environment is achieved using monitoring data of MEC and PNEC, giving a risk quotient necessary for risk characterisation. A literature survey of MEC for both polycyclic musk compounds were undertaken, and the values are presented in Table 1. Due to minimal effects detected in this study for all the species of microalgae, *Artemia*, sea urchin, mussels and fish, it was not possible to calculate the EC_{50} values and their respective confidence intervals, except for *P. lividus* larvae development tested with HHCB, producing an EC_{50} value and 95% confidence interval of 4.063 (0.963 – 120.731) µg/L. The IC_{10}/EC_{10} calculated for *P. tricornutum, I. galbana, P. lividus* and *M. galloprovincialis*, including the risk quotients using the MEC/PNEC ratio are presented in Table 2. The results of the microalgae growth inhibition and larval development tests showed that HHCB and AHTN posed high risk to the growth of *P. tricornutum* and *M. galloprovincialis* larval development at environmental relevant concentrations. Also, environmental concentrations of HHCB and AHTN pose ecological risk to *P. lividus* larval development and *I. galbana* growth, respectively (Table 2).

4. Discussion

The aim of this study was to evaluate the impacts of environmentally relevant concentrations of HHCB and AHTN on marine microalgae, *Artemia*, sea urchin, and mussels after short-term exposure and, where possible, characterise the risk following the ECHA guideline for ERA [35] Several *in vitro* and *in vivo* toxicity tests have been performed with these compounds using freshwater species [5], [24], [25], [28], [59], [60] and the only tests with marine species used high concentrations [22], [30], making it difficult to compare such results with actual environmental impacts. Furthermore, for evaluation of toxicity of

substances, it is imperative to use a varied battery of tests because organisms are not equally susceptible to the same toxic substance.

Due to the instability of these substances under laboratory exposure, it is difficult to estimate the exact concentration causing toxic effects. Although we could not measure the concentrations of the exposure water and bioaccumulation because of practical constraints, studies have shown that after 3 h of exposure, over 30% was lost and this reduced to ca. 80% after 96 h [22], [30], [51], [61]. Again, there are currently no techniques to measure the concentrations of these substances *in-situ*; therefore, there is a probability that reported environmental concentrations are a fraction of the actual concentration in the marine environments eliciting biological effects, given that some amount could be lost before or during sample analysis in the laboratory. Consequently, risk estimation was based on maximum concentration reported.

The effects of HHCB and AHTN on *P. tricornutum, T. chuii, I. galbana and R. subcapitata* showed that both compounds have limited effects (significantly similar to the control treatment) on microalgae growth (Figure 1). Although the statistical analysis indicated no differences between control with and without DMSO, the results for microalgae should be considered with caution, because in some situations inhibition or enrichment due to DMSO was observed. Therefore, we cannot reject the possibility that the effects observed in the presence of HHCB or AHTN was partially caused by DMSO.

Table 2: EC_{50} and EC_{10} (µg/L) and their respective confidence interval (CI), and estimated risk quotients of Galaxolide (HHCB) and Tonalide (AHTN) as the MEC (maximum value per location)/PNEC ratio.

		Microalgae growth		Embryo-Larval development	
		P. tricornutum	*I. galbana*	*P. lividus*	*M. galloprovincialis*
HHCB	EC_{50}	NC	NC	4.063 (0.963 – 120.731	NC
	EC_{10}	0.127(NC)	5.22(NC)	0.004 (0.000 – 0.025)	0.188(0.074 – 0.390)
	MEC/PNEC	0.378 – 18.110	0.009 – 0.440	1200 - 57500	25.532 – 1223.404
	Risk	Yes	No	Yes	Yes
ANTH	EC_{50}	NC	NC	NC	NC
	EC_{10}	0.002(0.000 – 0.014)	0.328(NC)		0.006 (NC)
	MEC/PNEC	24 - 1150	0.146 – 7.0122	NC	800 – 38333.330
	Risk	Yes	Yes	NC	Yes

NC = Not calculated.

Microalgae have been used in water quality assessments as *in-situ* bio-monitors because they are primary producers at the base of the ecological trophic arrangement and the basic supplier of oxygen in aquatic ecosystems [62]. Microalgae toxicity tests are useful in ERA and have gained international recognition leading to development of test guidelines for reliable and relevant toxicity data [63]. The potential of HHCB and AHTN to inhibit the growth of microalgae in the aquatic ecosystem have been scarcely reported. Previous studies revealed that significant acute toxicity of microalgae occurred at concentration greater than 100 µg/L or 500 µg/L depending on the species [5], [64] but our data suggest that even at low concentrations microalgae growth might be inhibited.

The microalgae growth inhibition tests were mildly sensitive to HHCB and AHTN; IC_{10} values for most of the microalgae could not be calculated, except for *P. tricornutum* and *I. galbana* exposed to HHCB with IC_{10} values of 0.127 and 5.220 µg/L, respectively (Table 2). In contrast, previous studies have reported higher EC_{50} values of 0.050 mg/L for *Navicula* spp. and 0.336 mg/L for *Scendesmus quadricauda* exposed to HHCB [64]. This is because microalgae are not equally sensitive to contaminants. For example, in all the microalgae tested, we found that differential sensitivity was exhibited, the order of sensitivity to HHCB was *P. tricornutum* > *I. galbana*, and to AHTN was *T. chuii* > *P. tricornutum* > *I. galbana*. Similarly, differential sensitivity of two microalgae to HHCB was recently reported, whilst the microalgae, *Navicula* spp. are more sensitive to HHCB than *S. quadricauda* [64]. The basis for the differential sensitivity of microalgae to these contaminants is beyond the scope of the current investigation. Furthermore, HHCB and AHTN are lipophilic and known to bioaccumulate in marine organisms [5], [15], [64]. Remarkably, the effects of these compounds on microalgae growth has been adduced to bioaccumulation, altering antioxidant enzymes and biochemical processes, resulting to decrease microalgae growth [64].

Artemia was not sensitive to either substance since the endpoints measured were not critically different from the control (Figure 2: A & B). This is because *Artemia* is known to be fairly resistant to toxic substances in comparison to other invertebrates and they respond differently to different environmental contaminants [65], [66].

HHCB significantly reduced the fertilization success of *P. lividus* as the concentrations increased. Meanwhile, AHTN had no significant effects on sea urchin fertilization success (Figure 2C). Sea urchin fertilization success depends on sperm fitness, motility potentiated by the axonemal engine in the flagellum, morphology and chemotaxic navigation [67]. For organoleptic substances, such as HHCB and AHTN, the latter might be the most compelling factor inducing reduction in sea urchin fertilization success since the exposure procedure involved prior treatment of sperm with the contaminants before the introduction of eggs. Between the two contaminants tested, HHCB had more significant effects on *P. lividus* fertilization by 19% reduction at 0.5 µg/L, and we observed that only 0.05 µg/L AHTN significantly reduced sea urchin fertilization by 16.67% (Figure 2C). Importantly, the effect exerted by both contaminants on sea urchin fertilization success was below 20%, presenting less toxic effects compared to other contaminants of emerging concern. For example, 500

ng/L propanol, 500 ng/L 17α-ethinylestradiol and 5000 ng/L gemfibrozil reduced sea urchin fertilization success by 24.1%, 36.9% and 26.9%, respectively [68]. Similarly, other contaminants of emerging concern belonging to pharmaceutical and personal care products have been reported to significantly affect sea urchin fertilization success at concentrations detected in the environment [69], [70].

Ecotoxicity studies with early life stages of aquatic organisms have been recommended as a faster and more cost-effective means of examining chemicals and environmental samples, because newly hatched larvae are sensitive to exogenous substances as the embryos lose their protective membranes and are fully exposed to potential xenobiotics [71]. *P. lividus* and *M. galloprovincialis* are well recognized in toxicity bioassays and are applied globally for the evaluation of toxicity of marine contaminants by the exposure of gametes to aqueous phases, such as surface waters and pore waters [69], [72] and elutriates [73]. Critical effects were detected in *P. lividus* and *M. galloprovincialis* larvae exposed to HHCB and AHTN compared to the control (Figure 2D and E). The effects of HHCB on sea urchin larvae development was significantly ($p < 0.01$) concentration dependent (Table S2 – Supplementary Materials) and significantly different ($p < 0.05$) compared to the control, with only 44.33% of larvae able to develop to pluteus stage after 48 h exposure to 5 µg/L HHCB. The sensitivity of sea urchins to environmental chemicals is widely reported and significant evidence showed that they represent an important biomonitoring tool for ecosystems health. Similarly, HHCB and AHTN significantly ($p < 0.05$) affected the development of *M. galloprovincialis* larvae when compared to the control (Figure 2E). However, when considered in relation to the number of oocytes exposed to each contaminant, the effects were minimal with the highest percentage of deformed and undeveloped oocytes being 19.88% and 17.60% for HHCB and AHTN, respectively. Although *P. lividus* and *M. galloprovincialis* larvae development tests were similar, the effects recorded were more pronounced in sea urchin larvae than mussels.

Significant ($p < 0.01$) concentration dependent responses (Table S2 – Supplementary Materials) were observed in *S. aurata* mortality tests with HHCB and AHTN (Table S2 – Supplementary Materials). Notwithstanding, the percentage mortality of *S. aurata* exposed to both fragrances not up to 20%, the highest effect being 13% for HHCB and AHTN at the highest concentration of 5 µg/L. Although this species and endpoints proved to be sensitive to other contaminants at low concentrations [40], [68], the impacts of environmental concentrations of the tested fragrances in this study were low. For chemical prioritization, fish early-life stage toxicity test is endorsed [47] because it's a reliable and reproducible risk assessment tool that requires shorter exposure time and lower cost to perform. However, the sensitivity of fish embryotoxicity to some emerging contaminants remain doubtful as previous studies reported low sensitivity [68], [74].

Risk characterization of contaminants is quantified using MEC/PNEC ratio and for aquatic environments (freshwater and marine), PNEC is estimated by dividing the EC_x value by an assessment factor of 1000 for acute toxicity test and 10 for chronic toxicity test [35]. Although short term toxicity tests were performed in this study, an assessment factor of 1000 was used only for larval development test while an assessment factor of 10 was used for the microalgae because the exponential phase of microalgae growth was regarded as a full

life stage and therefore considered as a chronic test. Analyzing the RQs, HHCB represented potential high risk for the marine environment based on the EC_{50} estimated for *P. lividus* larvae development. Also, HHCB and AHTN posed high ecological risk to *M. galloprovincialis* larval development at environmental relevant concentrations. The larval development of *P. lividus* and *M. galloprovincialis* seem to be very sensitive to chemical exposure because previous studies of environmental contaminants in coastal waters have reported significant toxicity of industrial and domestic effluent [40], pharmaceutically active products [68], [75], UV-filters [70] and organic pollutants [46], [72] to sea urchin and mussels larvae development. The quantitative risk estimate of HHCB obtained in this study for *P. lividus* using EC_{10} value (1200 - 57500) was higher than that of propanol (0.02 – 17.29), previously reported by Capolupo *et al*. [68]. We also found that both compounds posed high risk to *P. tricornutum* and *I. galbana* based on IC_{10} values recorded (Table 2). Microalgae have broadly been used in evaluation of ERA for other emerging contaminants. For example, *I. galbana* have been reported to be significantly affected by UV filters and pharmaceutical active ingredients [70], [75]. Fragrances have been demonstrated to pose high risk to microalgae in the marine environment, of which HHCB and AHTN were more toxic than musk xylene and musk ketone [7]. The adversity of measured environmental concentrations of HHCB and AHTN to *P. tricornutum* and *I. galbana* deserve attention because microalgae occupy the lowest trophic level of the marine food chain and, therefore, serve as food for higher trophic organisms. Again, HHCB and AHTN impacts on microalgae growth represent a potential bottom-up effect that might result in structural and functional disruption of the ecosystems [37]. Therefore, more studies are required to fully understand the environmental effects of these contaminants in the marine environments.

5. Conclusion

This study evaluated the potential risk of comparable measured environmental concentrations of HHCB and AHTN to microalgae and early life stages of marine organisms, including *A. franciscana, P. lividus, M. galloprovincialis* and *S. aurata*, adding to the dearth of information regarding the adverse effects of HHCB and AHTN in the marine environment. For all the species of microalgae, Artemia, sea urchin, mussels and fish tested, differential sensitivity was observed. Artemia motility and survival were the least sensitive endpoints affected by both substances and *P. lividus* larvae development was the most sensitive species/endpoint. From our data, the environmental risk of HHCB and AHTN was characterized as high for *P. tricornutum, I. galbana* growth, *P. lividus* and *M. galloprovincialis* larvae development. Therefore, more studies are required to understand the sub-lethal effects of these compounds in the marine environment.

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Conflict of Interest: The authors declare that there is no conflict of interest for the publication of this present work and are willing to provide any clarification whenever called upon to do so.

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3.2. Spatial avoidance as a complementary tool for environmental risk assessment of galaxolide and tonalide in marine environment

Summary

Ecotoxicological data obtained from laboratory studies rely on endpoints from bioassays using forced exposure systems. In this sense, the exposed organisms are constantly in contact with the tested compounds without any option of escape to safer environments. This is not the case for motile organisms in the wild, because under a heterogenous point source pollution where contaminant gradient is established, organisms will avoid more toxic environments to a safer area. In this section, the spatial avoidance of the shrimp *Palaemon varians* was investigated using a multi-compartmented non-forced exposure system (NFS) where the shrimps were exposed to a contaminant gradient of either HHCB or AHTN. The multi-compartmented NFS consist of six compartments made of plastic bottles which was constructed by connecting the cut-out bases and mouths using glue, to obtain a six-compartmented system. To allow introduction of the test chemicals and organisms, an opening was carefully cut-out at the top of each compartment. Validation tests were performed using seawater and seawater spiked with the organic solvent (0.001%v/v Dimethyl sulfoxide – DMSO) by adding 1 L of seawater and seawater spiked with DMSO, and 3 shrimps to each compartment. Before the start of the avoidance response assay with HHCB or AHTN, plasticine blocker was placed at the junction of each compartment and 1 L of seawater spiked with different concentrations (0.00, 0.005, 0.05, 0.5, 5 and 50 µg/L) of each compound was added to each compartment. The assay was performed in quadruplicate with 18 shrimps per system. The positions of the shrimps per system were recorded every 20 mins for 3-h in the dark. A 24-h acute toxicity in a forced exposure system (FS) was performed with shrimps from the same stock using laboratory beakers. The percentage avoidance response data from NFS and percentage mortality data from the 24-h acute toxicity test were integrated to compute the percentage of population immediate decline (PID).

The results from the 24-h acute toxicity test in a FS showed that HHCB and ANTN were not very toxic to the shrimps and no concentration-dependent toxicity was observed. HHCB and AHTN elicited significant concentration-dependent spatial avoidance in the shrimps. Furthermore, the percentage PID calculated from the mortality and avoidance responses showed that in a scenario of environmentally relevant heterogenous contamination, population decline at local scale will be primarily driven by the avoidance behaviour. Therefore, environmental risk assessment of contaminants should integrate data from NFS and FS to avoid overestimation or underestimation of the risks these substances might pose to marine environments.

This study has been published in a peer-reviewed journal with the title "Avoidance behaviour of the shrimp Palaemon varians regarding a contaminant gradient of galaxolide and tonalide in seawater" (**Ehiguese, *et al*.**, 2019. **Chemosphere** 232, 113–120. doi:10.1016/j.chemosphere.2019.05.196). The accepted manuscript formated to the journal recommended reference style is presented below.

The author's personal contribution included conceptualization, methodology, samples analyses, data curation, project management, original draft preparation, writing and editing of manuscript as well as first and corresponding authorship of the article.

The NFS and FS experiments were performed at the Institute of Marine Research of Andalusia, Puerto Real, and chemical analysis of the exposure media was performed at the Physical Chemistry Department, University of Cadiz, Puerto Real Campus, Spain.

1. Introduction

Maintenance and restoration of ecological integrity is the primary objective of environmental risk assessment (Ramesh and Kaplana, 2015). Consequently, various regulatory agencies have established guidelines to protect water bodies within their jurisdiction for the sustainable use of aquatic resources (EC, 2000; EPA, 2002) and, as a result, levels of individual contaminants, water quality parameters, or description of changes in the water bodies' conditions have been established in order to protect them. The United States Environmental Protection Agency's (USEEPA) aquatic life criteria is derived from the criterion maximum concentration, with the aim of protecting aquatic ecosystems from severe acute effects, and from the criterion continuous concentration, that has been set to protect against long term effects on survival, growth, reproduction etc. (Beaman *et al*., 2008). Data from ecotoxicity studies, through which the toxic effects of substances are assessed, are extrapolated from laboratory toxicity tests involving forced exposures, which implies a continuous exposure of the organisms to a specific concentration of chemicals or environmental samples (e.g. water, sediment, soil). The forced exposure approach makes it possible to identify the potential toxicity of contaminants, determine the concentration-response relationship and provide information about their mechanisms of action (Newman and Uger, 2002; Martinez-Haro *et al*., 2015). However, applying the forced exposure approach makes it difficult to check how contaminants affect the spatial distribution of organisms and their habitat selection processes for the cases in which they are able to flee from the contaminated areas, as it may occur (for mobile organisms and heterogeneous contamination scenarios) in natural environments (Åtland and Barlaup, 1995; Hansen *et al*., 1999; Moreira-Santos *et al*., 2008).

Spatial avoidance involves the emigration of organisms from a noxious environmental condition to a safer area, thus indicating the aversive character of the area (Jutfelt *et al*., 2017; Tierney, 2016). Experimentation concerning active avoidance was first performed by Shelford and Allee (1913), but spatial avoidance to assess organisms' behavioural response to contaminants was later performed with fish in tubes containing contaminants at one end and clean water at the other, thus allowing the fish to detect differences in contamination levels and to move to a less contaminated environment (Jones, 1947). Different exposure systems including two compartments, steep gradients, laminar flow chambers, avoidance/preference chambers, fluvarium systems and dilution gradients involving several compartments have been developed and employed to assess contamination-driven avoidance (Folmar, 1976; Gunn and Noakes, 1986; Hartwell *et al*., 1989; Moreira-Santos *et al*., 2008; Richardson *et al*., 2001; Smith and Bailey, 1990; see also review by Jutleft *et al*., 2017). The use of the free-choice, non-forced, multi-compartmented exposure system developed by Lopes *et al*. (2004), in which contamination gradients or patches are simulated, have also proven to be a suitable approach to assess how contaminants can interfere in the spatial distribution of organisms (see review by Araújo *et al*., 2016b; Araújo and Blasco, 2019). Laboratory exposure of fish, amphibians, decapods, molluscs, dipterans, copepods and annelids to fungicides, contaminated effluents, metals and organic compounds to determine avoidance responses at sublethal concentrations in a free-choice multi-compartmented exposure system have been reported, and have indicated that contaminants potentially can, to

some extent, drive the spatial distribution and habitat selection processes by organisms (Araújo *et al*., 2016a, 2014b, 2014c, 2014a; Dornfeld *et al*., 2009; Moreira-Santos *et al*., 2008; Rosa *et al*., 2012; Silva *et al*., 2017). This non-forced, multi-compartmented approach simulates a realistic heterogeneous contamination scenario where organisms are not restricted to continuous exposure as they are in forced exposure, thus providing a complementary approach to environmental risk assessment based not exclusively on toxicity, but also on the displacement patterns of organisms.

The polycyclic musk compounds (PMCs) – galaxolide (HHCB) and tonalide (AHTN) – are contaminants of emerging concern (CEC). They are applied in many personal care products including detergents, lotions, deodorants and shampoos, to mention just a few (Reiner and Kannan, 2011). In Europe, they constitute about 95% of the total fragrance materials in the perfumery industry (OSPAR, 2004). The use of these substances has reportedly increased in recent years and Southern European countries are the highest consumers (Cunha *et al*., 2018; European Commission, 2008a, 2008b). PMCs are lipophilic and possess high octanol/water partition coefficient (K_{OW}) values ranging from 5.4 – 6.3 (Cunha *et al.,* 2018), so they are not readily soluble in water. The presence of these substances in environmental samples has been reported and effluents of wastewater treatment plants have been identified as the primary route of entrance into aquatic ecosystems (Petrie *et al*., 2014). The concentrations recorded are highest at wastewater treatment effluent pools (Chase *et al*., 2012; Díaz-Garduño *et al*., 2017) and decrease along the water course (Sumner *et al*., 2010). Although most environmental measurements with high concentrations (6 – 13330 ng/l) were from effluents and rivers (Chase *et al*., 2012; Dsikowitzky *et al*., 2002; Fromme *et al*., 2001; Lee *et al*., 2010; Reiner and Kannan, 2011; Zhang *et al*., 2008), up to 2098 ng/l of HHCB and 159 ng/l of AHTN have been measured, respectively, in coastal waters (Sumner *et al*., 2010).

Contamination by PMCs can be considered ubiquitous as they have been detected in many environments such as: air (Peck and Hornbuckle, 2006), sediments (Fromme *et al*., 2001; Heberer, 2002; Zhang *et al*., 2008), particulate suspended matters (Gatermann *et al*., 2002) and human adipose tissue (Kannan *et al*., 2005; Moon *et al*., 2012b). Hence, bioaccumulation of PMCs in a wide diversity of freshwater and marine organisms including cetaceans, sharks, fish and shellfish in Europe, Japan, Korea, China and the USA has been extensively studied (Gatermann *et al*., 2002; Lee *et al*., 2014; Moon *et al*., 2012a; Nakata, 2005; Nakata *et al*., 2007; Picot Groz *et al*., 2014; Rüdel *et al*., 2006; Zhang *et al*., 2013). Assessment of the potential toxicity of HHCB and AHTN based on lethality from forced exposures, their chemical properties and ability to affect the human pheromone-endocrine system suggest they may alter the structure of a community and impoverish certain ecosystems (Breitholtz *et al*., 2003; Kallenborn *et al*., 1991; Rimkus *et al*., 1997; Wollenberger *et al*., 2003). However, despite the risks of contamination that PMCs might pose to organisms, ecotoxicological assessments of PMCs in freshwater (Balk and Ford, 1999) and marine environments (Ehiguese *et al,* unpublished) are still only incipient. Efforts to evaluate the potential of HHCB and AHTN to trigger avoidance in aquatic organisms has been even less explored. Therefore, the present study first addressed the potential of HHCB and AHTN to elicit avoidance response in the estuarine shrimp *Palaemon varians* causing emigration

to areas that are less contaminated. *P varians* is a euryhaline estuarine shrimp found mainly in shallow saltmarsh pools from west Baltic and British Isles southwards to the west Mediterranean. It is ecotoxicologically important for the trophic web as it can accumulate contaminants and acts as vector for the upper trophic levels (Rainbow *et al*., 2006). Previous studies in our laboratory have indicated that the presence of chemical contaminants may trigger their evasion to favourable areas. To this end, a free-choice, non-forced, multi-compartmented exposure system was used (Araújo *et al*., 2014c) because it allows a contamination gradient of compounds to be simulated. The second aim was to predict the population immediate decline (PID) (Rosa *et al*., 2012) of *P. varians* when exposed to HHCB and AHTN by integrating avoidance and lethality in short-term experiments to evaluate whether the shrimps were able to detect toxicity and avoid potentially toxic concentrations before suffering acute effects.

2. MATERIALS AND METHODS

2.1. Test organisms

Shrimps (1.0 to 1.5 cm length) were obtained from the Salina El Pópulo aquaculture farm in San Fernando (Southwest Spain) and immediately transported to the ecotoxicology laboratory of the Institute of Marine Sciences of Andalusia (ICMAN-CSIC), in Puerto Real (Spain). They were acclimated for 2 weeks in two 250 L aquaria supplied with filtered (0.5 µm) seawater (deep-well seawater: pH of 7.5 and salinity of 35) in a flow-through system with continuous aeration. The organisms were monitored regularly, and dead shrimps were removed immediately. No food was provided during laboratory acclimation. Laboratory conditions were 20±0.5 ºC temperature and a 12:12 h light/dark photoperiod.

2.2. Chemicals

Analytical grade HHCB and AHTN (85.0% and 97.0% GC, respectively) were purchased from Sigma Aldrich Spain. Due to their low solubility in water, stock solutions were prepared using dimethyl sulfoxide (DMSO 0.001%v/v). The stock solutions were diluted using nanopure water to reach the desired concentrations (0.005, 0.05, 0.5, 5 and 50 µg/L). The filtered seawater used in the culture was spiked with each concentration for the tests.

2.3. Multi-compartmented exposure system

A non-forced, multi-compartmented static assay system (Figure 1) was used in the avoidance experiments following the design used by Araújo *et al*. (2014c). In brief, six compartments made of plastic bottles were constructed (the dimensions are stated below), which were connected at the cut-out bases and mouths of the containers using glue (Sikaflex® construction sealant, Switzerland) to obtain a six-compartmented system. An opening was carefully cut out at the top of each compartment to facilitate the introduction of organisms and contaminant.

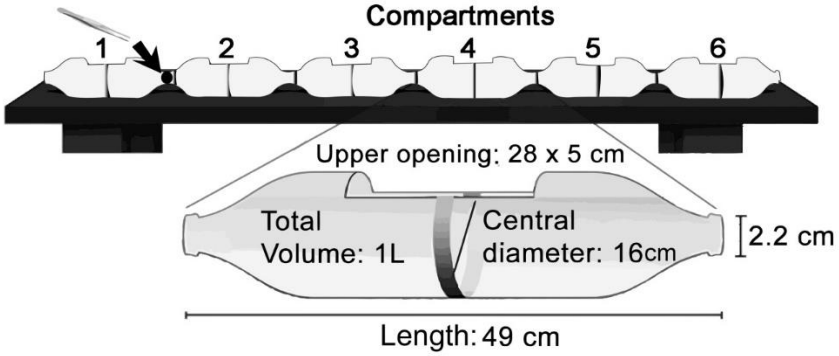

Figure 1: Non-forced multi-compartmented static exposure system used in the avoidance tests (Islam *et al*., 2019).

2.4. Avoidance Tests

To ascertain the non-interference of external factors in the avoidance tests and to verify random distribution of shrimps (no preference for any compartment), validation (control) tests with no contaminant were performed using seawater and seawater spiked with DMSO (0.001% v/v). The compartments were each filled with 1 L of seawater and then the shrimps (n = 3) were added to each compartment (18 shrimps in total per system) and the distributions of the organisms were recorded at each 20 min for 3 h. The tests were performed in quadruplicate.

For each avoidance test with HHCB and AHTN, plasticine plugs were positioned at the junction of each compartment before filling them with seawater spiked with HHCB and AHTN. The gradients of contaminants were in the order of 0 (seawater), 0.005, 0.05, 0.5, 5 and 50 µg/L. Three shrimps were added to each compartment and then the plasticine plugs were removed. The tests were conducted in quadruplicate for each substance with 18 organisms per system (three organisms × six concentrations). The distribution of the organisms in each compartment was recorded at each 20 min for 3 h. The tests were conducted in the dark at

20 ºC. At the end of each test, samples of the HHCB and AHTN in each compartment were collected using clean dark amber bottles and stored at -20 ºC for analyses.

2.5. Acute toxicity tests using forced exposure.

Acute toxicity tests (24 h exposure) were performed with HHCB and AHTN. The concentrations used for each contaminant were 0.005, 0.05, 0.5, 5 and 50 µg/L including seawater and solvent (DMSO) controls. The tests were carried out in quadruplicate. Each aquarium used in the tests was filled with 1 L of seawater spiked with the contaminants. Three shrimps were added per aquarium totalling 12 shrimps per treatment. Mortality was recorded at 1, 3, 7 and 24 h. No aeration was provided. The temperature was 20±0.1 ºC. Initial and final dissolved oxygen levels were 5.9±0.1 and 5.2±0.2 mg/L, respectively.

2.6. Sample collection and analyses

Samples taken at the beginning and end of all the tests were analysed for HHCB and AHTN concentrations using stir bar sorptive extraction (SBSE) following a modification of the methodology described by Pintado-Herrera *et al*. (2014). Prior to use, all polydimethylsiloxane bars (PDMS, 10 mm x 0.5 mm) were preconditioned by soaking them in a mixture of acetonitrile/methanol (80:20, v/v). Subsequently, these bars were placed in amber glass flasks containing the aqueous samples (350 ml). Internal standard (triphenylphosphate d15) was added to determine possible fluctuations during the extraction and analysis procedures and stirred at 900 rpm during 4 h at room temperature. After extraction, the bars were desorbed by liquid desorption (LD); the bars were sonicated for 30 min in vials containing 100 µL of ethyl acetate. Then, gas chromatography (SCION 456-GC, Bruker) and mass spectrometry (SCION TQ from Bruker with CP 8400 Autosampler) were used to identify and quantify the compounds. Capillary gas chromatography analysis was carried out on a HP-5MS column (30 m×0.25 mm i.d.×0.25 µm film thickness of 5 % phenyl, 95 % polydimethylsiloxane), keeping the helium carrier gas flow at 1 mL/min. The mass detector acquired in multiple-reaction monitoring (MRM) mode. Details of the detection methodology can be found in Pintado-Herrera *et al*. (2016). Calibration curves were constructed for each compound in the range of 0.005–50 µg/L. Method limits of quantification were calculated using a signal-to-noise ratio 10 to 1, respectively, for water samples that was lower than 0.04 ng/L. The recovery rate of the method was higher than 85% for both analytes. The mean concentration and standard deviation of the internal standard (triphenylphosphate d15) was 46.7±10.6 µg/L. Concentrations of the contaminants measured in all the tests are presented in Table S1 – Supplementary Material.

2.7. Statistical analysis

The percentage of organisms recorded in each compartment at different observation times were arcsine transformed. The random distribution of organisms in the seawater and solvent controls, and avoidance tests with the contaminants were evaluated using mixed designed (time as a repeated measure, within factor, and compartment as between factor) analysis of variance (ANOVA). Mauchly's test was used to check the sphericity. Where sphericity was violated (the variances of the differences are not equal: $p < 0.05$), Greenhouse-Geisser correction was applied (see Tables S2a, S3a, S4a and S5a – Supplementary Material). Statistical ($p < 0.05$) differences between factors were checked using the Bonferronni test. The avoidance to HHCB and AHTN was determined by calculating the difference between the number of organisms expected (N_E) and observed (N_O) as described in Moreira-Santos *et al*. (2008). N_E represents the number of organisms initially introduced into each compartment. As three shrimps were inserted into each compartment at the beginning of the test, for the compartment with the highest concentration N_E was 3. As the next compartment had initially 3 organisms plus 3 organisms introduced in the adjacent compartment with the highest concentration, N_E was 6. For the sixth compartment, which contained only control seawater with no contaminant, N_E was 18. N_O was calculated considering the number of organisms recorded per time per compartment including the organisms observed in the compartments with higher concentrations. The avoidance percentage for each compartment was computed as (Avoiders/N_E) *100.

Median lethal concentrations (LC_{50}) and their respective confidence intervals (CI) from the forced exposure experiments and median avoidance concentrations (AC_{50}) from the non-forced exposure ones were calculated using PriProbit 1.63 software (Sakuma, 1998). The population immediate decline (PID) was computed following the method described by Rosa *et al*. (2012). The avoidance and mortality percentages were integrated to calculate the PID (x in percent) induced by each HHCB and AHTN concentration that simultaneously caused a y mortality percentage (i.e. the 24 h LCy) and a w avoidance percentage (i.e. the 3 h ACw) as thus:

$$X = [1 - (1 - y/100 * (1 - w/100)] * 100$$

The PID was calculated on the premise that some organisms first emigrate (avoidance %) and that mortality is then calculated based on the remaining organisms that did not emigrate. PID_{50} was calculated following the above procedure for AC_{50} and LC_{50}.

3. RESULTS

3.1. Acute toxicity test

The shrimp mortality recorded during the 24 h acute test with HHCB was not concentration-dependent and was highest (17%) for 0.005 and 0.5 µg/L. For AHTN, no mortality was observed at 0 and 0.5 µg/L, and it was around 8% at 0.005, 5 and 50 µg/L (Table S2 – Supplementary Material).

3.2. Avoidance test

No mortality was recorded in the non-forced experiments. The distribution of shrimps between the compartments over time was not significantly different ($p < 0.005$) for both seawater ($p = 0.64$, $F_{(5,18)} = 0.693$) and DMSO ($p = 0.99$, $F_{(5,18)} = 0.085$) controls (Tables S3b and S4b – Supplementary Material) and the distribution of shrimps between the compartments was not significantly different ($p < 0.005$) for the seawater control and the DMSO control (Tables S3c and S4c – Supplementary Material). In the absence of contaminants, the distribution of shrimps in both seawater and DMSO controls (Figure 2) was random (showing no preference for any compartment) during the 3 h exposure.

In the tests with each contaminant, the shrimps' distribution did not vary over time (Tables S5b and S6b – Supplementary Material). The statistical analysis of the shrimps' distribution in the contaminant gradients of HHCB ($F_{(5,18)} = 7.388$, $p < 0.001$) and AHTN ($F_{(5,18)} = 6.127$, $p < 0.002$) revealed that the organisms significantly ($p < 0.05$) preferred the uncontaminated compartments (Tables S5c and S6c – Supplementary Material). Significant differences in the distribution of shrimps exposed to each substance are shown in Figure 3. The mean percentage distribution of *P. varians* exposed to gradient of HHCB and AHTN after 3 h was 28.9% and 30.2%, respectively, in uncontaminated compartments and it was over 4 times higher than the mean percentage distribution of organisms in compartment with the highest concentration (50 µg/L: 6.5% and 7.1% for HHCB and AHTN, respectively).

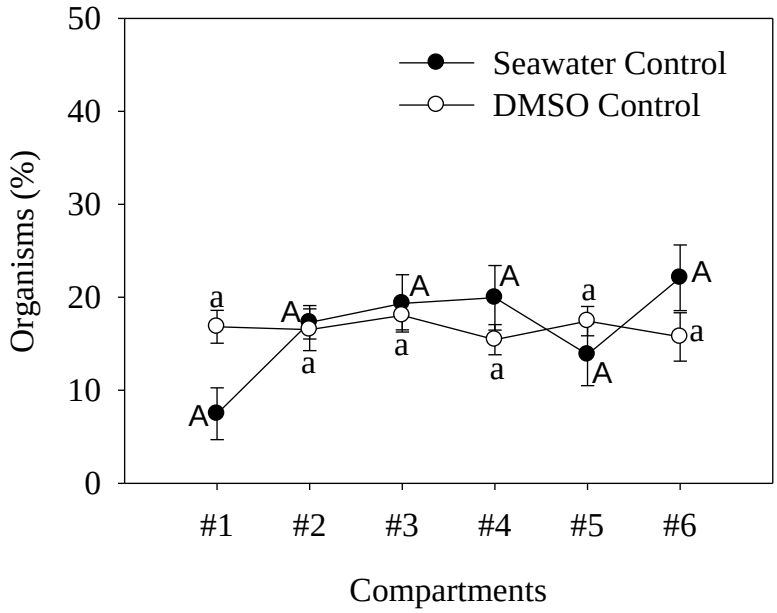

Figure 2: Mean percentage and standard deviation (n = 9 observation periods) of the number of shrimps *Palaemon varians* in the seawater and solvent (DMSO) control tests recorded in each compartment for 3 h. Different letters (upper case for seawater control and lower case for DMSO control) represent statistically significant difference.

The avoidance behaviour, mortality and PID data of the shrimps exposed to the contaminant gradients of HHCB and AHTN for 3 h are shown in Figure 4. For both contaminants, a concentration-dependent avoidance response was observed. For HHCB, the mean percentage avoidance for the lowest concentration (0.005 μg/L) was 14.6% and increased significantly to 60.5% ($p < 0.05$) for the highest concentration (50 μg/L). The same trend was observed for AHTN from 16.2% (at 0.005 μg/L) to 57.1% (at 50 μg/L). The PID curve (Figure 4) from the non-forced exposure data followed the same trend with the avoidance behaviour for both substances, as no mortality was recorded in the non-forced tests (Figure 4).

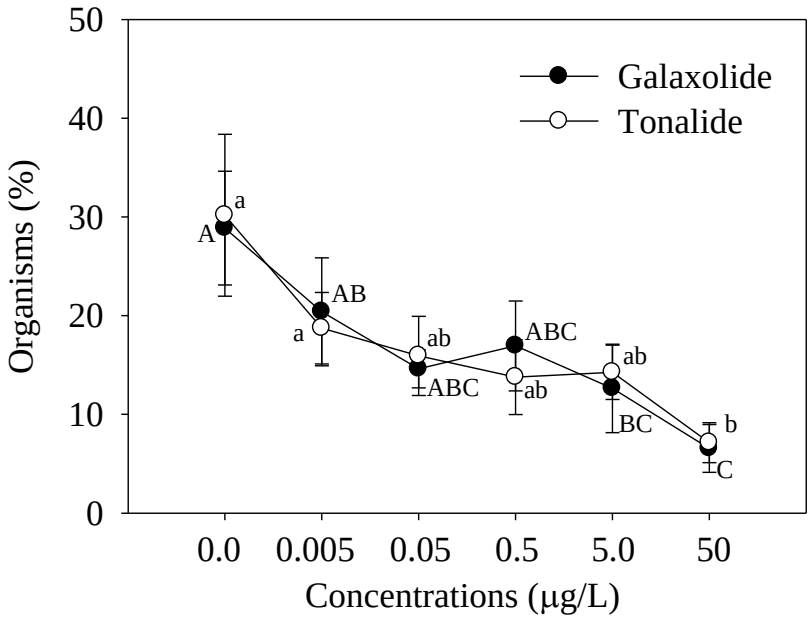

Figure 3: Mean percentage (n = 9 observation periods) of shrimps *Palaemon varians* exposed to gradient concentrations of galaxolide and tonalide recorded in each compartment for 3 h. Different letters (upper case for galoxide and lower case for tonalide) represent significant difference.

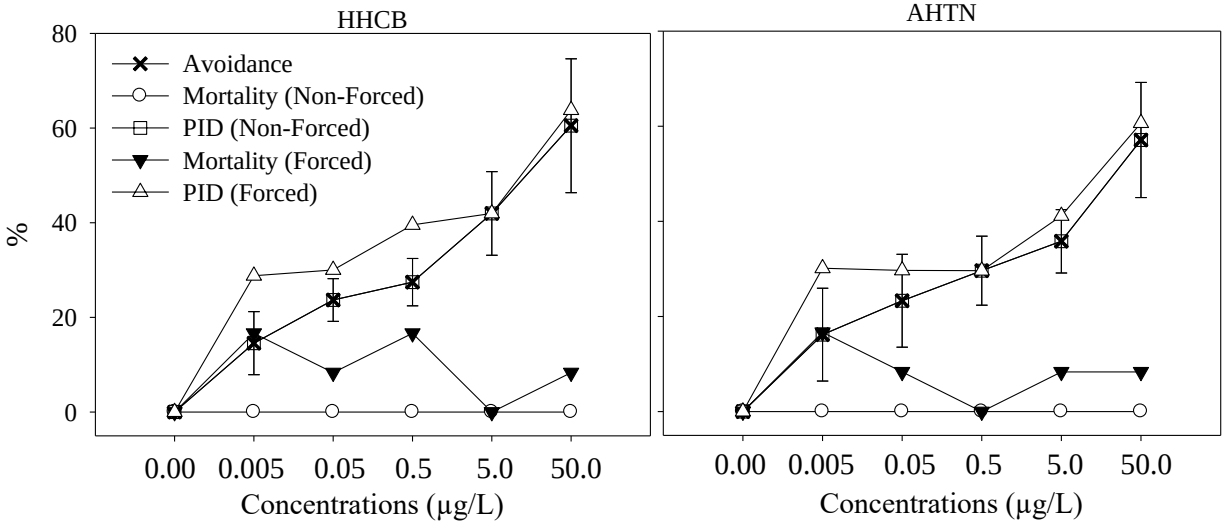

Figure 4: concentration-response curves for the avoidance (non-forced exposure) and mortality (forced exposure) responses, and the estimated PID (Population Immediate Decline) for *Palaemon varians* exposed to galaxolide (HHCB) and tonalide (AHTN). Standard deviations were not presented for forced tests, because the mortality was calculated based on the total number of exposed organisms.

3.3. AC_{50}, LC_{50}, and PID_{50}

The values of AC_{50}, LC_{50} and PID_{50} for HHCB and AHTN, based on mortality in the forced system and avoidance in non-forced exposure system, are shown in Table 1. The AC_{50} values (from non-forced exposure tests) obtained for HHCB and AHTN were 14.1 μg/L and 30.8 μg/L, respectively, and their corresponding LC_{50} values (from forced exposure tests) were >50 μg/L (no mortality was recorded). Since there was no mortality in the non-forced tests, the AC_{50} was equal to PID_{50}. However, the AC_{50} values recorded for the non-forced exposures were about twice as high as the PID_{50} values for both substances.

Table 1: Values (in μg/L) and their respective confidence intervals of AC_{50}, LC_{50}, and PID_{50} (concentrations that cause 50% of avoidance, mortality and population immediate decline of exposed organisms) for the shrimp *Palaemon varians* exposed to galaxolide (HHCB) and tonalide (AHTN) in non-forced (NFS) and forced (FS) exposure systems.

Substances	AC_{50} (NFS)	LC_{50} (NFS)	PID_{50} (NFS)	LC_{50} (FS)	PID_{50} (FS)
HHCB	14.1 (5.2 - 61.2)	>50	14.1 (5.2 - 61.2)	>50	6.3 (1.7 – 48.7)
AHTN	30.8 (7.7 - 345.6)	>50	30.8 (7.7 - 345.6)	>50	18.8 (3.9 - 381.4)

4. Discussion

The present study assessed the potential of the musk fragrances, galaxolide and tonalide, to trigger avoidance response in the estuarine shrimp *P. varians*. The results of the control tests showed that the displacement of the shrimps inside the system in the absence of contaminants was non-preferential. This indicates that the shrimps did not present aggregation behaviour that could condition, to some extent, their movement pattern (Araújo *et al*., 2016a). Previous studies with shrimp have also shown that the freshwater shrimp *Atyaephyra desmarestii* (Araújo *et al*., 2018a) and the marine shrimps *Litopenaeus vanamei* (Araújo *et al*., 2016a) did not aggregate when exposed to uncontaminated water using a similar non-forced, multi-compartmented system. Studies with other organisms such as the crustacean *Daphnia magna* (Rosa; *et al*., 2008; Rosa *et al*., 2012), the freshwater fish *Danio rerio* (Araújo *et al*., 2016b, 2014b) and *Poecilia reticulata* (Silva *et al*., 2017), exposed to uncontaminated water in non-forced, multi-compartmented systems, also did not display significant differences in the distribution of organisms throughout the different compartments. This random distribution in the absence of contaminants validates the suitability of the experimental system to study the spatial avoidance behaviour of organisms exposed to contaminant gradients.

Regarding the avoidance response, both compounds were detected by the shrimps and the potentially toxic concentrations were avoided. *Palaemonidae* are anatomically and physiologically adapted to detect the

presence of chemicals in their environment using both antennule and antennae (Machon *et al*., 2018), and as such, can select suitable habitats in the instance of chemical perturbation by avoiding contaminated area. Avoidance of both fragrances occurred at concentrations as low as 0.005 µg/L, indicating the high sensitivity of this response to reveal the risk of HHCB and AHTN. The mean avoidance percentages recorded for the lowest concentration (0.005 µg/L) of HHCB and AHTN were about 14 and 16%, respectively, and increased significantly in a concentration-dependent pattern to 60 and 57% at the concentration of 50 µg/L. Assessing the magnitude of this response, regarding how other marine species will react, is difficult due to the lack of studies on the avoidance responses of estuarine/marine organisms elicited by these compounds. The avoidance behaviour of marine shrimp and fish exposed in non-forced, multi-compartmented systems has been described for other compounds and it has revealed how suitable this system is for studying contamination-driven avoidance responses: around 80% of the estuarine shrimp *Litopenaeus vannamei* (whiteleg shrimp) and 60% of the marine fish *Rachycentron canadum* avoided contaminant gradients of copper assayed for 3 h (Araújo *et al*., 2016a). Regarding contaminants of emerging concern, studies of avoidance using non-forced, multi-compartmented exposure systems have been mainly performed with fish. For instance, around 50% of a population of zebrafish (*Danio rerio*) avoided 1.4 mg/L of the fungicide (pyrimethanil) (Araújo *et al*., 2014d, 2014b); 22% of the fish *P. reticulata* exposed under the same system for 4 h avoided triclosan concentrations as low as 0.2 µg/L (Silva *et al*., 2017); the AC_{50} for *P. reticulata* exposed to a bisphenol gradient was 0.15 µg/L, below the values considered safe for aquatic biota (Silva *et al*., 2018); *P. reticulata* also avoided environmentally relevant atrazine concentrations (0.02 µg/L; Araújo *et al*., 2018b). Organisms' behavioural response to the presence of a contaminant by avoiding contaminated sites is a protective strategy to prevent toxic impact and lethality (Gunn and Noakes, 1986; Lopes *et al.*, 2004; Oliveira *et al*., 2013; Silva *et al*., 2017). The use of a non-forced exposure approach provides an idea of the immediate response that organisms can present due to the aversive characteristic of contaminants and of the possible loss of organisms due to the massive emigration towards less aversive habitats (De Lange *et al*., 2006; Rosa *et al*., 2012; Araújo *et al*., 2016b). Although no effect at the individual level is expected to occur, the influence of a contaminant in the spatial distribution of organisms should be assessed carefully, as the consequences of the spatial avoidance are not restricted to the avoided ecosystem (Moreira-Santos *et al*., 2019). Whilst emigration from contaminated area is a solution for the avoiders (Moe *et al*., 2013), the consequences to ecosystem could lead to the loss of abundance and biodiversity (Lopes *et al*., 2004). The avoidance by the shrimps, that occupy an intermediate trophic level (Walker and Ferreira, 1985), might limit the amount of food available to organisms in the upper trophic strata and reduce the predation pressure on lower trophic levels.

Data of mortality recorded after forced exposure for 24 h suggest that the concentrations used were not acutely toxic, which is in accordance with the argument about the low acute toxicity of HHCB and AHTN (Breitholtz *et al*., 2003; Wollenberger *et al*., 2003). The 24 h-LC_{50} value recorded for HHCB (401.7 µg/L) was similar to the 48 h-LC_{50} (470 µg/L) for the marine copepod *Acartia tonsa* (Wollenberger *et al*., 2003), and to the 96 h-LC_{50} (288 µg/L) for *Chironomus riparus* (Artola-Garicano *et al*., 2003). However, a higher value (96 h-LC_{50}:

>1000 µg/L) for the adult harpaticoid copepod *Nitocra spinipes* has been reported (Breitholtz *et al*., 2003). The shrimp *P. varians* proved to be more sensitive to AHTN (24 h-LC_{50} value of 88.11 µg/L) compared to *N. spinipes* (96 h-LC_{50}: 610 µg/L; Breitholtz *et al*., 2003), *C. riparus* (96 h-LC_{50}: >460 µg/L; Artola-Garicano *et al*., 2003) and *A. tonsa* (48 h-LC_{50}: 2500 µg/L; Wollenberger *et al*., 2003). Although mortality was the only endpoint considered in the forced exposure systems, symptoms of stupefaction were also observed, which could impair the ability to avoid toxic environments. Subsequently, this and other potential sub-lethal effects under a forced exposure scenario should not be neglected.

To elucidate the immediate impact of contaminant dispersion on aquatic environments at the local level, the PID was estimated by integrating mortality and avoidance responses. Clearly, avoidance played a greater role in the PID than mortality. Although these substances are classified as low risk in terms of lethality and, therefore, are believed not to pose a significant threat at the present environmental concentration levels (European Commission, 2008a; 2008b), it is evident that they may contribute to a significant population decline at a local scale and for a short exposure period by triggering avoidance. For instance, at the lowest concentration (0.005 µg/L) the population of shrimps declined by 28 and 30% for HHCB and AHTN, respectively, as against 16% from mortality data from forced exposures. Several authors have recommended the inclusion of spatial avoidance data measured in non-forced, multi-compartmented exposure systems as a complementary tool for ecological risk assessment. This might help to predict the ecological risk of contaminants more accurately; thus avoiding overestimation or underestimation of risk prediction by either the forced or non-forced approaches (Araújo *et al*., 2016b; Moreira-Santos *et al*., 2008; Rosa *et al*., 2012; Silva *et al*., 2017). In other studies where PID was estimated, avoidance also tended to occur at concentrations lower than mortality (Araújo *et al*., 2014a; Rosa *et al*., 2012; Silva *et al*., 2017), indicating that under conditions of gradual and heterogeneous contamination, the contaminated area might lose part of the organism population due to its fleeing from contamination with possible longer-term consequences on local ecosystem structure and functioning. In cases where the contaminant (either due to its mode of action or to the concentrations) was observed to cause stupefaction in the organisms (Gutierrez *et al.*, 2012) and avoidance is prevented, the mortality in the short term might play a more important role for the PID (Araújo *et al*., 2014a, 2014c).

Particularly in the cases of HHCB and AHTN, the shrimps were not sensitive in the forced exposure tests, but they were able to detect and avoid concentrations of those compounds in the non-forced exposure tests. This might indicate that avoidance helps to prevent even sub-lethal effects that organisms could suffer after a continuous exposure. Under conditions in which an HHCB or AHTN gradient exists, it would be expected that the distribution of the shrimps was, at least to some extent, conditioned by the presence of those contaminants. Since the AC_{50} obtained for HHCB is 28 times lower than the 24 h-LC_{50} for forced exposure, the prediction of the ecological risks of HHCB and AHTN based exclusively on data from forced exposure assays might provide a relevant vision of the potential toxicity of both compounds but fails to assess the complete effects at the community structure level (spatial distribution of the shrimps). Therefore, for

contaminants of emerging concern, spatial avoidance using the non-forced, multi-compartmented approach should be considered as an additional line of evidence in environmental risk assessments.

5. Conclusion

Shrimps were not acutely (mortality response) sensitive to the concentrations of both compounds tested. However, under non-forced exposure to HHCB and AHTN contaminant gradients, the shrimps detected different concentrations and avoided those potentially harmful. The population decline for short exposure to environmentally relevant concentrations of HHCB and AHTN was proven to be driven by the avoidance behaviour. It is recommended that ecological risk assessments of compounds of emerging concern, as well as non-emerging and legacy contaminants, integrate data from both forced and non-forced exposure approaches to avoid underestimation of their full potential risks.

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3.3. Sub-lethal effects of environmental concentrations of galaxolide and tonalide on the manila clam Ruditapes philippinarum as bioindicator species

Summary

Many contaminants in the marine environments are present at levels where they cause no obvious instant decimation of marine biota but elicit sub-lethal effects, which might result in critical ecological disturbance. They may interfere with biochemical functions by causing alteration in enzymatic activities leading to physiological disorder and reduced biotic potential after chronic exposure. The presence of polycyclic musk compounds in the marine environments have been detected at nano to microgram levels and are reported not critically lethal to marine organisms at this level. Therefore, this section addressed the sub-lethal effects of galaxolide (HHCB) and tonalide (AHTN) in the marine environments after chronic exposure. The clams *Ruditapes philippinarum* were exposed to 0.005, 0.05, 0.5, 5 and 50 µg/L of the musks in a semi-static renovation bioassay for 21 days. The exposed clams were sampled on days 3, 7, 14 and 21, and digestive gland and gonad tissues were harvested and preserved prior to further treatment. A battery of biomarkers related to contaminants biotransformation (Ethoxyresorufin-O-deethylase – EROD and glutathione-S-transferase – GST), oxidative stress (glutathione peroxidase- GPx, glutathione reductase – GR and lipid peroxidation – LPO), DNA damage, neuroendocrine toxicity (acetylcholinesterase – AChE and cyclooxygenase - COX) and energy reserves measured as total lipids (TL) were measured at each sample time-points.

The results showed that environmental concentrations of HHCB and AHTN significantly induced EROD and GST activities and both compounds also significantly induced GPx activity assessed in the digestive glands of the clams. All the concentrations tested significantly increased lipid peroxidation on day 21 leading to significant DNA damage in the clams. Neuroendocrine function in the clams were modulated by both substances, HHCB induced AChE and COX activities and AHTN significantly inhibited AChE and COX activities at different time points. The impacts of the contaminants on energy reserve in clams was not very clear because variations in the amount of available energy in digestive gland and gonad were observed.

The results of the biotransformation enzymes, oxidative stress parameters and genotoxicity have been published as a peer-reviewed article entitled “Potential of environmental concentrations of the musks galaxolide and tonalide to induce oxidative stress and genotoxicity in marine environment” (**Ehiguese *et al*.,** 2020. **Marine Environmental Research.** 10.1016/j.marenvres.2020.105019). The accepted manuscript formated to the journal recommended reference style is presented below.

The author’s personal contribution included conceptualization, methodology, samples analyses, data curation, project management, original draft preparation, writing and editing of manuscript as well as first and corresponding authorship of the article.

The bioassay, biomarkers analyses and chemical characterization of the exposure water were performed entirely at the Physical Chemistry Department, University of Cadiz, Puerto Real Campus, Spain.

1. Introduction

The polycyclic musk compounds (PMCs) 1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethyl-cyclopenta-(g)-2-benzopyran (HHCB) branded Galaxolide® and 7-acetyl-1,1,3,4,4,6-hexamethyltetralin (AHTN) branded Tonalide® are bi-cyclic aromatic compounds. They are comprised of acetylated and highly methylated tetralin or indane skeletons (Sumner *et al*., 2010; Rick *et al*., 2003) and are lipophilic with high octanol-water partition coefficients (Kow) of 5.9 and 5.7 for HHCB and AHTN, respectively (Cunha 2012; Reiner and Kannan, 2011; Sumner *et al*., 2010). These substances are applied in a wide range of personal care products such as cosmetics, lotions, deodorants, detergents and more (Reiner and Kannan, 2011) because of their musky scent and fixative properties that enable them to bind to fabrics (Rimkus 1999; Reiner and Kannah, 2006). According to a European Commission risk assessment report, the production of HHCB and AHTN in Europe in the year 2000 was between 1000 to 5000 ton/year (EC, 2008a, 2008b) and accounted for approximately 95% of fragrance material in the perfumery industry (Balk and Ford, 1999; Pedersen *et al*., 2009). Reports have shown that, since 1990, all fragrances consumed in the United States have doubled (Roosens *et al*., 2007) and increased by 25% between 1996 and 2000 (Peck *et al*., 2006). Because of their high production they have been placed on the "High Production Volume List" by the United States Environmental Protection Agency (US EPA) (Peck, 2006).

Although HHCB and AHTN were first detected in aquatic environments (Eschke *et al*., 1994), recent studies have reported their presence in sediment and air (Fromme *et al*., 2001; Peck *et al*., 2006; Peck and Hornbuckle, 2006). In effluents from sewage treatment plants (STPs), concentrations of HHCB and AHTN have reached levels ranging from 1800 to 9000 $ng \cdot L^{-1}$ (Bueno *et al*., 2012; Díaz-Garduño *et al*., 2017). Those concentrations were similar to previous studies that reported environmental concentrations between 509 and 2337 $ng \cdot L^{-1}$ for AHTN and between 4750 and 13399 $ng \cdot L^{-1}$ for HHCB (Chase *et al*., 2012; Lee *et al*., 2010; Pintado-Herrera *et al*., 2014). Research has shown that approximately 50% to 90% of the total synthetic musks are eliminated, especially if tertiary treatments are employed, while the rest enters the receiving rivers and coastal waters (Heberer, 2002; Ricking *et al*., 2003; Lee *et al*., 2010). Since legislation requirements for effluents from Wastewater Treatment Plants (WWTPs) do not include tertiary depuration treatments [such as the multi-barrier treatment that can remove chemicals at 97%, and completely eliminate fragrances from wastewaters (Díaz-Garduño *et al*., 2017)] as mandatory, concentrations that could have been trapped during tertiary depuration escape into aquatic ecosystems.

To the best of our knowledge, only few studies have assessed PMCs' presence in the marine environment (Bester *et al*., 1998; Sumner *et al*., 2010). However, reports have demonstrated that they are found to bioaccumulate in marine organisms (Kannan *et al*., 2005; Moon *et al*., 2011, 2012). HHCB and AHTN have been measured in the tissues of mussels, crustaceans, fish, marine birds and mammals (Kannan *et al*., 2005; Moon *et al*., 2011; 2012; Nakata, 2005) coupled with bioaccumulation factors of toxic concern (Rimkus *et al*., 1997). The bioaccumulation factor values based on lipid weight calculated for HHCB in zebra mussels at the

Upper Hudson River (USA) was between 2610 and 4890 (Reiner and Kannan, 2011), and in Germany, bioaccumulation factor based on lipid weight calculated for AHTN was between 5100 and 40100 (Gatermann *et al*., 2002). Additionally, bioconcentration factor measured in *Lepomis machrochirus* for HHCB and AHTN in a laboratory study were 1584 and 597, respectively (Balk and Ford, 1999)Yet little is known about PMCs' toxicity in aquatic ecosystems and some of the studies have focused on freshwater ecosystems, while others used concentrations higher than those considered environmentally relevant (Wollenberger *et al*., 2003; Breitholtz *et al*., 2003; Carlsson and Norrgren, 2004; Chen *et al*., 2011; Gooding *et al*., 2006; Parolini *et al*., 2015; Pedersen *et al*., 2009). Environmental concentrations of HHCB and AHTN have been shown to experimentally elicit avoidance behaviour in the estuarine shrimps *Palaemon varians* when exposed to a gradient of both compounds in a free-choice multi-compartmented non-forced exposure system (Ehiguese *et al*., 2019). However, information about sub-lethal effects to marine organisms resulting from chronic exposure is incipient.

The aim of this study was to determine the potential risk of HHCB and AHTN to the clam *Ruditapes philippinarum* after chronic exposure to environmentally relevant concentrations. The risk to this species was based on a battery of biomarkers of exposure and effect (oxidative stress and genotoxicity), such as ethoxyresorufin-O-deethylase (EROD), glutathione-S-transferase (GST), glutathione peroxidase (GPx), glutathione reductase (GR), lipid peroxidation (LPO) and DNA damage. *R. philippanarum* is an important test organism in ecotoxicology because it is readily available, inexpensive and its biology is well understood. These euryhaline infauna suspension feeders have been used in studies related to bioaccumulation of contaminants (Moschino *et al*., 2012; Santana *et al*., 2017), metal toxicity (Aouini *et al*., 2018; Ji *et al*., 2019; X. Liu *et al*., 2011), Ocean acidification (De Marchi *et al*., 2017; Velez *et al*., 2016; Xu *et al*., 2016), wastewater effluents as contaminants mixture (Díaz-Garduño *et al*., 2017; Maranho *et al*., 2015) and pharmaceutical and personal care products (Aguirre-Martinez *et al*., 2013; 2016, 2015; Almeida *et al*., 2015; Trombini *et al*., 2019).

2. Materials and Methods

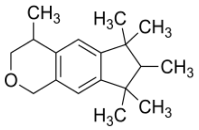
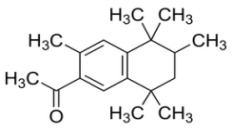
2.1. Selection and concentrations of the polycyclic musks

HHCB and AHTN were purchased from Sigma Aldrich, Spain. The characteristics of these substances are presented in Table 1. The concentrations (0.005, 0.05, 0.5, 5.0 and 50.0 µg/L) of HHCB and AHTN used in the experiments were carefully selected based on concentrations measured (maximum of 9 µg/L) in different environmental matrices (Bueno *et al*., 2012; Díaz-Garduño *et al*., 2017; Sumner *et al*., 2010). Initially, stock solutions were prepared and stored in dark bottles at 4 ºC. Each substance was dissolved in dimethyl sulfoxide (DMSO) in glass vial.

2.2. Organisms: acclimation and maintenance conditions

A total of 360 specimens of *R. philippinarum* (average size: 42 ± 0.9 mm; n = 120) were purchased from an aquaculture farm in the Bay of Cádiz, (Ctaqua, Cadiz, Spain). Organisms were immediately transported to the laboratory for acclimation and kept in a 300 L aquarium for seven days. The aquarium was supplied with constant aeration and the specimens were fed *ad libitum* once every day with the microalgae *Isochrysis galbana*. The physical and chemical parameters (photoperiod of 12 h light/12 h dark; temperature: 15 ± 1 ºC; salinity: 34.6 ± 0.3; pH: 7.8 – 8.2; dissolved oxygen: >5 mg/L) in the aquarium were monitored daily.

Table 1: Characteristics of HHCB and AHTN.

Characteristics	Galaxolide® (HHCB)	Tonalide® (AHTN)
Structural Formula		
Chemical Name	1,3,4,6,7,8-hexahydro-4,6,6,8,8-hexamethylcyclopenta-γ-2-benzopyran	6-Acetyl-1,1,2,4,4,7-hexamethyltetralin
CAS No	1222-05-5	21145-77-7
Log K_{ow}	5.9[a]	5.7[a]
Water Solubility	1.75 mg/l	1.25 mg/l

[a] Balk and Ford, 1999

2.3. Experimental approach

The bioassay was conducted in duplicates using a 10 L rectangular glass aquarium including sea water control and solvent control (DMSO, v/v 0.001%) to prevent any effect from the solvent (Aguirre-martínez *et al*., 2016). Sixteen individuals were exposed in each replicate (32 individuals per treatment). Exposure to compounds lasted 21 days and was performed in a semi-static regimen with total renewal of each test substance every 3 days (days 3, 6, 9, 12, 15 and 18). The glass aquariums were filled with 8 L of sea water spiked with the stock solution. Physiochemical parameters were similar to the acclimation conditions reported above. The specimens were sampled on days 3, 7, 14 and 21 (n=6).

2.4. Water samples collection and analyses

Water samples for each treatment were collected on days 0 and 3 using amber bottles and immediately stored at -20 ºC before determination of the concentrations of HHCB and AHTN. The methodology described by Pintado-Herrera *et al*. (2014) based on the SBSE-LD (stir bar sorptive extraction coupled to liquid desorption) by using polydimethylsiloxane bars (PDMS, 10 mm x 0.5 mm) was employed. The compounds were separated, identified and quantified using gas chromatography (SCION 456-GC, Bruker) coupled to a tandem mass spectrometry (SCION TQ from Bruker with CP 8400 Autosampler). Details of the detection and extraction efficiency of the methodology can be found in Pintado-Herrera *et al*. (2016) and (2014). The concentrations of HHCB measured in the exposure water ranged from 54% to 80% of the nominal concentration on day 0 and from 21% to 23% on day 3 just before renewal in the semi-static experiment. Regarding AHTN, concentrations on day 0 ranged between 73% to 97% of the nominal concentrations and from 23% to 29% on day 3, just before renewal. The differential between nominal and measured concentrations is consistent with other reported studies due to instability of HHCB and AHTN.

2.5. Biological samples preparation

Three individuals (n=6 per treatment) were sampled from each replicate after 3, 7, 14 and 21 days of exposure. Organisms were dissected on ice and digestive gland tissues were extracted. Homogenization buffer was prepared with 100 mM NaCl, 25 mM HEPES salt, 0.1 mM EDTA and 0.1 mM DTT. Digestive gland tissue (n=6) was homogenized following the procedure described by Lafontaine *et al*. (2000) and centrifuged at 15.000 *g* for 20 min at 4 ºC to obtain the supernatant fraction S_{15} and at 3000 *g* for 20 min at 4 ºC to obtain the supernatant fraction S_3. Supernatant fractions were carefully extracted and stored at −80 ºC. The total protein concentration (TP) (expressed as mg TP) was determined in the homogenized fraction and in S_{15} and S_3 fractions following an adaptation of the methodology by Bradford (1976). All biomarkers were measured using a kinetic microplate reader, Infinite® M200.

2.6. Biomarker Analyses

2.6.1 Ethoxyresorufin O-deethylase (EROD) Activity

EROD activity was measured using the method adapted from rainbow trout fingerling (Gagné and Blaise 1993). 50 µl of S_{15} samples (25 µl sample + 25 µl of MilliQ) were added in 96-well flat bottom dark microplates. 160 µl of 7-ethoxyresorufin and 10 µl of reduced NADPH were added with 100 mM K_2HPO4 buffer at pH 7.4. The reaction was activated using NADPH and allowed to proceed for 60 min at 30 ºC. 7-hydroxyresorufin was determined fluorometrically using 516 nm excitation and 600 nm emission filters and

readings were taken at 15 min intervals. The calibration curve was developed using concentrations of resorufin and results were normalized to their corresponding total protein expressed as pmol/min/mgTP.

2.6.2 Glutathione-S-Transferase Activity

Determination of GST activity was adopted from Boryslawskyj *et al*. (1998). In a transparent 96-well flat bottom microplate, 15 µl S_{15} samples were added to 200 µl reaction buffer of 10 mM HEPES salt, 125 mM NaCl and 1 mM glutathione reduce (GSH) normalized at pH 6.5. Subsequently, 15 µl of homogenization buffer was added to 2 wells and marked up with 200 µl reaction buffer to check the background reaction rate. Absorbance was measured at 340 nm, every 5 min for 30 min based on the appearance of the glutathione conjugate at 22 ºC. Results were expressed as microgram per minute per milligram total protein (µg/min/mgTP).

2.6.3 Glutathione peroxidase activity

Glutathione peroxidase activity was measured using the procedure adapted from Mcfarland *et al*. (1999). 20 µl S_{15} samples (10 µl + 10 µl homogenization buffer) were added to a transparent 96-well flat bottom microplate. 200 µl daily assay mixture prepared with GPx assay buffer (50 mM potassium phosphate, 0.1 mM EDTA and 0.15 mM sodium azide) and substrates (1 mM cumene hydrogen peroxide in 50 ml GPx assay buffer incubated at 30 ºC) was added to the microplate and incubated for 2 min at 30 ºC. A volume of 50 µl of substrate was then added to each well. The oxidation of NADPH to NADP was observed. Readings were taken at 340 nm for 3 min, at 10 sec intervals. 20 µl of homogenization buffer were added in 2 wells as a background corrected value. Results were expressed as nmol/min/mgTP.

2.6.4 Glutathione reductase (GR) activity

The method adapted by Martin-Diaz *et al*. (2007) was used to measure the activity of GR. In a transparent 96-well flat bottom microplate, 20 µl S_{15} sample (10 µl sample + 10 µl MilliQ) and 200 µl of incubated daily assay mixture (200 mM NaH_2PO4 and Na_2HPO_4 at pH 7.6, 10 mM oxidized glutathione, 1 mM NADPH) were added. A volume of 20 µl homogenization buffer was added to 2 wells as blank to check the background reaction rate. The reaction was measured spectrophotometrically using 340 nm emission at 30 ºC. The loss of NADPH was recorded at 2 min interval for 10 min. Results were expressed as pmol/min/mgTP.

2.6.5 Lipid peroxidation (LPO)

The thiobarbituric acid reactive substance (TBARS) procedure was used for LPO measurement (Wills 1987). 150 µl of diluted homogenate samples (90 µl samples + 60 µl MilliQ) were injected in 1.5 ml tubes. A standard solution of tetramethoxypropane (TMP) was prepared with 0.001% TMP and diluted serially with distilled water (0 – 15 µM TMP). 300 µl of 10% trichloroacetic acid (TCA), 1 mM $FeSO_4$ and 150 µl of 0.67% thiobarbithuric acid (TBA) were added to the sample and standard solution separately. They were then mixed and incubated for 10 min at 70 ºC in a J.P. Selecta® incubator. 200 µl of the precipitates (standard solution for the TMP standard curve and homogenate samples) were pipetted into a dark 96-well flat bottom microplate. Production of malondialdehyde (MDA), which is indicative of oxidative stress from the degradation of initial products of free radical attack on fatty acid (Janero, 1990), was measured spectrophotometrically at 516 nm (excitation) and 600 nm (emission) filter. Optical density values were converted to µgTBARS/mgTP.

2.6.6 DNA damage

The DNA precipitation assay methodology is based on 2% SDS-KCl precipitation of DNA-protein crosslink, which uses fluorescence to quantify the DNA strands (Olive, 1988; Gagne *et al*., 1995). When DNA breaks because of exposure to toxic chemicals, the strands are released from cellular protein into the supernatant when centrifuged at low speed (Olive, 1988). It becomes possible to quantify the amount of double and single stranded DNA at the end of the assay (Gagné *et al*., 1995). A volume of 25 µl of homogenate was mixed by inversion with 200 µl of SDS 2% prepared with 10 mM EDTA, Tris-Base and 40 mM NaCl. 200 µl of 0.12 mM KCl was added and mixed by inversion. The mixture was incubated for 10 min at 60 ºC, cooled at 4 ºC for 30 min and centrifuged 8000 x g at 4 ºC for 5 min. For DNA calibration, Salmon Sperm genomic DNA was dissolved in 1 ml TEIX (Tris-HCl and EDTA at pH 8.0) as a standard. In a dark 96-well flat bottom microplate, 50 µl of the supernatant was added to 150 µl of Hoescht dye 0.1 µg/mL diluted with sodium cholate containing 0.4 M NaCl, 4 mM sodium cholate and 0.1 M Tris-Acetate (pH 8.5). Fluorescence was measured at 360 nm (excitation) and 450 nm (emission) filters against blanks containing similar constituents without homogenate. Optical density values were converted to µg DNA/mgTP.

2.7. Statistical Analysis

Data for biomarker responses were analyzed using the SPSS®/PC + statistical package. Prior to parametric tests, the normality and homogeneity of the data were analyzed. Significant differences between controls and organisms exposed to polycyclic musk compound treatments were determined using one-way ANOVAs followed by Dunnett's comparison tests and significance levels were set at $p < 0.05$. In order to evaluate the

relationship between biomarker responses and musk concentrations over time, Spearman's rank order of correlation tests was run. Significance levels were set at $p < 0.05$ and $p < 0.01$ to obtain pairwise correlations.

3. RESULTS

3.1. Biomarker responses

During the 21 day exposure, there was no significant mortality (3%) and no significant difference in biomarker responses ($p < 0.05$) analysed in digestive gland tissues of clams exposed to the seawater control group and DMSO. The results of the biochemical biomarkers from digestive gland extracts are presented in Figures 1 and 2 and correlations between concentrations and biological responses can be found in Table 2.

3.1.1 Biomarkers of Exposure

The biotransformation enzymes, which involve EROD enzymatic activity, showed significant induction compared with control in organisms exposed to AHTN and HHCB. Significantly higher values compared with control ($p < 0.05$) were observed on days 3 and 7 (0.05, 0.5, 5 and 50 μg/L) and day 14 (0.05 and 0.5 μg/L) for AHTN (Figure 1). Regarding HHCB, significantly higher values ($p < 0.05$) were found on day 7 (0.05, 0.5, 5 and 50 μg/L), day 14 (0.005 μg/L) and day 21 (0.005, 0.5, 5 and 50 μg/L) (Figure 2).

3.1.2 Biomarkers of Effect

Induction of GST ($p < 0.05$) was observed in clams exposed to HHCB and AHTN. This induction was significant ($p < 0.05$) on days 7, 14 (0.005, 0.05, 0.5, 5 and 50 μg/L) and 21 (0.005, 0.05, 5 and 50 μg/L) in organisms exposed to AHTN (Figure 1) and from day 3 to 21 when clams were exposed to HHCB (Figure 2). When describing GPx activities, it was observed that significant induction ($p < 0.05$) was found on day 3 for HHCB (0.05, 0.5 and 5 μg/L) (Figure 2) and AHTN (0.05 and 0.5 μg/L) (Figure 1). This induction was not concentration dependent. Significant antioxidant induction was also observed on the last day of exposure to HHCB compared with control ($p < 0.05$) and was positively correlated with the concentrations 0.05, 0.5, 5 and 50 μg/L (Figure 2). Surprisingly, GR enzymatic activity decreased significantly compared with control ($p < 0.05$) in all treatments for both substances (Figures 1 and 2). These responses of the antioxidant enzymes showing oxidative stress corresponded with significant ($p < 0.05$) increasing lipid peroxidation in the digestive gland tissues of clams exposed to these polycyclic musk compounds after 21 days of exposure. Moreover, the increased LPO level was positively correlated on day 21 with concentration for HHCB. Significant increase in DNA strand break ($p < 0.05$) was observed on days 7, 14 and 21 (0.005, 0.05, 0.5 5 and 50 μg/L) compared

with control ($p < 0.05$) for HHCB (Figure 2) and only on day 21 (0.05, 0.5, 5 and 50 µg/L) for AHTN (Figure 1). This induction was positively correlated with the concentration on day 21 ($p < 0.05$).

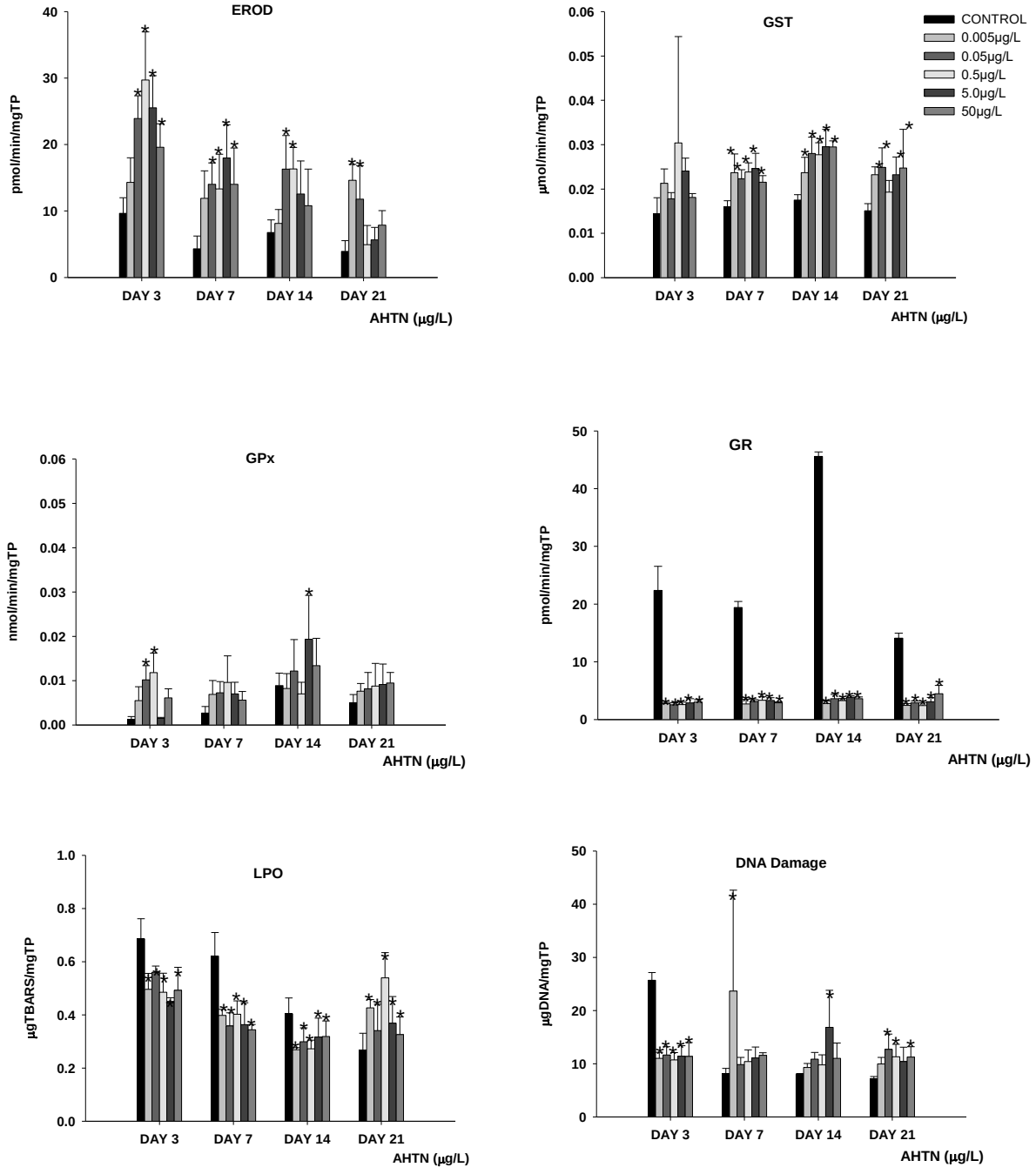


Figure 1: Biochemical biomarkers including ethoxyresorufin O-deethylase (EROD), glutathione-S-transferase (GST), glutathione peroxidase (GPX), glutathione reductase (GR) activities, lipid peroxidation (LPO) level and DNA damage (strand breaks) measured in the digestive gland tissues of *R. philippinarum* exposed to tonalide (ATHN) for 21 days. Asterisks (*) shows significant difference with control treatment ($p<0.05$).

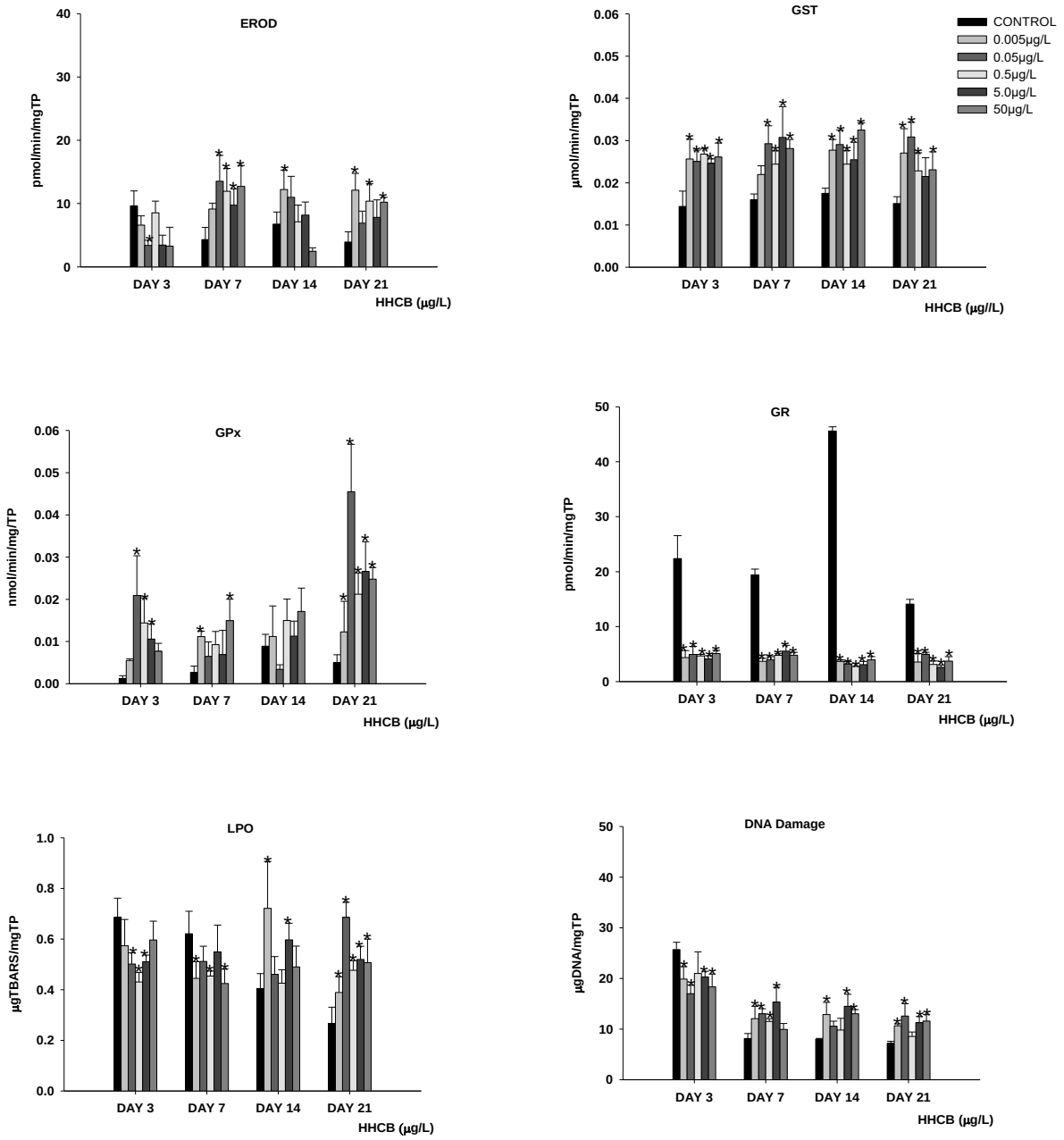


Figure 2: Biochemical biomarkers including ethoxyresorufin O-deethylase (EROD), glutathione-S-transferase (GST), glutathione peroxidase (GPX), glutathione reductase (GR) activities, lipid peroxidation (LPO) level and DNA damage (strand breaks) measured in the digestive gland tissues of *R. philippinarum* exposed to galaxoide (HHCB) for 21 days. Asterisks (*) shows significant difference with control treatment ($p<0.05$).

3.2 Correlations

Taking all biomarker responses into consideration, organisms showed positive and significant ($p < 0.01$) concentration-dependent response correlation regarding detoxification (GST), oxidative stress (GPx) and genotoxicity (DNA damage), and significant ($p < 0.05$) and negative correlation with GR (Table 2) in clams exposed to HHCB. For AHTN, concentration correlated significantly ($p < 0.01$) with induction of EROD, GST, GPx, DNA damage and significant ($p < 0.01$) inhibition of GR (Table 2) in clams exposed to AHTN. Positive correlation between biomarkers of exposure, GST with EROD, GPx ($p < 0.05$), and biomarkers of effects, DNA damage ($p < 0.01$), LPO ($p < 0.05$) in clams exposed to HHCB was determined (Table 2). Induction of GST significantly ($p < 0.01$) correlated with GPx and GR and negatively correlated with LPO, and EROD activation was significantly ($p < 0.01$) correlated with inhibition of GR corresponding to significant ($p < 0.01$) increased DNA damage (Table 2) in clams exposed to AHTN.

4. Discussion

Environmental risk assessments of PMCs have been mainly focused on toxicity for freshwater environments, and information about chronic toxicity in marine environments remains limited. In the present study, a battery of biomarkers related with xenobiotic detoxification (EROD and GST), oxidative stress (LPO, GPx and GR) and genotoxicity (DNA damage) have been tested to provide information regarding the potential toxicity of the fragrances tonalide and galaxolide for the marine clam *R. philippinarum*.

We observed dose-dependent significant induction of EROD activity ($p < 0.05$) in *R. philippinarum* exposed to AHTN and HHCB (Figures 1 and 2). The cytochrome P450 system has been described to be involved in the metabolism of detoxification of lipophilic compounds, while EROD enzymes catalyze the reactions of degradation of lipophilic compounds (van der Oost *et al*., 2003). EROD activity, measured in bioindicator species, has been established as a biomarker of exposure to lipophilic chemicals. The activity of P450 enzymes are key in phase I biotransformation of xenobiotics, specifically lipophilic compounds with aromatic backbone. EROD activity studied in aquatic organisms has shown high values after exposure to polycyclic aromatic hydrocarbons, polychlorinated biphenyls and some contaminants of emerging concern (Aguirre-Martínez *et al*., 2016; Luna-Acosta *et al*., 2015; Maranho *et al*., 2015; Park *et al*., 2009; Siebert *et al*., 2017; Tao *et al*., 2013). Consequently, HHCB and AHTN may have the capacity to bind to the aryl hydrocarbon (Ah) receptor in CYP450 1A1, because xenobiotics that fail to bind with the Ah receptor showed no induction of EROD activity (Petrulis *et al*., 2000). The induction of EROD activity recorded in this study is a confirmation of the bioavailability of HHCB and AHTN to marine organisms and thus requires urgent attention to address the potential toxicity in marine ecosystems.

Table 2: Spearman's rank order of correlation (rs) test between biomarkers measured in *R. philippinarum* digestive gland tissues after exposure to Galaxolide® (first value in bold) and Tonalide® (second value) concentrations (CON).

Biomarkers	CON	EROD	GST	GPx	GR	LPO	DNA Damage
EROD	**0.111**/0.333**	**1**					
GST	**0.378****/0.272**	**0.207***/0.083	**1**				
GPx	**0.484****/0.273**	**0.012**/-0.013	**0.196***/0.273**	**1**			
GR	**-0.196***/-0.335**	**-0.102**/-0.362**	**0.134**/0.268**	**-0.255****/-0.022	**1**		
LPO	**-0.018**/-0.393**	**-0.049**/-0.059	**0.021**/-0.252**	**-0.080**/-0.250*	**0.177***/0.154	**1**	
DNA Damage	**0.352****/0.310**	**0.015**/0.233**	**0.243****/0.136	**0.019**/-0.088	**0.193***/-0.054	**0.407****/0.096	**1**

Asterisks indicate the p values: * $p < 0.05$ and ** $p < 0.01$. Ethoxyresorufin O-deethylase (EROD), gluthathione-S-transferase (GST), gluthathione peroxidase (GPX), gluthathione reductase (GR), lipid peroxidation (LPO).

Aromatic xenobiotics are prooxidant chemicals, which increase intracellular generation of reactive oxygen species through the induction of cytochrome P450 pathway (Regoli and Giuliana, 2014) and antioxidant species, such as GST, GPx and GR; furthermore, they are bioindicators of contaminant-mediated oxidative stress that play significant roles in breaking down oxyradicals to less harmful products, consequently preventing oxidative damage (Wu *et al*., 2011). The significant increase in GST activity in the digestive gland of clams exposed to HHCB and AHTN suggests their capacity for inducing oxidative stress. GST is a phase II metabolic isoenzyme involved in the catalytic conjugation of reduced glutathione to xenobiotic substrates thus encouraging detoxification and preventing interactions with crucial cellular proteins and nucleic acids (Birben *et al*., 2012; Josephy, 2010). This detoxification process triggers GST induction and the increased GST activity observed in this study suggests that HHCB and AHTN possess electrophilic cores, with which glutathione conjugated (Hampel *et al.*, 2016). The activation of GPx activity in a concentration-dependent pattern as observed in clams exposed to HHCB and AHTN on day 21 indicated an effort to ameliorate oxidative stress, thus protecting the organism from cell damage. GPx activity is an important antioxidant biomarker with involvement in antioxidant metabolism because, in conjunction with GST, it reduces lipid hydroperoxides to alcohol, with some concomitant oxidation of reduced glutathione to oxidized glutathione (Regoli and Giuliana, 2014). GR activity in the present study was significantly inhibited by both HHCB and AHTN (Figures 1, 2) and cells need to maintain high levels of GR to function together with other enzymes in defending cells against degenerative attacks (Srikanth *et al*., 2013). GR acts as a substrate to other glutathione enzyme species to prevent oxidative stress and a balance in the amount of each glutathione enzymes is precursor for optimal cell defence. Therefore, GR inhibition may alter detoxification and antioxidant capacity of GST and GPx (McCay *et al*., 1976; Oxford Biomedical Research, 2001; Stojiljković *et al*., 2007). However, glutathione metabolism enzymes' pattern of responses is not always straightforward and depends on a series of factors, including the species (Antunes *et al*., 2013). Antioxidant defences can be overwhelmed by some chemical compounds which can depress the antioxidant capacity to remove oxyradicals and prevent cell damage (Regoli and Giuliani, 2014). Recent studies have shown alterations in antioxidant enzymes in aquatic and terrestrial organisms exposed to HHCB and AHTN, indicating the potential for oxidative stress. The goldfish *Carassius auratus* exposed to simulated urban runoff containing HHCB alone and HHCB mixed with cadmium showed a significantly increased antioxidant enzyme activity after 14 days and decreased significantly after 21 days of exposure (Chen *et al*., 2012). *Eisenia fetida* exposed to HHCB and AHTN upregulated antioxidant defence at a low dose of 0.6 µg cm^{-2} and significantly decreased at a concentration of 6.0 µg cm^{-2} after 48 h of exposure (Chen *et al*., 2011).

The alterations in the antioxidant enzymes activities could be linked to the significant increase in LPO recorded in this study after 21 days for clams exposed to AHTN and HHCB (Figures 1 and 2). Similarly, the zebra mussel, *D. polymorpha* exposed to 100 and 500 ng/L of HHCB and 20 and 80 ng/L of AHTN showed significant time-dependent lipid peroxidation after 21 days (Parolini *et al*., 2015). *E. fetida* exposure to HHCB and AHTN also induced a time-dependent significant increase in LPO due to oxidative stress (Chen *et al*., 2011; Liu *et al*., 2011). LPO is a self-propagating chain reaction, and thus, it is believed that the foremost oxidation of only a few lipid molecules can cause serious tissue damage (Mylonas and Kouretas, 1999).

Concentration-dependent significant DNA strand breaks in clams exposed to environmental concentrations of AHTN and HHCB were observed at the end of the exposure (Figures 1, 2 and Table 2). This is expected to occur as a result of significant oxidative stress, because direct reaction between DNA and free radical species can result in DNA damage including damaged bases, structural breaks and/or inter and intra strand crosslinks. It is possible that genetic damage was also as a result of the metabolites of the parent compounds, which in some cases could be more toxic to organisms than the parent compounds tested. Significant correlations between pollutant induced reactive oxygen species and DNA damage in marine invertebrates have been reported (Mamaca *et al*., 2005). Our result is supported by previous research, which documented significant time-dependent DNA fragmentation in the zebra mussel *D. polymorpha* after exposure to environmentally relevant concentrations (100 and 500 ng/L for HHCB) even after four days. The authors also demonstrated that 80 ng/L of AHTN induced significant time-dependent DNA damage with a value 3.6-fold higher than the control on day 21 (Parolini *et al*. 2015). These results are indicative of chronic effects of HHCB and AHTN in freshwater and marine ecosystems.

In addition, EROD correlated with GST ($p < 0.05$) and GST positively correlated with GPx ($p < 0.05$) and DNA damage ($p < 0.01$) in clams exposed to HHCB (Table 2). GST positively correlated with GPx and GR ($p < 0.01$) (Table 2). Taking these relationships into account, it is clear that the exposure of clams to these substances exerted significant activation of biomarkers of exposure and effect as observed in the significant correlation of HHCB and AHTN concentrations with DNA damage ($p < 0.01$) assessed in the digestive gland of clams (Table 2) which could be consequential to unsuccessful detoxification of contaminant-generated oxyradicals.

The biomarkers evaluated in *R. philippinarum* to understand the environmental risk of HHCB and AHTN have been found useful and sensitive to the exposure to both substances in the marine environment. Although the studies with HHCB and AHTN are incipient, our findings indicating a potential chronic risk of both compounds are corroborated by previous studies that examined oxidative damage by quantifying

the malondialdehyde level for LPO as a biomarker in the earthworm *E. fetida* (Chen *et al*., 2011) and *Dreissena polymorpha* (Parolini *et al*., 2015) for terrestrial and aquatic environments, respectively. Additionally, it has also been shown under laboratory conditions that environmental concentrations of HHCB and AHTN might elicit avoidance behaviour in the estuarine shrimps *P. varians*, probably due to their organoleptic characteristics (Ehiguese *et al*., 2019). Because of the volatile nature of these contaminants under laboratory exposure, the reported biological effects may be underestimated (Tumová *et al*., 2019). Taking into account the different types of chronic effect that HHCB and AHTN can potentially produce, either in terrestrial (Liu *et al*., 2011), freshwater (Parolini *et al*., 2015) or marine [Ehiguese *et al*., (2019) and the current study] organisms, it is crucial to review the regulatory status, when the environmental concentrations just indicate a slight risk before irreversible environmental effects are exerted.

5. Conclusions

The current research revealed that environmental concentrations of HHCB and AHTN might have adverse effects (oxidative stress and genotoxicity) on the marine clam *R. philippinarum*. Results from this study showed that, in particular, the activities of EROD, GST, GPX, GR, LPO levels and DNA damage were useful essential biomarkers to evaluate effects of PMCs at environmental concentrations in the marine environment because changes related with the concentrations of AHTN and HHCB were detected.

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3.4. Use of molecular biomarkers to assess the endocrine disrupting potential of galaxolide and tonalide in marine organisms

Summary

Overt contaminant driven ecological stress is initiated at a lower level of biological organization, because bioavailable environmental chemicals can interact with macromolecules to alter biomolecular structures that play an important role in regulating the physiology of living organisms. Ribonucleic acids (RNA), which are produced by cellular process of transcription, and messenger RNA (mRNA), which translates genetic messages from deoxyribonucleic acids (DNA) to protein, can be isolated in exposed organisms using tissues, organs, or whole organisms, and quantified to evaluate pollution driven molecular effects. This section addressed the effects of HHCB and AHTN at molecular level, using biomarkers related with endocrine disruptions. The screening for the potential of both substances to disrupt endocrine functions in fish is particularly important for the marine environment because the intrinsic properties of HHCB and AHTN suggest that they might mimic natural hormones and alter steroidogenesis in teleost fish. Consequently, yolk sac larvae of sheepshead minnow *Cyprinodon variegatus* were exposed for 3 days to 0.5, 5 and 50 µg/L of each compound, and the expression levels of *vtg1* and *cyp19* were determined using qPCR.

The results obtained from this study showed that HHCB and AHTN can modulate steroidogenesis in fish. It was observed that HHCB upregulated cyp19 and vtg1, while AHTN significantly downregulated both genes expression levels when compared with the control. These results represent the first evidence of the potential steroidogenic effects of HHCB and AHTN in the marine environments.

The results together with the neuroendocrine biomarkers assessed in clams have been published as a peer-reviewed article entitled "Galaxolide and tonalide modulate neuroendocrine activity in marine species from two taxonomic groups" (**Ehiguese *et al*.**, 2021. **Environmental Research 169,** https://doi.org/10.1016/j.envres.2021.110960**.** The accepted manuscript formated to the journal recommended reference style is presented below.

The author's personal contribution included conceptualization, methodology, samples analyses, data curation, project management, original draft preparation, writing and editing of manuscript as well as first and corresponding authorship of the article.

This study was entirely performed at the Division of Coastal Sciences, School of Ocean Science and Technology, University of Southern Mississippi, Ocean Springs MS, USA.

1. Introduction

Galaxolide (HHCB) and tonalide (AHTN) are polycyclic musk compounds (PMCs) used in household and personal care products and are included on the list of emerging contaminants of environmental concern due to their ubiquity in aquatic and terrestrial environments (Schreurs *et al*., 2004; Zhang *et al*., 2013). HHCB and AHTN consist of aromatic structures consisting of acetylated and extremely methylated pyran and tetralin bases (Sumner *et al*., 2010). They possess high *n*-octanol – water partition coefficients (K*ow*) akin to most persistent organic pollutants and may persist in the marine environment. The presence and toxicity of PMCs in transitional and coastal ecosystems is yet to be fully understood. Most research regarding these compounds has been focused on freshwater ecosystems (Balk and Ford, 1999a, 1999b; Parolini *et al*., 2015; Yamauchi *et al*., 2008) with only a few reports available on the toxicity of PMCs in the marine environment (Breitholtz *et al*., 2003; Luckenbach *et al*., 2004; Wollenberger *et al*., 2003). Recently, Ehiguese *et al*. (2019) studied the avoidance behavior of the shrimp *Palaemon varians* and found that HHCB and AHTN potentially elicited avoidance behavior in this shrimp. Chronic exposure to environmentally relevant concentrations of these substances suggests that they may alter antioxidant enzyme activity and potentially trigger oxidative stress in Manila clams (Ehiguese *et al*., 2020). To the best of our knowledge, the neurotoxicity and endocrine disrupting effects of these contaminants in the marine environment are yet to be addressed.

About 30% of commercially available chemicals are estimated to possess neurotoxic and endocrine disrupting properties (Tilson *et al*., 1995). These chemicals can target neurotransmitter pathways and their components such as neurotransmitters, receptors, biosynthetic enzymes, catabolic enzymes, and transporters (Basu, 2015). Neuroendocrine compounds promote diverse physiological and behavioral effects that alter the capacity of organisms to reach their biotic potential, cope with stress and other environmental challenges, and survive (Waye and Trudeau, 2011). Signals from environmental contaminants can interfere with neurotransmission and disrupt endocrine functions in marine organisms because of their potential to mimic the natural hormone estrogen, and can bind to estrogen receptors and influence estrogen biosynthesis (Waye and Trudeau, 2011). Many persistent organic pollutants have been implicated as neuroendocrine disruptors in the marine environment causing adverse effects related to changes in thyroid morphometry and functions, suppression of ovarian follicle development, altered sex differentiation, and mortality (Berg *et al*., 2016; Porte *et al*., 2006; Schnitzler *et al*., 2008). Furthermore, environmental concentrations of some pharmaceutical products have been shown to inhibit monoamine oxidase activity, increase plasma cortisol levels, and reduce feeding in aquatic organisms *(Maranho et al*., 2015; Melnyk-Lamont *et al*., 2014). Importantly, HHCB and AHTN have been demonstrated to disrupt

neuroendocrine activity in several *in vitro* studies(Li *et al*., 2013; Mori *et al*., 2007; Schreurs *et al*., 2005, 2004, 2002) and significantly alter gene expression levels in male medaka fish (Yamauchi *et al*., 2008).

The aim of this study was to investigate the neurotoxic and endocrine disrupting effects of HHCB and AHTN in the marine environment. We assessed biochemical activities in Manila clams (*Ruditapes philippinarum*) using biomarkers of neuroendocrine toxicity (AChE, COX) and energy reserves (total lipids; TL) in a 21 day exposure. We also assessed and gene expression levels of *cyp19* and *vtg1* in yolk-sac larvae of sheepshead minnow (*Cyprinodon variegatus*) after 3 days of exposure to HHCB or AHTN. The suitability of *R. philippinarum* for ecotoxicological studies has been previously stated by Ehiguese *et al*. (2020). Sheepshead minnow are a suitable marine model used in ecotoxicological studies because they are easy to breed under laboratory conditions and spawn continuously with relatively large demersal eggs (Cripe *et al*., 2009). They have been used in the assessment of endocrine disrupting chemicals in transitional and coastal waters (Bowman *et al*., 2000; Folmar *et al*., 2000; Hemmer *et al*., 2001) as well as to characterize alteration of immune pathways (Jones *et al*., 2017; Rodgers *et al*., 2020) and oxidative stress (Rodgers *et al*., 2018) after chemical exposures.

2. Materials and methods

2.1. Test chemicals

For the clam experiments, analytical grades of HHCB (85.0%) and AHTN (97.0%) were obtained from Sigma Aldrich Spain. The details of dissolution and preparations can be found in Ehiguese *et al*. (2020). In brief, stock solutions were prepared using DMSO (0.001%v/v) as the organic solvent to dissolve the test chemicals which were further diluted with distilled water to reach the concentrations needed (0.005, 0.05, 0.5, 5 and 50 µg/L). These concentrations were selected based on reported environmental concentrations measured in marine environments (Díaz-Garduño *et al*., 2017; Pintado-Herrera *et al*., 2013). For the fish experiments, 100 g of analytical grade AHTN (97%) was purchased from Sigma Aldrich, USA. 10 g of the product was dissolved in 0.001%v/v DMSO to form the stock solution. 25 g of HHCB dissolved in 50% diethyl phthalate containing 49% pure HHCB was purchased from TCI America, USA and the concentrations required were calculated based on the percentage of the active ingredient of HHCB in the solution. The stock solution was diluted using distilled water to create 0.5, 5.0 and 50.0 µg/L solutions for each compound.

2.2. Test organisms

The clams, *R. philippinarum* (550 specimens), were obtained from an aquaculture farm in the south-west of Spain and were transported to the laboratory of Marine Culture, Faculty of Marine and Environmental Science (University of Cadiz, Spain). The adult clams (average size of 43.2±1.6 mm) were acclimated in a 250 L aquarium and were fed with *Isochrysis galbana* once per day. During acclimation, aeration was provided to improve the oxygen content (dissolved oxygen >5 mg/L) of the medium and other physiochemical parameters in the aquarium were monitored and controlled. The temperature, salinity, pH and photoperiod during the acclimation were 15±1 ^{0}C, 34.7±0.4‰, 7.8 – 8.2 and 12 h light/12 h dark, respectively.

Adult sheepshead minnows (*C. variegatus*) previously purchased and kept in artificial seawater (15‰), between the temperature range of 25-27 ^{o}C and photoperiod (12:12 light/dark) in 300 L static recirculating raceways at the Toxicology Building, Gulf Coast Research Laboratory, University of Southern Mississippi (Ocean Springs MS, USA) were used as brood stocks. Before breeding, the brood stocks were fed daily with *Artemia* nauplii and commercial flake food. Four Spawntex® Mats (15-20 cm; Pentair Aquatic Eco-Systems) were placed in the two holding raceways overnight for spawning, two per raceway, containing gravid females with a female:male at a ratio of 2:1. The fertilized eggs were collected by gently tapping the Spawntex® Mats into a clean laboratory dish. The embryos were gently rinsed and transferred into a hatching jar supplied with aeration to aid suspension of the fertilized eggs in the water column and they were incubated in an ISOTEMP 115 (Fisher Scientific) at 30 ^{o}C until hatching (Dangre *et al*., 2010; Griffitt *et al*., 2012). These yolk-sac larvae were then carefully collected in 100 ml beakers filled with artificial seawater (15‰) prior to exposure.

2.3. Biochemical effects: Experimental approach

R. philippinarum were exposed aqueously to either HHCB or AHTN in 10 L rectangular glass aquariums. The bioassay experiment was carried out in duplicate for all treatments including the controls (seawater and DMSO). Natural, filtered seawater was obtained from the Marine Culture laboratory of the University of Cadiz, and 8 L of the seawater was mixed with each test chemical. 16 clams were added to each aquarium, totaling 32 per treatment except for the seawater and solvent controls with 14 specimens each. The treatments were renewed every three days, during which the water was siphoned out of the holding tanks and carefully cleaned and refilled with seawater spiked with freshly prepared contaminant. Any dead clams were immediately removed and recorded. The physical and chemical properties were adjusted to the same

conditions as reported above during the acclimation period. Three clams were randomly collected from each replicate on day 3, 7, 14 and 21, and tissues (digestive gland and gonads) were immediately harvested on ice and stored at -80 ºC in the laboratory prior to homogenization.

Buffer was prepared for sample homogenization using 0.1 mM EDTA, 100 mM NaCl, 25 mM HEPES salt, and 0.1 mM DTT. The samples stored in the freezer were thawed on ice and the digestive glands and gonads of three clams from each aquarium were pooled together for homogenization. The pooled samples were homogenized, and a fraction of each homogenate (HF) was centrifuged to obtain supernatant portions at a speed of 15.000 x g for 20 min at 4 ºC (S_{15}) and 3.000 x g for 20 min at 4 ºC (S_3). The Bradford (1976) methodology was adapted to determine the corresponding total protein (TP) concentration with values expressed as mg/mL for different extracts (HF, S_3 and S_{15}). The biochemical analyses of the biomarkers were quantified using a kinetic microplate reader, Infinite® M200.

2.4 Collection and analysis of exposure water

Exposure water for each concentration was sampled using clean amber bottles on day 0 and 3 for analysis of initial and final concentrations and the samples were kept in -20 ºC prior to the chemical analysis. Details of the methodology for the chemical analysis and the results of the detection and quantification can be found in Ehiguese *et al*. (2020).

2.5. Biochemical analyses

2.5.1. Acetyl Cholinesterase (AChE) Activity

AChE activity was measured in the post-mitochondria fraction of the digestive glands according to the methodology described in Guilhermino *et al*. (1996). 20 µL of the centrifuged S_{15} fraction was added to 20 µl of 50 mM potassium phosphate buffer at a pH of 7.5 in 96 transparent, flat bottom wells. 130 µL of 5,5-Dithiobis (2-nitrobenzoic acid) (DTNB) solution was further added with 50 µL of acetylthiocholine iodide solution. The activity of AChE was determined by absorbance measured at 405 nm at every 40 – 52 sec for 5 – 7 min. The data were expressed as the formation of thiols in pmol DTNB/min/mg TP.

2.5.2. Cyclooxygenase (COX) Activity

COX activity was measured according to Gagné *et al*. (2015), following the oxidation of 2,7-dichlorofluoresceine in the presence of arachidonate (Fujimoto *et al*., 2002). Briefly, 96 dark, flat bottom well microplates were coated with 50 µL of the S15 sample. 200 µL of the assay buffer containing 50 µM arachidonic acid and 2 µM dichlorofluoresceine was added with 0.1 µg/mL horseradish peroxidase containing 50 mM Tris-HCl, pH 8.0 and 0.05% Tween 20. The reaction was incubated at 30 ºC for 0, 5, 10, 15, 20, 25 and 30 min and the fluorescence were measured at 485 nm (excitation) and 520 nm (emission). The data were expressed as µmol fluorescein/min/mg TP.

2.5.3. Total lipids (TL)

TL were measured in gonad and digestive gland tissues following the phosphovanilin method by Frings *et al*., (1972). Dark microplates with 96 flat-bottom wells were coated with 10 µL of samples diluted with 10 µL of MilliQ. 30 µL of concentrated sulphuric acid and 150 µL of phosphovanilin prepared with vanillin and phosphoric acid in water were added and incubated for 10 min at 80 ºC and cooled at 4 ºC for 2 min. The absorbance was determined at 540 nm. A standard solution of Triton X-100 was used for calibration and the results were expressed as µg TL/mg TP.

2.6. Molecular effects: Experimental approach

All tests were performed in static renewal bioassays in triplicate; 200 mL of seawater spiked with each treatment (0.5, 5.0 and 50 µg/L) was transferred into laboratory dishes. Then, 10 yolk-sac larvae of *C. variegatus* were randomly selected and transferred into each dish including the controls (seawater and DMSO). They were incubated in a Precision Scientific Incubator (Thermo, MA, USA) at 30 ºC and the exposure water in each dish was renewed every 24 h during the 3 days exposure. Upon termination of the experiment, the larvae were inserted into 1.5 mL tubes containing 500 µL RNALater® solution and stored at -80 ºC prior to RNA extraction.

2.7. Molecular analysis: Quantitative PCR (qPCR)

For RNA extraction, six larvae per replicate were pooled from each treatment and both controls (seawater and DMSO) for homogenization. Total RNA was extracted using RNeasy kits (Qiagen, Hilden, Germany)

as described in the manufacturer's protocol, and the total RNA quantity and quality was assessed spectrophotometrically using a NanoDrop™ 2000 (Thermo Scientific, Wilmington, DE, USA). Reverse transcription of total RNA to single stranded cDNA was performed using RevertAid First Strand cDNA Synthesis Kit following the manufacturer's instructions (Thermo Scientific, Wilmington, DE, USA).

Real-time qPCR was performed using the primers listed in Table 1. 18s was used as an endogenous control, while *cyp19* and *vtg1* were used as biomarkers of endocrine disruption. All qPCR reactions were performed in triplicates using an Applied Biosystems 7500 Fast Cycler with Fast SYBR Green Master Mix (Life Technologies, Carlsbad, CA). Relative quantification values compared to the control samples were determined by applying the ΔΔCT method. Fold changes in *cyp19* and *vtg1* genes were log transformed to normalize the data.

Table 1. Details of forward and reverse primers

Gene	Primers	Amp. Length	Ref
18s	F: GCTGAACGCCACTTGTCC R: ATTCCGATAACGAACGAGACTC	100	Simning *et al*., 2019
cyp19	F: CTGTCCCCTGCAATCCCAAT R: AAAGGGGACCCAAACCCAAG	72	This study
vtg1	F: ATGTCACTGTGAAGGTCAACGAA R: ACCTGTTGGGTGGCGGTAA	68	Knoebl *et al*., 2004

2.8. Statistical Analysis

All data were analyzed using SPSS (16.0) statistical package. Data normality and homogeneity were assessed before statistical tests were performed. One-way analysis of variance (ANOVA) coupled with Dunnett's multiple comparison tests were performed and significant differences between controls and clams treated with HHCB and AHTN were determined at $p < 0.05$. The relationships between effects and concentrations were checked using Spearman's rank order of correlation, and significant points were set at $p < 0.05$ and $p < 0.01$. For gene expression levels, significant differences were determined using a least-square difference test and significant difference was set at $p < 0.05$.

3. Results

3.1. Biochemical effects

No mortality was recorded in control clams, meanwhile 3% mortality was recorded in clams treated with HHCB and ~ 4% mortality in the clams treated with AHTN during the experiment.

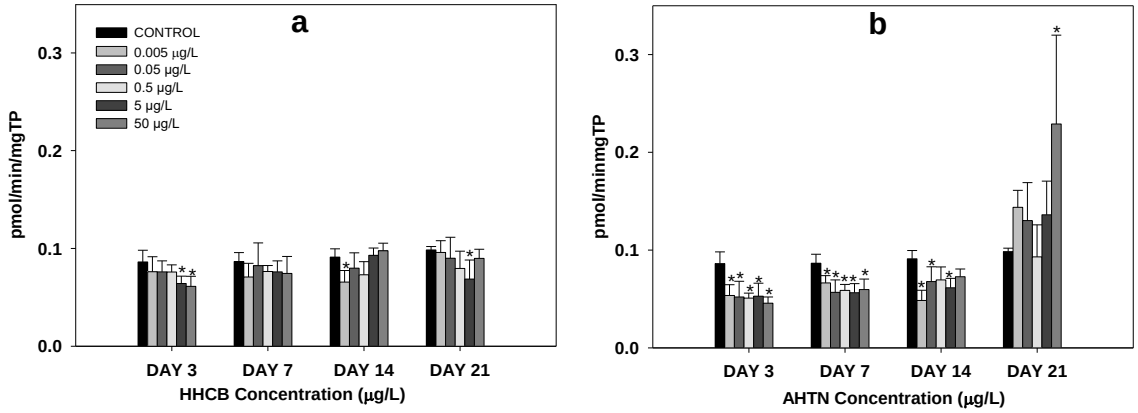

Figure 1: Acetyl Cholinesterase (AChE) activity measured in the digestive gland tissues of *R. philippinarum* exposed for 21 days to (a) galaxolide (HHCB) and (b) tonalide (AHTN). Asterisks (*) show significant differences from control ($p<0.05$)

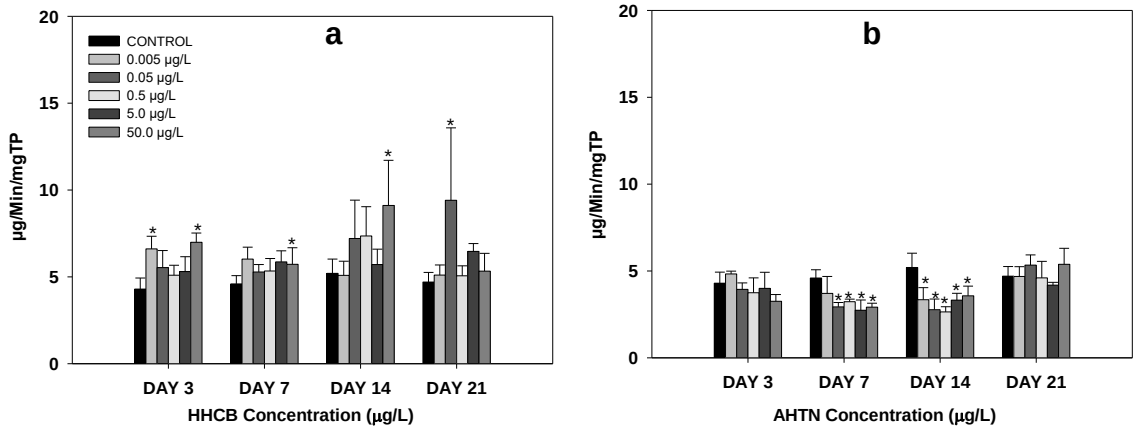

Figure 2: Cyclooxygenase (COX) activity measured in the digestive gland tissues of *R. philippinarum* exposed for 21 days to (a) galaxolide (HHCB) and (b) tonalide (AHTN). Asterisks (*) show significant differences from control ($p<0.05$).

The effects of HHCB and AHTN on AChE activity are presented in Figure 1. There was significant inhibition ($p < 0.05$) of AChE activity in the clams treated with HHCB at 5.0 µg/L and 50 µg/L after 3 days (Figure 1a). As the exposure continued, significant differences in AChE activity were further seen in the clams treated with 0.005 and 5 µg/L HHCB on days 14 and 21, respectively (Figure 1a). Prolonged significant inhibition of AChE ($p < 0.05$) was triggered by all concentrations of AHTN tested until day 7, but only 0.005, 0.05 and 5.0 µg/L concentrations produced significant AChE inhibition by day 14 (Figure 1b). Interestingly, at the end of the experiment (day 21), the 50 µg/L AHTN exposure showed a significant increase in AChE activity. (Figure 1b).

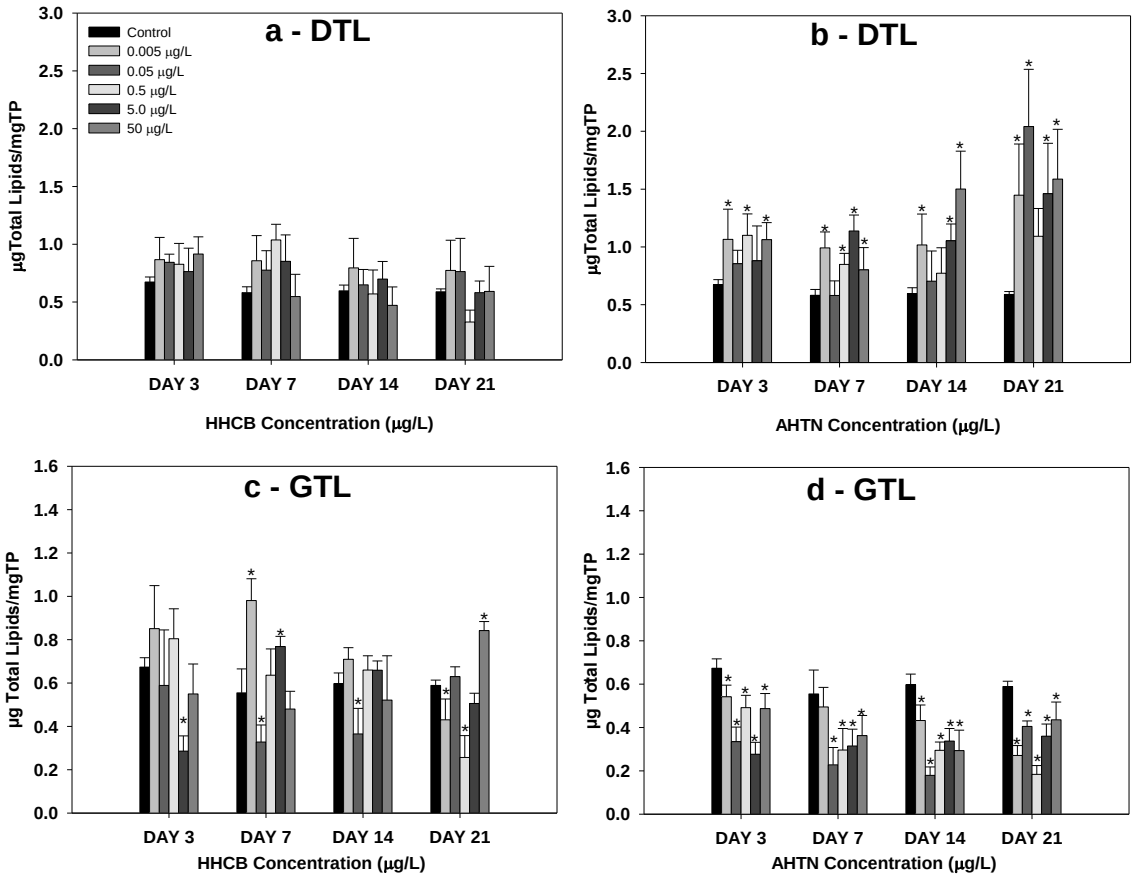

Figure 3: Energy reserves measured as total lipids in digestive gland (a & b - DTL) and gonad (c & d - GTL) tissues of *R. philippinarum* exposed for 21 days to galaxolide (HHCB) and tonalide (AHTN). Asterisks (*) show significant differences from control ($p<0.05$).

The impact of HHCB and AHTN on COX activity measured in *R. philippinarum* after 21 days exposure in a semi-static bioassay is presented in Figure 2. COX activity was induced in the clams treated with HHCB and this induction was significant in the 0.005 and 50 µg/L exposures on day 3. Continuous exposure to HHCB up to day 14 produced significant effects on COX activity at the highest concentration (50 µg/L) tested, but at day 21 only the 0.05 µg/L concentration significantly increased ($p < 0.05$) COX activity (Figure 2a). On the other hand, AHTN inhibited COX activity in *R. philippinarum*, and the inhibition was significantly different from the control group ($p < 0.05$) on days 7 and 14 in all the concentrations tested except for 0.005 µg/L on day 7(Figure 2b).

The energy reserves, measured as total lipids, were determined in the digestive gland (DTL) and gonad (GTL) tissues of clams exposed to HHCB and AHTN. There was no significant difference in DTL in the clams exposed to HHCB (Figure 3a). For the clams exposed to AHTN, DTL increased significantly ($p < 0.05$) throughout the exposure period with the exception of the 0.05 µg/L treatment group on days 3, 7, and 14, and the 0.5 µg/L treatment on days 14 and 21 (Figure 3b). There was no clear pattern in GTL of the clams exposed to HHCB, as we observed both significant increases and decreases in GTL depending on the treatment and time point (Figure 3c). However, GTL decreased significantly ($p < 0.05$) after exposure to AHTN in all treatment groups except at the lowest concentration (0.005 ug/L) measured on day 7 (Figure 3d).

The activities of neuroendocrine biomarkers (AChE and COX) measured in the clams correlated significantly with the concentrations of HHCB ($p < 0.01$) over time (Table S1 – Supplementary Materials). For AHTN, a significant time and concentration-dependent correlation of AChE and COX was observed. In addition, the inhibition of AChE and COX activities correlated significantly ($p < 0.01$) (Table S2 – Supplementary Materials). Finally, gonad energy reserves (GTL) were significantly depleted over time (Table S2 – Supplementary Materials).

3.2. Molecular effects

There was no mortality in the yolk sac larvae of *C. variegatus* exposed to HHCB; for AHTN exposures, 3% mortality was recorded in the fish exposed to the 50 µg/L treatment.

The expression of *cyp19* in yolk sac larvae of *C. variegatus* exposed to HHCB was slightly upregulated, though not significantly in any of the treatments (Figure 4a). However, concentration-dependent downregulation of *cyp19* was observed in the larvae exposed to AHTN and was significantly different ($p <$

0.05) at the highest concentration (50 µg/L) with more than a 3-fold change in expression compared to the controls (Figure 4b).

Expression of *vtg1* measured in the yolk-sac larvae of *C. variegatus* exposed to both substances had a similar pattern to *cyp19* expression (Figure 5). HHCB slightly induced the expression of *vtg1* and the induction was highest at 5.0 µg/L, though not significant (Figure 5a). For AHTN, a concentration-dependent downregulation of *vtg1* was observed and decreased significantly ($p < 0.05$) by 3.40-fold versus the controls at the 50 µg/L exposure concentration (Figure 5b).

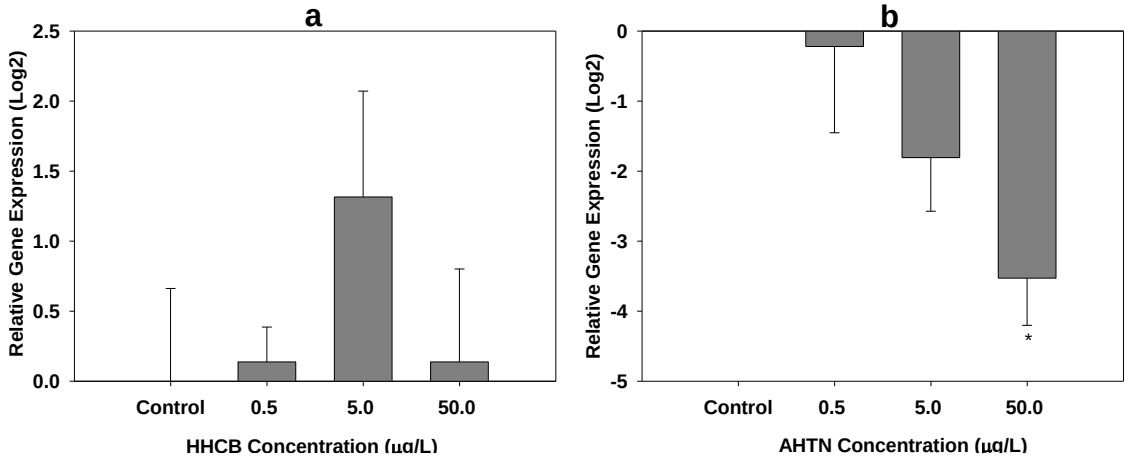

Figure 4: Relative gene expression s for cyp19 in *C. variegatus* yolk sac larvae exposed to (a) galaxolide (HHCB) and (b) tonalide (AHTN) for 96 h. Asterisks (*) show significant differences from control ($p<0.05$).

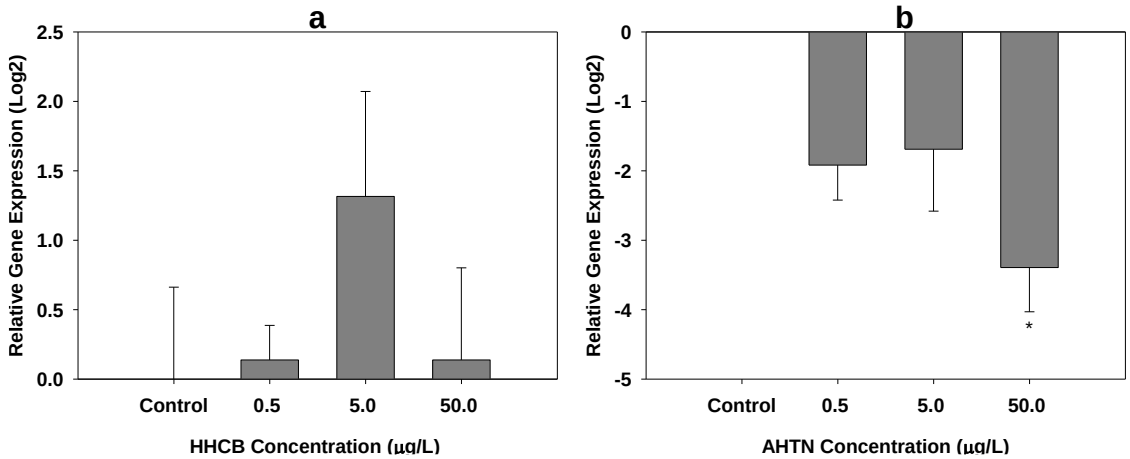

Figure 5: Relative gene expression for vtg1 in *C. variegatus* yolk sac larvae exposed to (a) galaxolide (HHCB) and (b) tonalide (AHTN) for 96 h. Asterisks (*) show significant differences from control ($p<0.05$)

4. Discussion

The current study assessed the neuroendocrine effects of environmental concentrations of HHCB and AHTN in the marine environment by assessing enzyme activities and gene expression levels in marine organisms from two taxonomic groups. Biomarkers of endocrine disruption (*cyp19* and *vtg1*) were measured in *C. variegatus* and neurotoxicity (AChE) was measured in *R. philippinarum* together with assessments of neuroendocrine and inflammation responses (COX) and energy reserves (TL).

4.1. Biochemical effects

Significant concentration-dependent AChE inhibition was observed in the clams treated with environmentally comparable concentrations of HHCB and AHTN, at the first time point (3 days of exposure - Figure 1). AChE is a well-established biomarker in toxicological studies of neurotoxicity and is the enzyme responsible for the deactivation of acetylcholine at the cholinergic synapses, preventing a build-up of acetylcholine, which is necessary for the normal functioning of sensory and neuromuscular systems (van der Oost *et al*., 2003; Sturm *et al*., 2000). AChE is also a target of many organic pollutants, toxic metals, human pharmaceuticals and personal care products, which have all been reported to inhibit AChE activity (Aguirre-Martínez *et al*., 2016; Maranho *et al*., 2015; Matozzo *et al*., 2005; Stefano *et al*., 2008). Our observations showed that these substances might possess the ability to bind with cholinesterase, preventing the breakdown of acetylcholine. It appears that HHCB and AHTN can inhibit AChE after a short exposure

to environmental concentrations, but this does vary with the concentration and duration of the exposure (Figure 1). For HHCB, limited AChE inhibition was observed at days 14 to day 21 with no significant inhibition on day 7 (Figure 1a). This may be because the clams were able to metabolize HHCB to less toxic metabolites during the exposure (Balk and Ford, 1999a). On the other hand, all the AHTN exposure concentrations inhibited AChE activity until day 14 when all but the highest concentration of AHTN significantly ($p < 0.05$) inhibited AChE activity (Figure 1b). Consequently, chronic inhibition of AChE in clams could lead to high levels of acetylcholine, over-stimulation of cholinergic receptors, alteration of postsynaptic cell function, and signs of cholinergic toxicity such as morphological and behavioral changes may start to manifest (Nallapaneni *et al*., 2008; Pope *et al*., 2005; Song *et al*., 2004; Waseem *et al*., 2010). Furthermore, fatality may occur if AChE activity is depressed during exposure to cholinesterase-inhibiting chemicals due to the overstimulation of the target cells (Sancho *et al*., 2000). The nitro musk compound ambrette was previously reported to possess neurotoxic potential as well as elicit carcinogenesis in organisms, leading to its prohibition (Nair *et al*., 1986; Spencer *et al*., 1984). Our results are comparable with other studies reporting inhibition of AChE in bivalves after chronic exposure. Shan *et al.* (2020) reported significant inhibition of AChE in the digestive gland of Asian clams (*Corbicula fluminea*) exposed to 20 - 2000 µg/L imidacloprid for 30 days. Similarly, 0.1 – 1 µg/L carbamazepine, 5 and 50 µg/L caffeine, and 50 µg/L ibuprofen significantly decreased AChE activity assessed in the digestive gland of *C. fluminea* after 21 days exposure (Aguirre-Martínez *et al*., 2018). In contrast, environmental concentrations (15 µg/L) of ibuprofen and carbamazepine reportedly increased AChE activity assessed in the gills of *R. philippinarum* after 7 days exposure (Trombini *et al*., 2019). Although both HHCB and AHTN inhibited AChE activity at various points, AHTN appears to be more robust at inhibiting AChE because it exerted prolonged inhibition of AChE activity until day 14, although the clams seem to have recovered by day 21 (Figure 1b). These results demonstrate the potential of polycyclic musk compounds as neuroinhibitors and provide a baseline upon which neurotoxicity of HHCB and AHTN could be further investigated.

HHCB increased COX activity in clams, though this inhibition was also concentration and time-dependent (Figure 2a). COX catalyzes arachidonic acid to form prostaglandins which are responsible for several physiological and reproductive functions in aquatic organisms (Di Costanzo *et al*., 2019). It is an important indicator of inflammation in aquatic organisms exposed to environmental stressors (Gagné *et al*., 2015). Clams exposed to wastewater effluents in the Bay of Cadiz (Spain) exhibited significantly inhibited COX activity and triggered inflammatory responses in the gonad tissues, which correlated significantly with general stress, measured as lysosomal membrane stability (Díaz-Garduño *et al*., 2018). Recent studies have demonstrated that both HHCB and AHTN induce oxidative stress in clams (Ehiguese *et al*., 2020). In addition, COX synthesis of prostaglandins is involved in the control of oogenesis and spermatogenesis in

aquatic invertebrates (Di Costanzo *et al*., 2019) and controls the effect of serotonin in the spawning process of bivalves (Matsutani and Nomura, 1987). Prameswari *et al*. (2017) demonstrated that arachidonic acid induced a significantly ($p < 0.001$) increased ovarian index, oocyte diameter and ovarian vitellogenin in the freshwater crab (*Oziothelphusa senex senex*). The authors reported that COX inhibitors, including indomethacin and aspirin, significantly ($p < 0.001$) reduced ovarian index, oocyte diameter and ovarian vitellogenin levels, corroborating the involvement of COX in the regulation of female reproduction in crabs. Given the concentration-dependent alteration of COX activity in clams exposed to HHCB and AHTN, reproductive success could be at risk, but additional research is needed to validate this hypothesis. Furthermore, the functions of COX activity in marine bivalves are not fully understood but the significant correlation of COX and AChE activities in clams exposed to AHTN (Table S2 – Supplementary Materials) suggests that COX activity may be involved in neuroendocrine functions in marine bivalves. Consequently, AHTN might be a neuroendocrine disruptor in the marine environment.

To understand toxicity-driven energy deficit in clams, TL in the digestive gland and gonadal tissues were measured. It was hypothesized that chemical stress may trigger significant energy demand due to relatively high enzyme activities. For *R. philippinarum* exposed to HHCB, total lipids measured in DTL were unaffected and we did not observe any clear tendency in energy level measured in the gonads (Figure 3 a, b). The DTL measured in the clams exposed to AHTN was significantly ($p < 0.05$) higher than the control for most treatments and time points (Figure 3b), but GTL was significantly reduced in most treatments and time points (Figure 3d). Total lipids assessed in the gonad of *R. philippinarum* exposed to wastewater effluents was significantly reduced in other studies (Díaz-Garduño *et al*., 2018; Maranho *et al*., 2016). The variation in total lipids assessed in this study, especially for HHCB, did not reveal any clear trend.

4.2. Molecular effects

Exposure of fish to endocrine disrupting chemicals in the marine environment has serious consequences concerning survival and reproduction. In yolk-sac *C. variegatus* larvae exposed to HHCB, the expression of *cyp19* showed no concentration-dependent transcriptional effect (Figure 4a). Meanwhile, we observed concentration-dependent downregulation of *cyp19* expression levels after 3 days of exposure to AHTN (Figure 4b). Similarly, significant downregulation of *cyp19b* expression levels were measured in juvenile salmon exposed to 0.04 – 1 mg/L of the organophosphate flame retardant, tris(2-cloroethyl) phosphate for 7 days (Arukwe *et al*., 2016), and significant dose-dependent downregulation of *cyp19a* and *cyp19b* were recorded in the ovaries of adult marine medaka exposed to 2 – 5 ng/L 17β-trenbolone for 21 days (Zhang *et al.*, 2020). In contrast, *cyp19a* and *cyp19b* expression levels were upregulated in adult male and female

Danio rerio exposed to 1 mg/L perfluorodecanoic acid (Jo *et al.*, 2014) and perfluorononanoate (Zhang *et al*., 2016). *cyp19* is an important biomarker of endocrine disruption in teleosts because aromatase, the enzyme involved in the conversion of androgen to estrogen, plays an essential role in sexual differentiation, maturation, and reproduction (Cheshenko *et al*., 2008). *cyp19* is regarded as a major target for endocrine disrupting chemicals because modulation of its expression and function may potentially disrupt estrogen production (Cheshenko *et al*., 2008; Kazeto *et al*., 2004). Our results suggest that AHTN may be a more potent modulator of *cyp19* expression than HHCB (Figure 4). Previous reports in an *in vitro* study using the H295R cell line exposed to 25 µM HHCB demonstrated upregulation of *cyp19*, while AHTN downregulated *cyp19* by 43% of the basal control (Li *et al*., 2013). The modulation of *cyp19* by HHCB and AHTN in *C. variegatus* larvae may affect estrogen biosynthesis and, as a result, alter the survival, sexual behavior, and sex differentiation in fish. Although most studies of estrogen as an endocrine disruption biomarker in fish are related to reproductive functions or tissues, estrogen alteration may also affect tissue mineralization and mineral homeostasis (Suzuki *et al*., 2009; Yoshikubo *et al*., 2005), as well as delay development in early life stage fish (Rawson *et al*., 2006).

The induction and inhibition of *vtg1* in *R. philippinarum* exposed to HHCB and AHTN, respectively, was similar to *cyp19* (Figure 5). The basis for this similarity is not well understood but it appears that because vitellogenin is induced by estrogen (which is biosynthesized by the enzyme complex aromatase that converts androgen into estrogen), effects on *cyp19* may trickle down to have an impact on vitellogenin (Andersen *et al*., 2003). Previous studies have demonstrated correlations between aromatase and vitellogenin in fish exposed to endocrine disrupting chemicals (Andersen *et al*., 2003; Bizarro *et al*., 2014). However, further investigation is needed to help understand the relationships between the effects of contaminants on both biomarkers. Similar to our observation for *vtg1* measured in *C. variegatus* yolk-sac larvae exposed to HHCB, there was no statistically different increase in plasma vitellogenin levels in rainbow trout intraperitoneally injected with 1.41 X 10^{-5} mol/Kg of HHCB for 5 days (Simmons *et al*., 2010). Previous research on the effects of both contaminants have showed that a three day exposure of male medaka to 5, 50 and 500 µg/L of HHCB and AHTN led to a significant induction in the expression of *vtg1* at 500 µg/L (Yamauchi *et al*., 2008). Meanwhile, the concentration of AHTN that elicited significant inhibition of *vtg1* in our study was much lower (50 µg/L), which may be attributed to age differences as fish larvae tend to be more sensitive to environmental contaminants than adults (Hutchinson *et al*., 1998). Reports of other chemicals inducing differential *vtg1* expression levels in fish abound. For example, three generations of *Oryzias melastigma* exposed to 20 and 200 µg/L benzo[a]pyrene demonstrated significant downregulation of *vtg1* expression (Sun *et al.*, 2020). In addition, female *Oryzias melastigma* exposed to lower concentrations (2 and 10 ng/L) of 17β-trenbolone (Zhang *et al*., 2020) and F1 generation male

Oryzias latipes exposed to metformin (Lee *et al.*, 2019) all significantly downregulated *vtg1* expression levels. Contrarily, exposure of male *Oryzias melastigma* to 10 and 50 ng/L of 17α-ethynylestradiol significantly upregulated *vtg1* expression level (Zhang *et al*., 2020).

Generally, the concentrations of PMCs causing effects should be interpreted with caution due to their high volatility and potential to adsorb to aquaria walls. From our studies, the concentrations of HHCB and AHTN measured on day 0 were 74.26±18.38% and 88.50±19.09%, respectively and degraded to 22.00±1.41% and 26.37±4.24%, respectively on day 3 (Ehiguese *et al*., 2020). Tumová *et al.* (2019) suggested that the toxicity of PMCs for aquatic organisms under semi-static conditions could be underestimated due to the potential volatility in the aquarium, significantly lowering the concentration over time.

5. Conclusion

This study assessed the neuroendocrine effects of HHCB and AHTN using the marine bivalve *R. philippinarum* and yolk-sac larvae of an estuarine species, the sheepshead minnow, *C. variegatus*. Changes in AChE and COX activities as biomarkers of neuroendocrine effects were observed in the clams, though the exact effects varied with concentration and duration of exposure. *cyp19* and *vtg1* gene expression in yolk-sac *C. variegatus* larvae after 3 days of exposure to HHCB revealed no effect for the expression of either gene, but AHTN significantly downregulated the expression of both genes at 50 µg/L. The biomarkers studied provided useful insights to understand the potential neuroendocrine toxicity of both substances in the marine environment. Although significant changes were detected in some of the biomarkers after exposure to each contaminant, AHTN seems to be a more potent inhibitor of neuroendocrine functions in marine organisms than HHCB.

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Chapter 4

General Discussion and future perspectives

4.1 General discussion

The main objective of this thesis was to assess the environmental risk of the PMCs (HHCB and AHTN) in marine environments because evidence have shown that these substances are present in different compartments of the ecosystems. Moreover, as enumerated in chapter 1, they have been found to bioaccumulate in marine organisms of different trophic levels (Moon *et al*., 2012a, 2012b; Nakata, 2005; Nakata *et al*., 2007). Therefore, hypotheses were developed to address the risks of those musks to marine organisms at molecular, biochemical, and individual levels. Research experiments were designed to test different hypotheses. Environmental risk assessments of these substances were performed determining dose-response relationships where acute and chronic effects were studied after exposure to different concentrations of HHCB and AHTN. Different species, representing different trophic levels and levels of biological organization as well as different endpoints were examined. Experimental design involved short-term impacts of HHCB and AHTN on the growth of the microalgae *Phaeodactylum tricornutum, Tetraselmis chuii, Isochrysis galbana* and *Raphidocelis subcapitata*, the fertilization success, larval development of sea urchin (*Paracentrotus lividus*) and mussels (*Mytilus galloprovincialis*), motility and survival of artemia (*Artemia franciscana*), and survival of fish yolk sac larval (*Sparus aurata*). Other hypotheses tested were related to the loss of biodiversity due to the effects on the spatial displacement of organisms and the possible immediate population decline of marine organisms (namely the shrimp *Palaemon varians*) under a heterogeneous chemically exposure scenario, biochemical changes in the clams *Ruditapes philippinarum* after chronic exposure and modulation of gene expression of endocrine biomarkers in yolk sac larval of the fish *Cyprinodon variegatus*.

HHCB and AHTN have low molecular weight (<300 g/mol) and high octanol-water partition coefficient of 5.9 and 5.7, respectively (Balk and Ford, 1999). These substances are lipophilic, and they are frequently detected emerging contaminants in aquatic ecosystems because they are incorporated in household and personal care products, which are used only for external purposes (Sumner *et al*., 2010). Therefore, this PhD thesis investigated the effects of HHCB and AHTN by exposing different organisms under laboratory conditions to environmental relevant concentrations of these substances. Because of the inherent lipophilicity of HHCB and AHTN, it is suspected that under laboratory condition they may adsorb to aquaria walls and bioaccumulate in the exposed organisms but, due to practical limitations, the bioaccumulation of both compounds was not assessed. Except for the acute toxicity study reported in chapter 3, the exposure water for the chronic toxicity and the spatial avoidance assays were measured and the results showed that HHCB and AHTN degrade in the laboratory a few hours after exposure to as low as 23% after 3 day. Therefore, the effects recorded were cautiously interpreted because the concentration causing the effects may be much lower than the nominal concentrations.

The current chapter focuses on the general discussion of the results found in this PhD research. Different assay methodologies were used including forced exposures in a static and semi-static bioassay, and non-forced exposure systems to understand the range of effects these compounds might exert on marine ecosystems. Furthermore, a range of effects were detected in the various studies that were conducted at different levels of biological organization, from molecular levels to effects related to spatial distribution at the landscape level (e.g., avoidance studied in a non-forced exposure system). The general discussion in this chapter is therefore attempted to elucidate the stress and damage that HHCB and AHTN could provoke after exposure to marine organisms, from different levels of biological organization. The analysis of dose response might help in the prediction of adverse effects that these substances may cause at higher levels of the biological organization.

4.2 Marine environmental risk of HHCB and AHTN measured at the molecular level.

Overt contaminant driven ecological stress is initiated at a lower level of biological organization because bioavailable environmental chemicals can interact with macromolecules to alter biomolecular structures that play an important role in regulating the physiology of living organisms. Ribonucleic acids (RNA), which are produced by cellular process of transcription, and messenger RNA (mRNA), which translates genetic messages from deoxyribonucleic acids (DNA) to protein, can be isolated in exposed organisms using tissues, organs, or whole organisms, and quantified to evaluate pollution driven molecular effects (Lowe *et al*., 2017). In addition, significant improvement in bio-analytical technology has made it possible to assess and quantify these alterations and provide understanding of the mode of action of contaminants in biological systems, because molecular mechanism of toxic effects is fundamental to understanding the detrimental biochemical and physiological effects contaminants can exert on organisms.

Molecular effects of HHCB and AHTN in post hatched larval *C. variegatus* using biomarkers related to endocrine disruptions were measured. Relative expression of *cyp19* and *vtg1* in the fish were quantified after 3 days exposure to environmental concentrations of the test chemicals because these genes are regarded as important biomarkers of aromatase inhibition (Muth-Köhne *et al*., 2016). *Cyp19* gene codes for the cytochrome P450 enzyme aromatase, an important biomarker of endocrine disruption in teleost (Cheshenko *et al*., 2008). Aromatase is the enzyme involved in the conversion of androgen to estrogen, which play a key role in the control of sexual differentiation, maturation and reproduction (Cheshenko *et al*., 2008). *Cyp19* is regarded as a major target for endocrine disrupting chemicals because modulation of its expression and function may potentially disrupt the level of estrogen production (Cheshenko *et al*., 2008; Kazeto *et al*., 2004) and it is frequently assessed in fish because its susceptibility to endocrine disruptors is

conserved throughout life (Le Page *et al*., 2011). On the other hand, *vtg1* is an estrogen receptor element gene that regulates vitellogenin synthesis in its promoter region and it is largely expressed in the liver of adult fish (Tingaud-Sequeira *et al*., 2012; Tran *et al*., 2019); although, studies have shown that this gene is active in 24 h post fertilized embryos (Hao *et al*., 2013; Muth-Köhne *et al*., 2016). Therefore, alteration in *vtg1* expression will reduce vitellogenin production, which could lead to significant reduction in oocyte quality and maturation in female teleost (Muth-Köhne *et al*., 2016). Also, expression of both genes provides understanding of mechanism of action and represent molecular initiating events in the adverse outcome pathways of endocrine disrupting chemicals.

The expression of *cyp19* and *vtg1* in the yolk sac larvae of the sheepshead minnow exposed to AHTN and HHCB was similar. Both *cyp19* and *vtg1* genes were weakly upregulated after exposure to HHCB except for larvae exposed to 5 µg/L HHCB that were moderately upregulated by approximately 1.8-fold change in both genes. Contrarily, AHTN downregulated the expression of both gene in a concentration dependent pattern, being significantly different from the control by more than 3-folds at 50 µg/L. The expression of both genes in the early life stage of *C. variegatus* is indicative of the potential endocrine disrupting effects of these polycyclic musk compounds. Although the expression of these genes in yolk sac larvae of *C. variegatus* have not been previously reported, the current study attested that these genes are active in sheepshead minnow from post-hatch stage, and it is conserved in its entire life. Similarly, *vtg1* expression has been reported in zebrafish and found to be active around 24 h post fertilization (hpf) (Jarque *et al*., 2019; Muth-Köhne *et al*., 2016).

The expression of *cyp19* in teleost by environmental chemicals is well reported and such alteration leads to skewed sex ratio because inhibition of estrogen favors over production of androgen, which leads to masculinity (Muth-Köhne *et al*., 2016). For example, *Cyprinus carpio* and *Oreochromis niloticus* treated with the *cyp19* inhibitor letrozole resulted in 79.39±1.09% and 87.91±1.39% masculinization, respectively, when compared to negative control with sex ratio of 48.28% male to 50.78% female *C. carpio* and 46.38% male to 53.62% female *O. niloticus* (Singh and Srivastava, 2015). Similarly, Afonso *et al*. (2001) reported that the nonsteroidal aromatase inhibitor fadrozole favored increased masculinization in *O. niloticus* after treatment for 30 days in dose dependent manner. These results combined with significant down regulation of *vtg1* and *cyp19* in different life stages of zebrafish exposed to fadrozole (Muth-Köhne *et al*., 2016), a pattern comparable to this study, confirms the endocrine disrupting potential of HHCB and AHTN.

4.3 Marine environmental risk of HHCB and AHTN measured at cellular level.

Sublethal effects of HHCB and AHTN were measured after chronic exposure using as bioindicator species the clam *R. philippinarum*. Organisms were exposed to environmental concentrations of HHCB and AHTN during 21 days in a semi-static renovation bioassay. In that study, it was intended to understand the mechanism of defense by exploring a suite of biomarkers related with biotransformation of contaminants (ethoxyresurofin-*O*-deethylase – EROD and glutathione-*S*-transferase – GST), oxidative stress (glutathione peroxidase – GPx, glutathione reductase – GR and lipid peroxidation - LPO), genotoxicity (DNA damage), neuroendocrine toxicity (acetylcholinesterase – AChE and cyclooxygenase -COX) and energy reserve disruption measured as total lipids content (TL). The use of *R. philippinarum* as a model organism for biomarker-based assessment of marine contamination gained attraction in the last decades and has widely been used to investigate the potential toxicity of pesticides (Tao *et al*., 2013), metals (Aouini *et al*., 2018; Ji *et al*., 2019; Santana *et al*., 2017; Wang *et al*., 2011), wastewater effluent (Díaz-Garduño *et al*., 2018, 2016; Maranho *et al*., 2015a; Maranho *et al*., 2015b), and pharmaceutical products (Aguirre-Martínez *et al*., 2013; Aguirre-martínez *et al*., 2016; Almeida *et al*., 2015; Matozzo *et al*., 2012; Milan *et al*., 2013; Trombini *et al*., 2019; Volland *et al*., 2015) and nanoparticles (De Marchi *et al*., 2017; Marisa *et al*., 2018). Therefore, this research explored the possibility of the clams to provide useful information for the ERA (Environmental Risk Assessment) of HHCB and AHTN in marine environments after chronic exposure by evaluating biochemical alterations in digestive gland and gonad tissues.

The impacts of HHCB and AHTN in digestive gland and gonad tissues of *R. philippinarum* using biotransformation enzymes revealed toxic influence of the tested compounds as both EROD and GST activities were significantly induced. Biotransformation process involves conversion of hydrophobic substances capable of depolarizing the permeability of cell membrane to excretable hydrophilic substances. The two phases of the biotransformation process initiated by oxidation in phase 1 to conjugation of phase I metabolite in phase II were activated in the clams, thus defending the organism from toxic effects of contaminants. However, the capacity to metabolize xenobiotics to non-toxic products could be overwhelmed and in that case the species involved may undergo oxidative stress. Secondly, the metabolites of the transformed substances may be more toxic than the parent compounds, consequently leading to severe damage in the cellular components of the organism. Significant changes in EROD and GST activities at each sample time-point is indicative of prolonged detoxification activities in the clams and correlated with antioxidants enzyme activities because clams exposed to HHCB and AHTN significantly induced GPx activities. Contrarily, GR enzyme activity was significantly decreased in clams exposed to HHCB and AHTN measured in each time-point and this is very important because deactivation of GR activity proportionally affects the capacity of other antioxidants enzymes to prevent the cell from oxidative attack

caused by ROS (Regoli and Giuliani, 2014). HHCB and AHTN are known to modulate antioxidants activities in both aquatic and terrestrial ecosystems. Reports showed that these compounds significantly affect antioxidants enzymes in *Danio rerio* after chronic exposure to concentration range of 50 – 50000 ng/L (Blahova *et al*., 2018), *Carassius auratus* after chronic exposure to concentration range of 15 – 150 µg/L (Chen *et al*., 2012) and earthworms after 48-h exposure to concentration range of 11.87 – 20.76 µg/cm^2 (Chen *et al*., 2011).

In clams exposed to HHCB and AHTN, positive correlations were detected in detoxification, oxidative stress, and DNA damage biomarkers, linking the impacts of both substances on the antioxidant enzymes to cellular damage. Similarly, the degradation of the phospholipid bilayer of the cell membrane due to lipid peroxidation in clams exposed to both compounds showed to exerted direct DNA damage. However, it is not clear if lipid peroxidation in the clams directly led to DNA damage found in the exposed organisms. Although that is a possible hypothesis, it was not tested in this research. Also, DNA damage in clams exposed to both substances for 21 days could be caused by the formed metabolites because the metabolites of these chemicals may be more toxic than the parent compounds (Gao *et al*., 2016). Other research on the peroxidation and cellular damage caused by HHCB and AHTN have shown that both substances significantly increased lipid peroxidation and DNA damage in zebra mussel *Dreissena polymorpha* at environmental concentration (Parolini *et al*., 2015), rainbow trout (Hodkovicova *et al*., 2020) and higher concentrations significantly increased lipid peroxidation in earthworm (Chen *et al*., 2011; Liu *et al*., 2011).

Other biochemical indicators of ecotoxicity of HHCB and AHTN were measured using neuroendocrine parameters such as AChE and COX activities. Both substances significantly inhibited AChE activity in clams after 3 days of exposure and this impact was observed throughout the exposure period for HHCB, whereas AHTN showed recovery after day 21. Contrarily, COX activity was significantly induced in clams exposed to HHCB and significantly inhibited in clams exposed to AHTN. Similar behavior of AChE and COX activities were observed in clams exposed to AHTN as the inhibition of both enzymes lasted till day 14 and reversed on day 21, indicating recovery from the suppression imposed by AHTN. This is the first time these parameters have been reported in marine environments and serve as baseline information for future investigation regarding the neuroendocrine effects of HHCB and AHTN. The effects detected in clams are similar to those of pharmaceutical products previously reported. For example, ibuprofen, carbamazepine, and propranolol decreased COX activity in the marine polychaetes *Hediste diversicolor* after 14 days exposure (Maranho *et al*., 2015) and environmental concentration of caffeine significantly induced AChE and COX activities in *Corbicula fluminea* (Aguirre-Martínez *et al*., 2018).

Chronic inhibition of AChE in clams could lead to high levels of acetylcholine, over-stimulation of cholinergic receptors, alteration of postsynaptic cell function and signs of cholinergic toxicity such as morphological and behavioral changes may start to manifest (Nallapaneni *et al*., 2008; Pope *et al*., 2005; Song *et al*., 2004; Waseem *et al*., 2010). Furthermore, fatality may occur if AChE activity is depressed on exposure to cholinesterase inhibiting chemicals due to overstimulation of the target cells (Sancho *et al*., 2000). In addition, COX synthesis of prostaglandins is involved in the control of oogenesis and spermatogenesis in aquatic invertebrates (Di Costanzo *et al*., 2019) and controls the effect of serotonin in the spawning process in bivalves (Matsutani and Nomura, 1987). Prameswari *et al*. (2017) demonstrated that the freshwater crabs (*Oziothelphusa senex senex*), induced with arachidonic acid, significantly ($p < 0.001$) increased the ovarian index, oocyte diameter and ovarian vitellogenin. The authors reported that those induced with COX inhibitors, including indomethacin and aspirin, significantly ($p < 0.001$) reduced ovarian index, oocyte diameter and ovarian vitellogenin levels in crabs, corroborating the involvement of COX in regulation of female reproduction in crabs. Given the significant concentration-dependent alteration of COX activity in clams exposed to HHCB and AHTN, reproduction and survival of this organism might be at risk, posing potential threat to marine biodiversity.

4.4 Spatial avoidance as a complementary tool for environmental risk assessment of HHCB and AHTN in marine environment

The study of avoidance behavior was used as an additional line of evidence to determine the potential risk of HHCB and AHTN for the spatial distribution of populations in marine environments. The shrimps *P. varians* exposed to a contaminant gradient of each substance (0.0 – 50 µg/L) in a non-forced multi-compartmented exposure system significantly avoided toxic areas to less toxic compartments. It was possible to see that when shrimps were exposed in a forced system, even for a longer time than the avoidance, lethality was minimal. Furthermore, both avoidance responses under a non-forced system and lethality under a forced system were integrated to estimate the population immediate decline and overt population immediate decline driven largely by avoidance behavior was detected. This line of evidence demonstrates the potential repellent of HHCB and AHTN for the marine environments. Thus, it would be expected that in a scenario of heterogeneous contaminant dispersion motile organisms will evade contaminated areas towards safer conditions (Araújo and Blasco, 2018). In addition, contaminated areas may lose some part of its population due to escape from noxious substances with possible long-term impacts on structure and functioning of ecosystems at the local level. However, under higher concentration where

the organisms are stupefied before they could emigrate from the contaminated area, the lethality in a short term might contribute more to population immediate decline at local scale (Araújo *et al*., 2014a, 2014b).

In particular, the shrimps were not lethally sensitive to HHCB and AHTN after a 24-h exposure in a forced exposure condition, but they were highly sensitive and able to avoid concentrations of those compounds in the non-forced tests. Therefore, avoidance helps to prevent sub-lethal effects that organisms could suffer after continuous exposure. Also, because the median avoidance behavior estimated for shrimps exposed to HHCB was 28 times lower than median lethal concentration after 24-h exposure in a forced system, the prediction of the ecological risk of HHCB and AHTN based only on data from forced exposure tests might give an insight to the potential toxicity of these substances, but it fails to examine the total impacts at the community level. The escape of the population due to the repellence of a contaminant might bring serious consequences for the ecosystems, even similar to the death of the populations, because part of or the entire population emigrated (Lopes *et al*., 2004; Moreira-Santos *et al*., 2008). When organisms are propelled to move towards other areas, even if no toxic consequences could be observed, the ecosystem is suffering with an imbalance in the ecological interactions due to the loss of biodiversity. Therefore, avoidance response is a response that might prevent organisms of suffering, but not the ecosystem. Under real conditions, great part of the reduction in the biodiversity that could not be explained by toxic effects, as the concentrations are not very high, might be a consequence of this escape behavior. The repellence of both musks might influence the spatial distribution of organisms, affect the quality of ecosystems to be considered habitable and restrict the number of potentially colonized areas (Araújo *et al*., 2020). Consequently, spatial avoidance as presented in this thesis should be regarded as a complementary line of evidence in estimating the environmental risk of HHCB and AHTN.

4.5 Acute toxicity and overview of the potential risks of HHCB and AHTN on structure and functioning of marine ecosystems

The acute toxicity of environmental concentrations of HHCB and AHTN were performed in a static bioassay using as test organisms the microalgae *P. tricornutum, T. chuii, I. galbana* and *R. subcapitata* for 72 h and early life stage of *A. franciscana, P. lividus, M. galloprovincialis* and *S. aurata* to determine the impacts of PMCs on their growth performance, development, and survival. The use of microalgae in ecotoxicology to predict the impacts of contaminants in marine ecosystems is highly significant because they are primary producers, occupying the base of the food pyramid and providing stability for the entire ecosystem. Contaminants impact on microalgae can exert direct effects on the marine ecosystems by

reducing microalgae biomass and increasing competition for scarce food resources in dependent trophic levels or indirectly at population or community levels (Fleeger *et al*., 2003). Because of the low impact of both substances on microalgae growth it was only possible to calculate the IC_{10} for *P. tricornutum* with a value of 0.127 and 0.002 µg/L for HHCB and AHTN, respectively, and *I. galbana*, 5.22 and 0.328 µg/L for HHCB and AHTN, respectively. For the other species, the IC_{10} values were out of the range of concentrations that were used in this study and as such were not recorded, although they were useful to understand the species-specific sensitivity to PMCs. Furthermore, a recent study of the impact of HHCB to the microalgae *Navicula* sp. and *Scenedesmus quadricauda* recorded EC_{50} values of 0.050 and 0.336 mg/L after 3 days exposure (Ding *et al*., 2020). This together with this study corroborated the differential sensitivity of HHCB and AHTN toxic impacts to microalgae growth. Again, comparing the degree of toxicity of HHCB and AHTN to microalgae, it becomes apparent that AHTN inhibited the growth of *I. galbana* 15 times more than HHCB. *I. galbana* seems to be very sensitive to emerging contaminants because previous studies have reported IC10 value of 3.69 µg/L of the UV-filter, benzophenone-3 (Paredes *et al*., 2014). From the data obtained in this study, the impacts of HHCB and AHTN to microalgae growth could be regarded as low. However, this should be carefully examined because the nominal concentrations of HHCB and AHTN may not be available to the microalgae as the inherent lipophilicity of these substances favor adsorption to the aquaria walls and possible bioaccumulation in the microalgae. Although in this thesis the exposure water and bioaccumulation could not be measured due to practical limitations, previous study has shown that HHCB bioaccumulated in microalgae (Ding *et al*., 2020).

The impacts of the tested PMCs on early life development of marine organisms as presented in chapter 3 revealed significant concentration dependent toxicity in early life stages of sea urchin, mussels, and fish. However, the effects were more pronounced in sea urchin and mussel larval development than in Artemia and fish survival tests. Significant concentration dependent effects were observed in sea urchin fertilization, mussel larvae development and fish yolk sac larvae survival tests. Sea urchin fertilization success was significantly reduced by 0.05, 0.5 and 5 µg/L of HHCB, whereas only 0.05 µg/L AHTN affected sea urchin fertilization success. It appears that the sperm may have been incapacitated by HHCB and AHTN since the spermatozoa were first exposed for about 10 min before the eggs were added; thus, reducing fertilization success in sea urchin. Compared to other substances, the effects of HHCB and AHTN on sea urchin fertilization was lower. For example, same concentration of propanol and 17 α-ethinylestradiol reduced sea urchin fertilization success by 24.1% and 36.9%, respectively, (Capolupo *et al*., 2018) as against 19% and 16.67% for HHCB and AHTN, respectively.

Early life stages of aquatic organisms are useful tools for toxicity testing and have been approved as a faster and more cost-effective means of screening chemicals and environmental samples, because newly hatched

larvae are sensitive to exogenous substances as the embryos lose their protective membranes and are fully exposed to potential toxicants (Beiras *et al*., 2003). *P. lividus* and *M. galloprovincialis* are well recognized in toxicity bioassays and are applied globally for the evaluation of toxicity of marine contaminants by the exposure of gametes to aqueous phases, such as surface waters, pore waters (Aguirre-Martìnez *et al*., 2015; Bellas *et al*., 2005) and elutriates (Losso *et al*., 2007). Significant effects were observed in *P. lividus* and *M. galloprovincialis* larvae exposed to HHCB and AHTN compared to the control. The effects of HHCB on sea urchin larvae development was significantly different ($p < 0.05$) compared to the control, and only 44.33% of larvae were able to develop to the pluteus stage after 48 h exposure to 5 µg/L HHCB. However, the sensitivity of sea urchins to AHTN was observed at 0.5 µg/L, reducing larval development by approximately 45%. The impact of contaminants on sea urchin larval development is well documented and significant reports have proven that they represent an important biomonitoring tool for ecosystem health. Similarly, HHCB and AHTN significantly ($p < 0.05$) affected the development of *M. galloprovincialis* larvae when compared to the control. However, when considered in relation to the number of embryos exposed to each contaminant, the effects were incipient with the highest percentage of deformed and undeveloped embryos being 19.88% and 17.60% for HHCB and AHTN, respectively. Although *P. lividus* and *M. galloprovincialis* larvae development tests were similar, the effects recorded were more pronounced in sea urchin larvae than mussels.

Fish early life stage is well recommended for chemical screening and is popularly used for regulatory purposes because the test is quick, affordable and provides reliable information on the environmental impacts of contaminants. The post hatched larvae of *S. aurata* exposed to environmental concentrations of HHCB and AHTN was moderately affected. Although significant differences were observed, it was primarily due to the high survival rate recorded in the control group. The highest mortality was recorded at 5 µg/L for each contaminant and the percentage mortality was 14%. Compared to early life stages of the invertebrates tested, fish survival test was among the least sensitive species and this has been confirmed by recent investigations, which support high sensitivity of invertebrates' early life development to contaminants of emerging concern as against fish early life development (Aguirre-Martínez *et al*., 2015; Capolupo *et al*., 2018; Paredes *et al*., 2014).

Generally, the bioaccumulation and toxicity of PMCs in microalgae, invertebrates and fish may pose a threat to the stability of ecosystem structure and functioning; firstly, because trophic transfer of contaminants may have higher risk to organisms at successive trophic level due to biomagnification (Figure 7.1). For example, HHCB was bioaccumulated in *S. quadricauda* at intracellular content of 86±1.8 mg/L after 3 days exposure to 2 mg/L HHCB (Ding *et al*., 2020), which could be ingested by organisms depending on it as food source following the trophic transfer pathway presented in Figure 7.1. However, this is only

conjectural consequent to paucity of research to validate the biomagnification of synthetic musks in aquatic organisms. Field investigation of bioaccumulation of PMCs and to elucidate possible trophic transfer was conducted by Nakata *et al*. (2007) and found that the concentrations of HHCB and AHTN in different marine organisms were negatively correlated. Again, it is important to state that the organisms assessed by the authors were both pelagic and benthic which means that, though some of the organisms belong to lower trophic levels, they may be exposed to higher concentrations due to interaction with sediments, if they are benthos. Notwithstanding, more investigation at the community level is still needed to understand the trophic transfer of these substances.

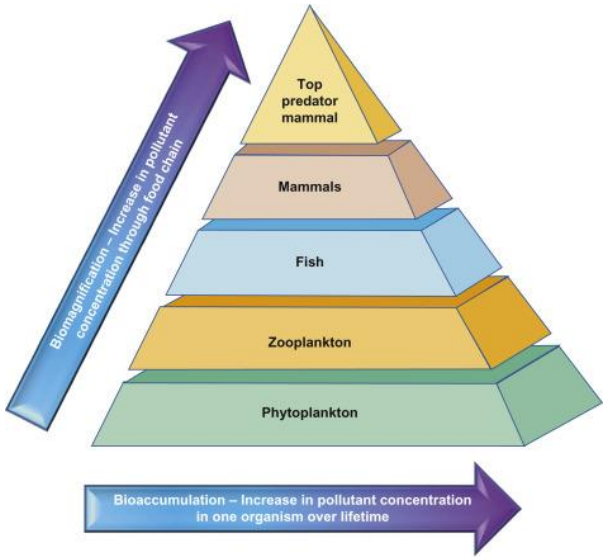

Figure 7.1: Description of how HHCB and AHTN might bioaccumulate and biomagnify through trophic transfer. Adapted from Popek (2018).

Secondly, population and natural communities may be affected directly or indirectly by contaminants because sensitive species may be destroyed by lethality or impaired by sub-lethal effects, which could lead to changes in the ecology of that system. Contaminant-induced changes may also initiate trophic cascade or a release from competition that alternatively results to responses in tolerance species. Also, contaminant driven alteration in nutrient and oxygen fluxes may alter ecosystem function (Fleeger *et al*., 2003). The direct effects of HHCB and AHTN presented in this study may reduce abundance by mortality or reduced recruitment at population level. For example, the significant abnormal larval development influenced by

HHCB on *P. lividus* (predator/grazer) might impact the population dynamics of sea urchin in the marine environment that could lead to cascading indirect effects on resistant species in other trophic levels. Furthermore, HHCB and AHTN may influence those organisms that positively affect the fitness of other species as a result of changes in their environment, and consequently result in alteration in abundance of related species. Lastly, the indirect effects of these contaminants, may increase or decrease abundance at the community level due to increase competition for nutrients and/or food resources.

4.6 Future perspectives

Generally, this thesis evaluated the environmental risk of HHCB and AHTN in marine environments by providing relevant information to bridge important ecotoxicity data gap needed. For that propose, laboratory bioassays under forced and non-forced exposure systems were conducted using both invertebrates and fish as test organisms. The results obtained in this research provide baseline information for future investigations of the impacts of these substances and to design environmental management programmes regarding HHCB and AHTN contamination of marine environments. The high differences in the sensitivity of the endpoints of the different species tested allow to choose suitable species for environmental monitoring of these compounds in marine ecosystems.

Future research on the impacts of HHCB and AHTN in the marine environments should take into consideration the following aspects:

- To the best of our knowledge, most research on the impacts of HHCB and AHTN are tested as single contaminant exposure even though they are often detected as a mixture of contaminants in environmental matrices. Therefore, both HHCB and AHTN should be tested as a contaminant mixture to understand the possible synergistic effects they might impose on marine organisms.

- Assessment of the toxicity of HHCB and AHTN should measure the metabolites of these compounds in test media and accumulated in the test organisms and investigate the potential toxicity arising from the metabolites.

- HHCB and AHTN are lipophilic and adsorb to aquaria wall and it does mean that they may adsorb to other marine contaminants like nanoparticles and microplastics, which could serve as vehicles, transporting these substances at higher concentrations than measured environmental concentrations. Therefore, research should be designed to test the impacts of these vehicular transport, together with the inherent toxicity of the vehicles.

- Future research on the effects of HHCB and AHTN should consider a full life cycle assessment and intergenerational effects to understand the life stage that is most sensitive and possible susceptibility at subsequent generations.

- The use of combined ‘omic’ tools such as transcriptomic and proteomic should be explored to understand if effects at the transcription level leads to alterations at the translational level after exposure to HHCB and AHTN.

- For more ecological relevant research, HHCB and AHTN impacts on the ecosystem should be tested by exposing multispecies at the same time to understand if impacts on a trophic level results in cascade effects at successive trophic levels.

- For management plans and policy making regarding the environmental management of HHCB and AHTN, the data presented in this thesis and other available data involving different species and endpoints should be integrated for overall understanding of exposure and effects of HHCB and AHTN.

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Chapter 5

Conclusions / Conclusiones

5.1 Conclusions

This PhD thesis assessed the environmental risk of galaxolide (HHCB) and tonalide (AHTN) as contaminants of emerging concern in marine ecosystems by measuring biological effects at various biological levels and endpoints with different ecological relevance. The conclusions derived from the various laboratory experiments are summarized below:

i. The results presented in this memory showed the sensitivity and dose response relationship of different acute endpoints measured in microalgae, crustaceans, bivalves, echinoderms, and fish early life stages to environmental concentrations of HHCB and AHTN. The microalgae tested were moderately sensitive to HHCB and AHTN, and the estimated risk quotient showed that both polycyclic musk compounds (PMCs) pose risk to *P. tricornutum* and *I. galbana* growth. *Artemia* motility and survival were the least sensitive endpoints while *P. lividus* larval development represented the most sensitive endpoints detected. HHCB and AHTN were characterized to pose high risk to *P. lividus* and *M. galloprovincialis* larval development. Even though fish survival test is a regulatory tool for chemical screening and prioritization, it was found to be less sensitive to environmental concentrations of HHCB and AHTN. Since ecological integrity is achieved by protecting the most sensitive species in the community, *P. lividus* and *M. galloprovincialis* larval development tests should be included in chemical screening and prioritization of contaminants of emerging concern.

ii. The results of this study highlighted the aversiveness and potential of HHCB and AHTN to elicit avoidance response and lethality in the estuarine shrimps *P. varians* after exposure in a non-forced and forced exposure systems, respectively. HHCB and AHTN lethality to shrimps were minimal after a 24-h exposure in a forced system. However, shrimps exposed to contaminant gradients of HHCB and AHTN under a multi-compartmented, non-forced system were able to detect the different concentrations and emigrated from those that were potentially toxic. The AC_{50} obtained for both substances were about 28 times higher than the LC_{50}. Population immediate decline (PID), calculated by integrating both avoidance and lethality responses, was driven primarily by the avoidance response rather than mortality. Therefore, ecological risk assessment of contaminants should integrate data from spatial avoidance study in a non-forced system and lethality in a forced system to avoid overestimation or underestimation of their full potential risks.

iii. The determination of a battery of biomarkers in the clams *R. philippinarum* after chronic exposure to environmental concentrations of HHCB and AHTN revealed alterations in enzymes activities,

indicating that both substances are bioavailable to marine organisms and might have the potential to elicit oxidative stress and genetic damage in the marine environment. Also, the biomarkers (EROD, GST, GPx, GR, LPO and DNA damage) assessed in this study are useful tools for the environmental monitoring of PMCs.

iv. Determination of the potential of HHCB and AHTN to disrupt neuroendocrine activities in marine environment were assessed using as bioindicator species the adult clam *R. philippinarum*. HHCB and AHTN significantly altered AChE and COX activities biochemically measured in clams after chronic exposure which indicate that both substances are disruptors of neuroendocrine activities in clams. The substance AHTN was observed to elicit higher neuroendocrine disruption than HHCB.

v. Disruption at the transcription level of neuroendocrine activity was studied in yolk sac larvae of sheepshead minnow *C. variegatus* after exposure to HHCB and AHTN, which indicates disruption of genes encoding for sex-hormone involved in steroidogenesis. Despite the significant alterations in neuroendocrine biomarkers in clams and fish exposed to both substances, AHTN appeared to be a more potent disruptor of neuroendocrine activity.

vi. Since the presence of HHCB and AHTN in marine environments have been demonstrated by different research works to cause adverse effects to marine organisms, t**he ecotoxicological information presented in this research work should be incorporated into policy decision for the conservation and management of marine environment.**

5.2 Conclusiones

En la presente Tesis Doctoral se evaluó el riesgo ambiental del HHCB y el AHTN como contaminantes de interés emergente en ecosistemas marinos mediante la determinación de los efectos en diferentes especies que representaban diferentes niveles de la cadena trófica y en diferentes estadios de desarrollo. Además, se tuvieron en cuenta diferentes respuestas a tiempo final. Las conclusiones derivadas de los resultados obtenidos se resumen a continuación:

i. Los resultados presentados en esta memoria mostraron sensibilidad y relación dosis-respuesta en microalgas, bivalvos, equinodermos, crustáceos y peces en sus primeras etapas de vida a las concentraciones ambientales de HHCB y AHTN. Las respuestas a tiempo final medidas en microalgas fueron moderadamente sensibles al HHCB y al AHTN, y el cociente de riesgo estimado mostró que ambos almizcles policíclicos PMCs suponen un riesgo para el crecimiento de *P. tricornutum* e *I. galbana*. La movilidad y la supervivencia de *Artemia salina* fueron las respuestas a tiempo final menos sensibles, mientras que el desarrollo larvario de *P. lividus* representó las respuestas a tiempo final más sensibles detectadas. Las sustancias HHCB y AHTN se caracterizaron por constituirr un alto riesgo para el desarrollo larvario de *P. lividus* y *M. galloprovincialis*. A pesar de que la prueba de supervivencia de larvas de peces es una herramienta reguladora para el cribado y la priorización de sustancias químicas, se comprobó que era menos sensible a las concentraciones ambientales de HHCB y AHTN. Dado que la integridad ecológica se consigue protegiendo a las especies más sensibles de la comunidad, las pruebas de desarrollo larvario de *P. lividus* y *M. galloprovincialis* deberían incluirse en el cribado químico y la priorización de contaminantes de interés emergente.

ii. Los resultados de este estudio pusieron de manifiesto el potencial del HHCB y el AHTN para provocar una respuesta de evitación y letalidad en el camarón de estuario *P. varians* tras la exposición en un sistema de exposición no forzada y forzada, respectivamente. La letalidad del HHCB y el AHTN para los camarones fue mínima tras una exposición de 24 horas en un sistema forzado. Sin embargo, los camarones expuestos a gradientes de contaminantes de HHCB y AHTN en un sistema multicompartimental no forzado fueron capaces de detectar las diferentes concentraciones y emigraron de las que eran potencialmente tóxicas. Los AC50 obtenidos para ambas sustancias fueron aproximadamente 28 veces superiores a los LC50. La disminución inmediata de la población (PID), calculada integrando las respuestas de evitación y de letalidad, fue impulsada principalmente por la respuesta de evitación más que por la de mortalidad. Por lo tanto, la evaluación del riesgo ecológico de los contaminantes debería

integrar los datos del estudio de evitación espacial en un sistema no forzado y de letalidad en un sistema forzado para evitar la sobreestimación o subestimación de todos sus riesgos potenciales.

iii. La determinación de una batería de biomarcadores en la almeja *R. philippinarum* tras la exposición crónica a concentraciones ambientales de HHCB y AHTN reveló inducción en las actividades enzimáticas, lo que indica que ambas sustancias son biodisponibles para los organismos marinos y podrían tener el potencial de provocar estrés oxidativo y daños genéticos en el medio marino. Además, los biomarcadores (EROD, GST, GPx, GR, LPO y daño en el ADN) evaluados en este estudio mostraron ser herramientas útiles para la monitorización ambiental de los azmilcles policiclícos (PMCs).

iv. La determinación del potencial del HHCB y el AHTN para perturbar las actividades neuroendocrinas en el medio ambiente marino se evaluó utilizando como especie bioindicadora la almeja adulta *R. philippinarum*. El HHCB y el AHTN alteraron significativamente las actividades AChE y COX medidas bioquímicamente en las almejas tras una exposición crónica, lo que indica que ambas sustancias son perturbadoras de las actividades neuroendocrinas en las almejas. Se observó que la sustancia AHTN provocaba una mayor alteración neuroendocrina que la sustancia HHCB.

v. Se observó la alteración a nivel de transcripción de la actividad neuroendocrina en las larvas del saco vitelino *C. variegatus* tras la exposición a HHCB y a AHTN, lo que indica la alteración de los genes que codifican las hormonas sexuales que intervienen en la esteroidogénesis. A pesar de las alteraciones significativas en los biomarcadores neuroendocrinos en almejas y peces expuestos a ambas sustancias, la sustancia AHTN pareció ser un disruptor más potente de la actividad neuroendocrina.

vi. Dado que la presencia de HHCB y AHTN en el medio marino ha sido demostrada por diferentes trabajos de investigación como causante de efectos adversos para los organismos marinos, la información ecotoxicológica presentada en este trabajo de investigación debería incorporarse a la toma de decisiones políticas para la conservación y gestión del medio marino.

Annex I

Published Articles

processes

MDPI

Article

Effects and Risk Assessment of the Polycyclic Musk Compounds Galaxolide® and Tonalide® on Marine Microalgae, Invertebrates, and Fish

Friday Ojie Ehiguese [1,*], M. Judit González-Delgado [1], Carmen Garrido-Perez [2], Cristiano V. M. Araújo [3] and M. Laura Martin-Diaz [1]

1 Physical Chemistry Department, University Institute of Marine Research (INMAR), International Campus of Excellence of the Sea (CEI.MAR), University of Cádiz, 11510 Puerto Real (Cádiz), Spain; juditgonzalezdelgado@hotmail.com (M.J.G.-D.); laura.martin@uca.es (M.L.M.-D.)
2 Environmental Technology Department, University Institute of Marine Research (INMAR), International Campus of Excellence of the Sea (CEI.MAR), University of Cádiz, 11510 Puerto Real (Cádiz), Spain; carmen.garrido@uca.es
3 Department of Ecology and Coastal Management, Institute of Marine Sciences of Andalusia (ICMAN-CSIC), 11510 Puerto Real, Cádiz, Spain; cristiano.araujo@icman.csic.es
* Correspondence: friday.ehiguese@alum.uca.es

Abstract: The current research investigated the potential environmental risk of the polycyclic musk compounds, Galaxolide® (HHCB) and Tonalide® (AHTN), in the marine environments. These substances are lipophilic, bioaccumulated, and potentially biomagnified in aquatic organisms. To understand the toxicity of HHCB and AHTN, acute toxicity tests were performed by exposing marine microalgae (*Phaeodactylum tricornutum*, *Tretraselmis chuii*, and *Isochrysis galbana*), crustaceans (*Artemia franciscana*), echinoderms (*Paracentrotus lividus*), bivalves (*Mytilus galloprovincialis*), fish (*Sparus aurata*), and a candidate freshwater microalga (*Raphidocelis subcapitata*) to environmentally relevant concentrations (0.005–5 µg/L) following standardized protocols (US EPA, Environment Canada and OECD). *P. tricornutum* and *I. galbana* were sensitive to both substances and for *P. tricornutum* exposed to HHCB and AHTN, the IC_{10} values (the inhibition concentration at which 10% microalgae growth inhibition was observed) were 0.127 and 0.002 µg/L, respectively, while IC_{10} values calculated for *I. galbana* were 5.22 µg/L (a little higher than the highest concentration) and 0.328 µg/L, for HHCB and AHTN, respectively. Significant ($p < 0.01$) concentration dependent responses were measured in *P. lividus* and *M. galloprovincialis* larvae developments, as well as *S. aurata* mortality tested with HHCB. The effect of HHCB on *P. lividus* larvae development was the most sensitive endpoint recorded, producing an EC_{50} value (the effect concentration at which 50% effect was observed) of 4.063 µg/L. Considering the risk quotients both substances seem to represent high environmental risk to *P. tricornutum* and *M. galloprovincialis* in marine environments.

Keywords: environmental risk assessment; polycyclic musk compounds; acute toxicity; growth inhibition; larvae development

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1. Introduction

Polycyclic musk compounds (PMCs) have been included in the priority lists of the European Commission existing substances regulation [1]. PMCs are synthetic chemicals, produced in large quantities as a replacement for nitro musks, which have been banned because of their environmental persistence and adverse effects on humans and the environment [2]. They have wide applicability in household and personal care products such as detergents, shampoos, lotions, perfumes, as well as additives in cigarettes and fish baits [2,3]. Due to their high water solubility, inherent lipophilicity, and biological stability, coupled with external application and the fact that they do not undergo biotransformation,

it is not surprising to find them as contaminants in aquatic ecosystems at concentrations ranging from ng/L to µg/L [4].

In particular, the PMCs marketed as Galaxolide® (HHCB) and Tonalide® (AHTN) represent about 95% of total fragrance materials in the perfume industry [5] and are the most commonly detected PMCs in environmental compartments and biological tissues [6–12]. Fromme et al. [8] detected HHCB and AHTN in surface water in Berlin (Germany) at concentration values ranging from 70 to 1590 ng/L and 20 to 530 ng/L, respectively. Similarly, Heberer [3] also reported high levels of HHCB and AHTN in surface water in Berlin at concentrations ranging from 20 to 12,500 ng/L and 30 to 1100 ng/L, respectively. Moreover, these substances have been detected in surface waters in almost every country in Europe [8,12,13]. Although most studies were for the freshwater environment, there are data confirming their presence in the marine environment [6,7,11,14], and reported values over 5 µg/L depending on the proximity to a sewage treatment plants (STP). Sumner et al. [11] studied the transport of PMCs from an STP effluent into coastal waters and reported that the concentrations of HHCB and AHTN in the open sea depends on the distance from STP and the dilution power towards the open sea [11]. For instance, the authors measured the concentration of HHCB in STP effluent ranging from 987 to 2098 ng/L, being diluted towards the sea over 2 km, to 6 to 28 ng/L. However, this level could be higher near urban coasts where STP effluents are directly discharged into coastal waters, which could represent a potential threat to the survival of nearshore organisms, an important part of the marine ecosystems.

Bioaccumulation of PMCs, particularly HHCB and AHTN, have also been reported in marine organisms including crustaceans, bivalves, fish, marine birds, and mammals [9,15–17] at levels of environmental concern. The concern over the potential effects is not only related to the environment, but also the impacts on food safety and, consequently, public health. Therefore, Vandermeersch et al. [18] reviewed emerging contaminants in seafood, acknowledging that HHCB and AHTN were the most commonly detected PMCs, with concentrations reaching 160 and 45 µg/g lipid weight in mollusks and fish, respectively [19]. Similarly, in the framework of the FP7 ECsafefood project, where 62 commercial seafood samples (mackerel, tuna, salmon, seabream, cod, monkfish, crab, shrimp, octopus, perch, and plaice) in the European Union were analyzed for residues from personal care products, HHCB was detected at concentrations ranging from 2.5 to 414.4 µg/kg dry weight, with the highest concentration measured in fish (sole) and AHTN found at concentrations ranging from 2.5 to 12.2 µg/kg dry weight [20]. Furthermore, HHCB and AHTN have the potential to elicit adverse effects in marine organisms due to the bio-concentration factors (based on lipid content) of 3504 and 5017 [8] as consequence of high octanol water partition coefficients (LogKow) of 5.9 and 5.7 [21], respectively.

Data on the acute, sub-chronic, and chronic aquatic toxicity of HHCB and AHTN are available for algae, crustaceans, mollusks, bivalves, and various fish [22–31]. Although the majority of the studies were focused on freshwater ecosystems, Breitholtz et al. [22] and Wollenberger et al. [30] investigated the acute, sub-lethal and lethal effects of these substances on the marine copepods, *Nitocra spinipes* and *Acartia tonsa*, respectively. While Wollenberger et al. [30] concluded that HHCB and AHTN inhibited larval development in *A. tonsa* at low concentration and should be considered very toxic, Breitholtz et al. [22] reported low adverse effects in *N. spinipes*.

Several attempts have been made to assess the environmental risk of HHCB and AHTN in the aquatic environment. Balk and Ford [5] provided an insight by using acute and chronic toxicity data from freshwater and terrestrial organisms to estimate the environmental risk, but with no reference to the marine environment. Other environmental risk assessments (ERA) performed so far [1,32–34] revealed a paucity of ecotoxicity data for the marine environment needed to effectively assess the risk of these substances.

The aim of this research was to assess the potential environmental risk of HHCB and AHTN in the marine environment by exposing organisms from different trophic levels at early life stages to environmental concentrations of HHCB and AHTN. Therefore, acute

toxicity tests were performed using marine organisms such as microalgae (*Phaeodactylum tricornutum*, *Tretraselmis chuii* and *Isochrysis galbana*), crustaceans (*Artemia franciscana*), echinoderms (*Paracentrotus lividus*), bivalves (*Mytilus galloprovincialis*) and fish (*Sparus aurata*), and a candidate freshwater alga—*Raphidocelis subcapitata*. Secondly, the potential risks these substances might exert on the marine ecosystems were estimated following European Chemical Agency (ECHA) guidelines [35]. Furthermore, understanding the toxicity of HHCB and AHTN in lower trophic organisms will help to predict possible bottom-up and top-down effects, which could lead to a functional and structural disruption of the ecosystems [36–39]. The species used in the current study have been used to examine the effects of effluents from sewage treatment plants [40,41], metals [42,43], organic solvents [44], and inorganic chemicals [45,46] in marine environments. Additionally, these species have been endorsed by international organizations for ERA of contaminants due to their sensitivity [35,47–50].

2. Materials and Methods

2.1. Chemical Selections

Analytical grade HHCB and AHTN were purchased from Sigma–Aldrich, Spain. The physiochemical properties, preparations in organic solvent and determination of exposure concentrations followed details found in Ehiguese et al. [51]. In brief, HHCB and AHTN were dissolved in dimethyl sulfoxide (DMSO) (0.001% *v/v*) in glass vials to form a stock solution. Concentrations (0.005, 0.05, 0.5, and 5.0 µg/L) of each substance were prepared by diluting the stock solutions in 18.2 MΩ-cm Nanopure water.

2.2. Acute Toxicity Test

2.2.1. Microalgae Growth Inhibition Test

Microalgae growth inhibition tests were performed using three marine and one freshwater species, following the procedure reported by Garrido-Perez et al., [52]. Inocula of *P. tricornutum*, *T. chuii* and *I. galbana* (marine species) were provided by the Laboratory of Marine Culture of the University of Cádiz, Spain, and *R. subcapitata* (strain 61.81) was provided by the Culture Collection of Algae at Goettingen University, Germany. Seawater microalgae culture media was prepared by adding nutrients (macro- and micronutrients and vitamins) according to f/2 medium [53] to synthetic seawater according to the formula from USEPA [50]. For the freshwater species, the culture media were prepared according to the concentrations of COMBO Media [54] in Nanopure water. Before exposure to HHCB and AHTN, an inoculum from each species of microalgae was cultured in fresh media and in the same chamber of assay (19 ± 1 °C; 11,000 lux; photoperiod 14/10 light/day). Inocula were maintained for three days to reach the exponential growth phase, in order to provide acclimatized and healthy cells for the tests.

An aliquot of 0.25 mL of each inoculum was added to 3.75 mL of the culture media spiked with different concentrations of contaminants. The exposure was performed in triplicates including two controls: (1) culture media without contaminants (normal growing of the microalga in a fresh medium), and (2) culture media without contaminants but including DMSO (to test the toxic effects of the organic solvent). The addition time of the microalgae was as short as possible, to ensure that they had similar biomass at the initial time. After 30 min inoculation, the initial biomass (B_0) was measured, representing time zero (T_0). The biomass was measured indirectly through absorbance at 680 nm (maximum chlorophyll peak in a fresh culture of microalga), with a TECAN 2000 micro-well plate reader. This measurement was repeated at 24, 48, and 72 h. The endpoint observed in this test was the inhibition of biomass growth at 72 h compared to the control. The minimal growth acceptable for the control was 16 times higher than the initial density.

2.2.2. Artemia Toxicity Test

The cysts of *A. franciscana* are certified biological material (AF450, INVE) and were acquired from Acuazul, S.C. (Spain). Before starting the trial, dehydrated artemia cysts

were induced to hatch. In a one-liter Erlenmeyer flask containing 300 mL of natural seawater (35 g/L salinity), approximately 100 mg of cysts were resuspended and left with aeration and continuous illumination throughout the hatching time, which occurs within 24 to 36 h. Once the artemia hatched, the aeration was removed. The hatched nauplii were placed in clean seawater to avoid moving empty or unhatched cysts. Therefore, all nauplii used in the test belong to the same cohort. The test was carried out in triplicates using Petri dishes made of glass, and 60 mm diameter. On each plate 10 nauplii were placed using a glass Pasteur pipette to avoid stress to the nauplii. Subsequently, seawater spiked with each contaminant was added to a total volume of 10 mL. The test was conducted at 20 ± 0.4 °C and in the dark to minimize the swimming of the nauplii and the consumption of energy. The test lasted for 72 h and records were taken at 24, 48, and 72 h. The *Artemia* were counted using a stereomicroscope at a magnification of 2× and 4×, identifying dead specimens (mortality was recorded when they showed no sign of any movement of their limbs for 30 s) and those that presented motility problems such as movements in circles, asynchronous, among others. The test was considered acceptable when survival of over 80% was recorded in the control group after 72 h.

2.2.3. Sea Urchin Toxicity Test

The sea urchin *P. lividus* fertilization and larval development tests were performed following procedures described by Fernandez and Beiras [55], and Environment Canada [48]. Individuals were collected from the uncontaminated rocky subtidal environment off the Bay of Cádiz (Spain) at 1.5–2 m depth. They were immediately transported to the laboratory in a cooler box. Matured individuals were dissected, and eggs and sperm were collected using a micropipette. For the fertilization test, 10 µL of sperm were added to the aliquots containing 10 mL of the test concentrations arranged in sequence for 10 min; then, 1 mL of eggs was added to each test tube, swirled gently, and allowed to proceed for 10 min. The endpoint for fertilization success was the presence of a fertilization membrane. A larval development test was performed by adding 1 mL of fertilized eggs to beakers containing test solutions in triplicate, including the controls (seawater and DMSO), in dark conditions for 48 h. The test was considered valid when development in the control was ≥80% and the result expressed as percentage of normal pluteus stage, normalized to the corresponding seawater control. Both fertilization and larval development tests were conducted in a controlled chamber at a temperature of 20 °C.

2.2.4. Mussels Larvae Development Test

The mussels *M. galloprovincialis* were purchased from an aquaculture farm in northwestern Spain. The depurated mussels were transported to the laboratory at 8 °C. Animals with matured gonads were held at 9 °C for two weeks to acclimate to the laboratory condition. The toxicity test was performed following the ASTM protocol for acute toxicity of saltwater bivalves [56]. Mussels were placed each in a beaker containing filtered seawater and induced to spawn by thermal stimulation at 19 °C. Eggs and sperm were filtered to remove debris using 75 and 37 µm screens, respectively. Before fertilization, the egg and sperm quality and density were evaluated under a microscope. Aliquots of 10 µL of sperm were added to the eggs for fertilization (10^6/egg) and fertilization success was assessed under the microscope. The fertilized eggs were added to test solutions at 50 eggs/mL and incubated for 48 h at 16 ± 0.2 °C. The test was performed in triplicate including seawater and solvent controls (DMSO). Samples were fixed with 40% formalin at the end of the test and 100 larvae were counted under the microscope (×40 magnification) per replicate distinguishing between normal developed larvae (D-shaped) and malformed larvae. Test results were accepted when normal developed larvae in control was ≥ 80%.

2.2.5. Fish Larva Mortality Test

The effects of environmental concentrations of HHCB and AHTN on the survival of seabream (*S. aurata*) larvae was tested following OECD guidelines for fish early-life stage

toxicity test [47]. Yolk-sac larvae (3–5 h post hatch) of *S. aurata* were obtained from the laboratory of Marine Culture, Faculty of Marine and Environmental Sciences, University of Cádiz, Spain. Individuals (n = 70) of the yolk-sac larvae were added to each beaker (Pyrex©) containing 600 mL of seawater spiked with the concentrations of contaminants, and each treatment was run in triplicate. Seawater and solvent (DMSO) controls were also tested. The exposure proceeded for 96 h, physiochemical parameters were monitored using the CRISON CM35+ and 40MM+ multiparameter probes (Crison–Hachs Lange S.L.U., Spain) and values recorded during the exposure were: Temperature (16.01 ± 0.2 °C), salinity (34.2 ± 0.3‰), pH (7.7 ± 0.2), and oxygen (>5 mg/L). The test was considered valid if mortality did not exceed 10% in the control group. Mortality in each treatment group was recorded and data were expressed as the percentage of survived larvae at each experimental condition.

2.3. Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics, version 24.0. Significant differences between organisms exposed to the different concentrations of PMCs, or to the organisms from control, were checked using a one-way ANOVA followed by multiple comparisons of Turkey's or Dunnett's test. Statistically significant differences were set at $p < 0.05$. Spearman's rank order of correlation was calculated between the measured effects and the exposure concentrations for pairwise comparison, setting significance levels at $p < 0.05$ and $p < 0.01$. In addition, the SPSS Probit response model and PriProbit 1.63 software [57] were used to calculate the IC_x/EC_x (concentrations that cause growth inhibition or any effect, respectively, to x% of the population).

The Risk Quotient (RQ) was derived from the ratio of the measured environmental concentrations (MEC) listed in Table 1 to the predicted no effect concentration (PNEC). PNEC is the effect concentrations (EC) or inhibition concentration (IC) obtained from the tests divided by an assessment factor (European Comission Joint Research Centre, 2003).

$$RQ = \frac{MEC}{PNEC} = \frac{MEC}{\frac{EC}{1000}} \text{ or } \frac{MEC}{IC/10} \quad (1)$$

where:

If RQ < 1 = no risk expected, and no further evaluation required

If RQ > 1 = potential risk and further evaluation is required

3. Results

3.1. Microalgae Growth Inhibition Tests

Data of growth inhibition for microalgae are presented in Figure 1. There was no critical difference between microalgae exposed to seawater control and solvent control, although microalgal growth response was observed in the group treated with DMSO. In brief, it is possible to observe that toxicity of both compounds was minimal for the four microalgae, although AHTN seemed to be slightly more toxic. For *P. tricornutum*, growth inhibition for both compounds were observed in some concentrations. However, significant differences ($p < 0.05$) in growth in relation to the control only occurred with exposure to AHTN at 0.005 µg/L by 16.2% (Figure 1A). On the one hand, *T. chuii* growth was inhibited by AHTN and the decrease in biomass was significantly ($p < 0.01$) concentration dependent (Table S1 in Supplementary Materials). On the other hand, the exposure to HHCB produced a stimulation (hormesis) in the growth (Figure 1B) and similar biphasic responses was seen in *I. galbana* biomass after exposure to both musk compounds (Figure 1C). The freshwater microalgae, *R. subcapitata* growth decreased after exposure to HHCB and AHTN. In the case of AHTN, significant ($p < 0.05$) concentration dependent growth inhibition of *R. subcapitata* was observed (Table S1 in Supplementary Materials), and the highest inhibition occurred at 5 µg/L by 23.5%. In contrast, *R. subcapitata* growth inhibition by HHCB was more severe at 0.05 µg/L by 14.5% in relation to the control (Figure 1D).

3.2. Artemia, Sea Urch, Mussels, and Fish Early Life Stage Toxicity Tests

For the organisms tested for fertilization, larvae development, motility, and mortality, the validity criteria for the control experiments (seawater control and solvent control—DMSO) were not exceeded.

Table 1. Measured Environmental Concentrations (MEC) of Galaxolide (HHCB) and Tonalide (AHTN) in seawater from different locations. Measured values are in ng/L. Maximum values (bold) were used in calculating risk quotient (RQ).

Country/Location	HHCB	AHTN	Reference
Germany (North Sea)	0.09–4.8	0.08–2.6	[6]
Germany (Elbe Estuary)	95–136	65–200	[6]
United Kingdom (Tamar Estuarine—Plym Sound)	6.00–30	3.00–15	[11]
Spain (Bay of Cadiz)	230 ± 0.1	NA	[14]
Singapore (Coastal water)	1.66–21.8	0.244–1.85	[58]

NA = Data not available.

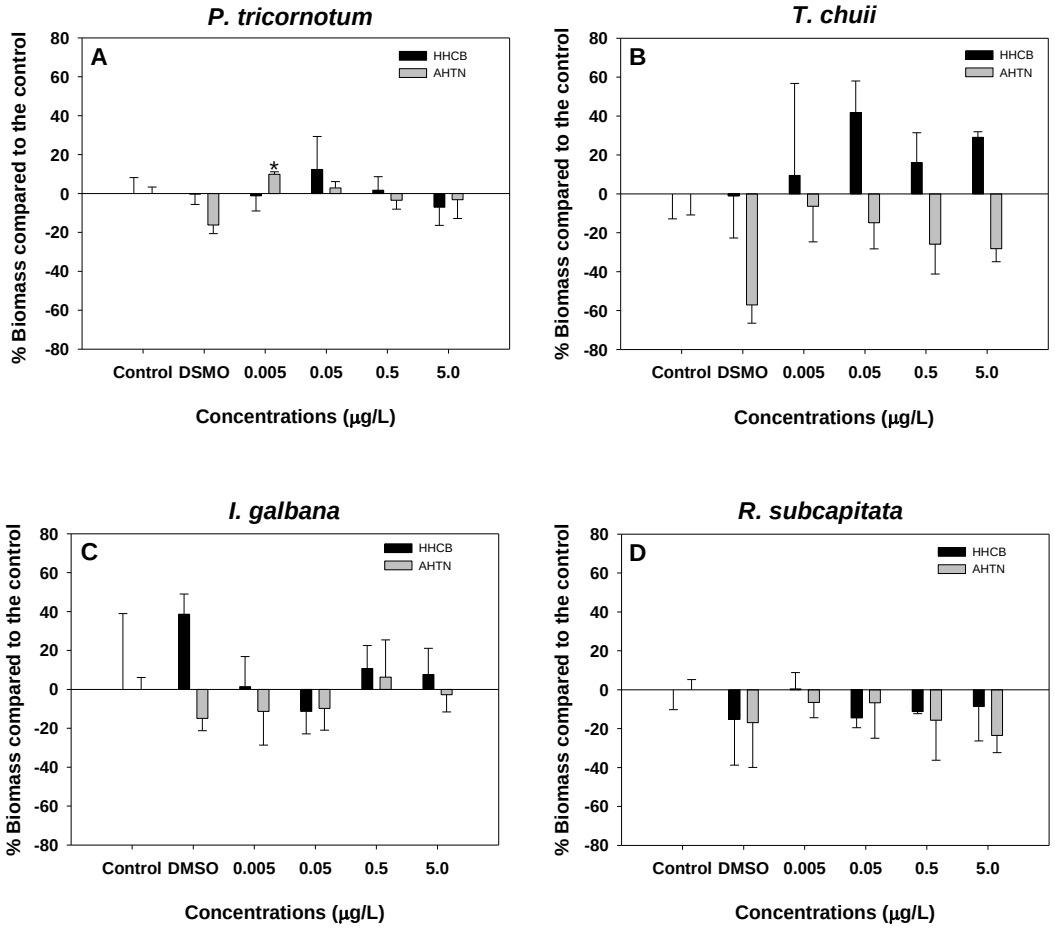

Figure 1. A-1D: Microalgae exposed to galaxolide and tonalide for growth inhibition tests after 72 h. Biomass (%) compared to the control of (**A**) *P. tricornotum*, (**B**) *T. chuii*, (**C**) *I. galbana*, and (**D**) *R. subcapitata* (negative values corresponding to % growth inhibition). Asterisks (*) indicate significant differences ($p < 0.05$) in relation to control.

Moreover, there was no significant difference ($p < 0.05$) in responses detected in organisms exposed to seawater control and DMSO.

The effects of HHCB and AHTN on survival and motility of *A. franciscana* are shown in Figure 2A and 2B, respectively. Both responses showed no significant difference ($p < 0.05$)

compared to the control. For the *A. franscana* mortality test, percentage mortalities in the controls were 3.33 and 6.67% for seawater and DMSO, respectively. The effect of HHCB on the survival of *A. franciscana* was very minimal with the highest mortality of 10% recorded at a 0.5 µg/L HHCB. The effect of AHTN on *A. franciscana* mortality was also very low, with only 3.33% mortality at 0.05 and 0.5 µg/L. Other concentrations of AHTN tested had no effect on *A. franciscana* survival. Artemia motility test was not sensitive to either compound (Figure 2B).

The effects of HHCB and AHTN on *P. lividus* fertilization and larval development tests are presented in Figure 2C and 2D, respectively. The percentage of sea urchin that were able to fertilize in the controls were 89.50 and 90.00% for seawater and DMSO, respectively. Percentage fertilization of sea urchin tested with 0.005, 0.05, 0.5, and 5 µg/L HHCB were 86.33, 82.33, 81.00, and 82.33%, respectively. Under exposure to AHTN, sea urchin fertilization success recorded at 0.005, 0.05, 0.5, and 5 µg/L were 89.00, 83.33, 86.33, and 86.33%, respectively. Although the effect is minimal, *P. lividus* fertilization failure induced by both substances was more pronounced for HHCB with significant effects at 0.05–5 µg/L, while a significant difference ($p < 0.05$) was only observed at 0.05 µg/L AHTN compared to control (Figure 2C). The results for the sea urchin larval development tested with HHCB and AHTN are presented in Figure 2D. The percentages of larval development in the seawater and solvent controls were 83.50 and 81.50%, respectively. The number of oocytes that were able to develop to pluteus stage was significantly ($p < 0.01$) concentration dependent (Table S2 in Supplementary Materials) and a significant ($p < 0.05$) decrease in larvae development was recorded at 5 µg/L HHCB compared to the control (Figure 2D). The percentage of larval that were able to develop from morula stage to normal pluteus stage for 0.005, 0.05, 0.5, and 5 µg/L HHCB were 85.00, 85.00, 68.00, and 44.33%, respectively, while similar concentrations of AHTN reduced larvae development by 63.00, 75.33, 54.67, and 62.33%, respectively.

The results for the larval development toxicity test for *M. galloprovincialis* exposed to HHCB and AHTN are presented in Figure 2E. The number of fertilized eggs that developed after 48 h to D-veliger stage in the control was 98%. However, the embryotoxicity of HHCB to mussels was significantly ($p < 0.01$) concentration dependent (Table S2 in Supplementary Materials). From 0.05 to 5 µg/L HHCB, the percentage of abnormal larvae development was significantly ($p < 0.05$) different from the control (Figure 2E) and the percentage effect increased to 19.88% in the highest concentration tested (5 µg/L). Similarly, significant toxicity of AHTN to embryos of *M. galloprovincialis* was observed in 0.5 and 5.0 µg/L with percentages of abnormal larvae of 8.36 and 11.63%, respectively (Figure 2E).

The results of the effect of HHCB and AHTN on the survival of yolk-sac larvae of *S. aurata* larval after 96 h exposure was significantly ($p < 0.01$) concentration dependent (Table S2 in Supplementary Materials) and the measured effect is presented in Figure 2F. After 96 h, the percentage mortality of yolk-sac larvae of *S. aurata* in seawater and DMSO controls was 5.33% each. Percentages of mortality in fish exposed to 0.005, 0.05, 0.5, and 5 µg/L of HHCB were 8.67, 12.00, 10.67, and 13.33%, respectively. On the other hand, the percentage mortality of fish tested with the same range of concentrations of AHTN were 10.00, 7.33, 12.67, and 13.33%, respectively.

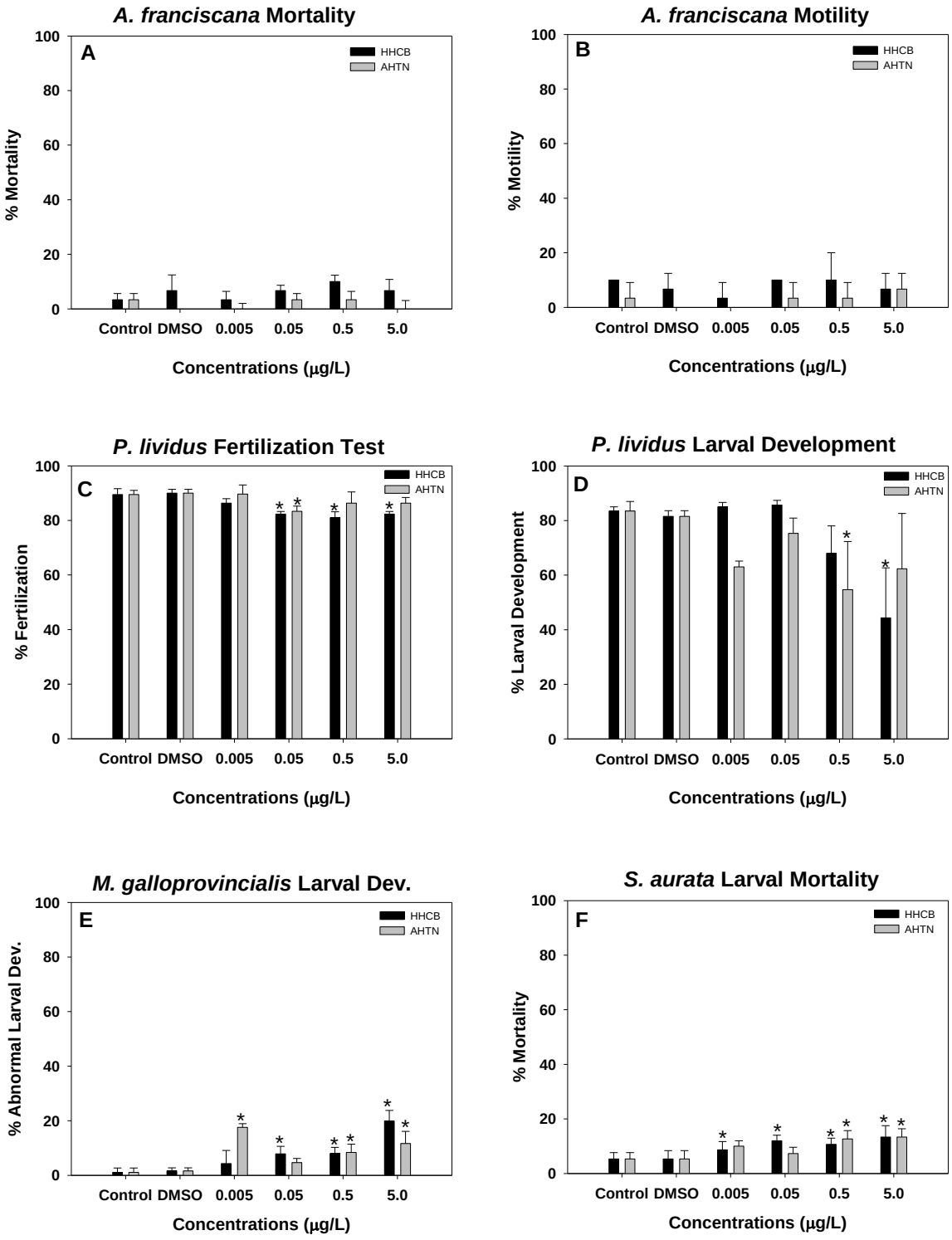

Figure 2. *A. franciscana* 72 h mortality and motility, *P. lividus* fertilization and 48 h larval development, *M. galloprovincialis* 48 h larval development and *S. aurata* 96 h larval mortality tests exposed to galaxolide and tonalide. Asterisks (*) indicate significant differences ($p < 0.05$) in relation to control.

3.3. Risk Quotient (RQ)

Quantitative risk estimation of chemicals in the environment is achieved using monitoring data of MEC and PNEC, giving a risk quotient necessary for risk characterization. A literature survey of MEC for both polycyclic musk compounds were undertaken, and the values are presented in Table 1. Due to minimal effects detected in this study for all the

species of microalgae, *Artemia*, sea urchin, mussels, and fish, it was not possible to calculate the EC_{50} values and their, respective, confidence intervals, except for *P. lividus* larvae development tested with HHCB, producing an EC_{50} value and 95% confidence interval of 4.063 (0.963–120.731) µg/L. The IC_{10}/EC_{10} calculated for *P. tricornutum, I. galbana, P. lividus,* and *M. galloprovincialis*, including the risk quotients using the MEC–PNEC ratio are presented in Table 2. The results of the microalgae growth inhibition and larval development tests showed that HHCB and AHTN posed high risk to the growth of *P. tricornutum* and *M. galloprovincialis* larval development at environmental relevant concentrations. Additionally, environmental concentrations of HHCB and AHTN pose ecological risk to *P. lividus* larval development and *I. galbana* growth, respectively (Table 2).

Table 2. Median lethal concentration (EC50) and EC10 (µg/L) and their respective confidence interval (CI), and estimated risk quotients of Galaxolide (HHCB) and Tonalide (AHTN) as the MEC (maximum value per location)—PNEC ratio.

		Microalgae Growth		**Embryo-Larval Development**	
		P. tricornutum	*I. galbana*	*P. lividus*	*M. galloprovincialis*
HHCB	EC50	NC	NC	4.063 (0.963–120.731)	NC
	EC10	0.127(NC)	5.22(NC)	0.004 (0.000–0.025)	0.188(0.074–0.390)
	MEC/PNEC	0.378–18.110	0.009–0.440	1200–57500	25.532–1223.404
	Risk	Yes	No	Yes	Yes
ANTH	EC50	NC	NC	NC	NC
	EC10	0.002(0.000 – 0.014)	0.328(NC)		0.006 (NC)
	MEC/PNEC	24–1150	0.146–7.0122	NC	800–38333.330
	Risk	Yes	Yes	NC	Yes

NC = Not calculated.

4. Discussion

The aim of this study was to evaluate the impacts of environmentally relevant concentrations of HHCB and AHTN on marine microalgae, *Artemia*, sea urchin, and mussels after short-term exposure and, where possible, characterize the risk following the ECHA guideline for ERA [35] Several in vitro and in vivo toxicity tests have been performed with these compounds using freshwater species [5,24,25,28,59,60] and the only tests with marine species used high concentrations [22,30], making it difficult to compare such results with actual environmental impacts. Furthermore, for evaluation of toxicity of substances, it is imperative to use a varied battery of tests because organisms are not equally susceptible to the same toxic substance.

Due to the instability of these substances under laboratory exposure, it is difficult to estimate the exact concentration causing toxic effects. Although we could not measure the concentrations of the exposure water and bioaccumulation because of practical constraints, studies have shown that after 3 h of exposure, over 30% was lost and this reduced to ca. 80% after 96 h [22,30,51,61]. Again, there are currently no techniques to measure the concentrations of these substances in-situ; therefore, there is a probability that reported environmental concentrations are a fraction of the actual concentration in the marine environments eliciting biological effects, given that some amount could be lost before or during sample analysis in the laboratory. Consequently, risk estimation was based on maximum concentration reported.

The effects of HHCB and AHTN on *P. tricornutum, T. chuii, I. galbana,* and *R. subcapitata* showed that both compounds have limited effects (significantly similar to the control treatment) on microalgae growth (Figure 1). Although the statistical analysis indicated no differences between control with and without DMSO, the results for microalgae should be considered with caution, because in some situations inhibition or enrichment due to DMSO was observed. Therefore, we cannot reject the possibility that the effects observed in the presence of HHCB or AHTN was partially caused by DMSO.

Microalgae have been used in water quality assessments as in-situ bio-monitors because they are primary producers at the base of the ecological trophic arrangement and the basic supplier of oxygen in aquatic ecosystems [62]. Microalgae toxicity tests are useful in ERA and have gained international recognition leading to development of test guidelines for reliable and relevant toxicity data [63]. The potential of HHCB and AHTN to inhibit the growth of microalgae in the aquatic ecosystem have been scarcely reported. Previous studies revealed that significant acute toxicity of microalgae occurred at concentration greater than 100 or 500 µg/L depending on the species [5,64] but our data suggest that even at low concentrations microalgae growth might be inhibited.

The microalgae growth inhibition tests were mildly sensitive to HHCB and AHTN; IC_{10} values for most of the microalgae could not be calculated, except for *P. tricornutum* and *I. galbana* exposed to HHCB with IC_{10} values of 0.127 and 5.220 µg/L, respectively (Table 2). In contrast, previous studies have reported higher EC_{50} values of 0.050 mg/L for *Navicula* spp. and 0.336 mg/L for *Scendesmus quadricauda* exposed to HHCB [64]. This is because microalgae are not equally sensitive to contaminants. For example, in all the microalgae tested, we found that differential sensitivity was exhibited, the order of sensitivity to HHCB was *P. tricornutum* > *I. galbana*, and to AHTN was *T. chuii* > *P. tricornutum* > *I. galbana*. Similarly, differential sensitivity of two microalgae to HHCB was recently reported, whilst the microalgae, *Navicula* spp. are more sensitive to HHCB than *S. quadricauda* [64]. The basis for the differential sensitivity of microalgae to these contaminants is beyond the scope of the current investigation. Furthermore, HHCB and AHTN are lipophilic and known to bioaccumulate in marine organisms [5,15,64]. Remarkably, the effects of these compounds on microalgae growth has been adduced to bioaccumulation, altering antioxidant enzymes and biochemical processes, resulting to decrease microalgae growth [64].

Artemia was not sensitive to either substance since the endpoints measured were not critically different from the control (Figure 2A,B). This is because *Artemia* is known to be fairly resistant to toxic substances in comparison to other invertebrates and they respond differently to different environmental contaminants [65,66].

HHCB significantly reduced the fertilization success of *P. lividus* as the concentrations increased. Meanwhile, AHTN had no significant effects on sea urchin fertilization success (Figure 2C). Sea urchin fertilization success depends on sperm fitness, motility potentiated by the axonemal engine in the flagellum, morphology and chemotaxic navigation [67]. For organoleptic substances, such as HHCB and AHTN, the latter might be the most compelling factor inducing reduction in sea urchin fertilization success since the exposure procedure involved prior treatment of sperm with the contaminants before the introduction of eggs. Between the two contaminants tested, HHCB had more significant effects on *P. lividus* fertilization, causing a 19% reduction at 0.5 µg/L, and we observed that only 0.05 µg/L AHTN significantly reduced sea urchin fertilization by 16.67% (Figure 2C). Importantly, the effect exerted by both contaminants on sea urchin fertilization success was below 20%, presenting less toxic effects compared to other contaminants of emerging concern. For example, 500 ng/L propanol, 500 ng/L 17α-ethinylestradiol and 5000 ng/L gemfibrozil reduced sea urchin fertilization success by 24.1, 36.9, and 26.9%, respectively [68]. Similarly, other contaminants of emerging concern belonging to pharmaceutical and personal care products have been reported to significantly affect sea urchin fertilization success at concentrations detected in the environment [69,70].

Ecotoxicity studies with early life stages of aquatic organisms have been recommended as a faster and more cost-effective means of examining chemicals and environmental samples, because newly hatched larvae are sensitive to exogenous substances as the embryos lose their protective membranes and are fully exposed to potential xenobiotics [71]. *P. lividus* and *M. galloprovincialis* are well recognized in toxicity bioassays and are applied globally for the evaluation of toxicity of marine contaminants by the exposure of gametes to aqueous phases, such as surface waters and pore waters [69, 72] and elutriates [73]. Critical effects were detected in *P. lividus* and *M. galloprovincialis* larvae exposed to HHCB and AHTN compared to the control (Figure 2D and E). The effects of HHCB on sea urchin

larvae development was significantly ($p < 0.01$) concentration dependent (Table S2 in Supplementary Materials) and significantly different ($p < 0.05$) compared to the control, with only 44.33% of larvae able to develop to pluteus stage after 48 h exposure to 5 µg/L HHCB. The sensitivity of sea urchins to environmental chemicals is widely reported and significant evidence showed that they represent an important biomonitoring tool for ecosystems health. Similarly, HHCB and AHTN significantly ($p < 0.05$) affected the development of *M. galloprovincialis* larvae when compared to the control (Figure 2E). However, when considered in relation to the number of oocytes exposed to each contaminant, the effects were minimal with the highest percentage of deformed and undeveloped oocytes being 19.88 and 17.60% for HHCB and AHTN, respectively. Although *P. lividus* and *M. galloprovincialis* larvae development tests were similar, the effects recorded were more pronounced in sea urchin larvae than mussels.

Significant ($p < 0.01$) concentration dependent responses (Table S2 in Supplementary Materials) were observed in *S. aurata* mortality tests with HHCB and AHTN (Table S2 in Supplementary Materials). Notwithstanding, the percentage mortality of *S. aurata* exposed to both fragrances not up to 20%, the highest effect being 13% for HHCB and AHTN at the highest concentration of 5 µg/L. Although this species and endpoints proved to be sensitive to other contaminants at low concentrations [40,68], the impacts of environmental concentrations of the tested fragrances in this study were low. For chemical prioritization, fish early-life stage toxicity test is endorsed [47] because it is a reliable and reproducible risk assessment tool that requires shorter exposure time and lower cost to perform. However, the sensitivity of fish embryotoxicity to some emerging contaminants remain doubtful as previous studies reported low sensitivity [68,74].

Risk characterization of contaminants is quantified using MEC–PNEC ratio and for aquatic environments (freshwater and marine), PNEC is estimated by dividing the EC_x value by an assessment factor of 1.000 for acute toxicity test and 10 for chronic toxicity test [35]. Although short term toxicity tests were performed in this study, an assessment factor of 1000 was used only for larval development test while an assessment factor of 10 was used for the microalgae because the exponential phase of microalgae growth was regarded as a full life stage and therefore considered as a chronic test. Analyzing the RQs, HHCB represented potential high risk for the marine environment based on the EC_{50} estimated for *P. lividus* larvae development. In addition, HHCB and AHTN posed high ecological risk to *M. galloprovincialis* larval development at environmental relevant concentrations. The larval development of *P. lividus* and *M. galloprovincialis* seem to be very sensitive to chemical exposure because previous studies of environmental contaminants in coastal waters have reported significant toxicity of industrial and domestic effluent [40], pharmaceutically active products [68,74], UV-filters [70] and organic pollutants [46,72] to sea urchin and mussels larvae development. The quantitative risk estimate of HHCB obtained in this study for *P. lividus* using EC_{10} value (1200–57,500) was higher than that of propanol (0.02–17.29), previously reported by Capolupo et al. [68]. We also found that both compounds posed high risk to *P. tricornutum* and *I. galbana* based on IC_{10} values recorded (Table 2). Microalgae have broadly been used in evaluation of ERA for other emerging contaminants. For example, *I. galbana* have been reported to be significantly affected by UV filters and pharmaceutical active ingredients [70,74]. Fragrances have been demonstrated to pose high risk to microalgae in the marine environment, of which HHCB and AHTN were more toxic than musk xylene and musk ketone [7]. The adversity of measured environmental concentrations of HHCB and AHTN to *P. tricornutum* and *I. galbana* deserve attention because microalgae occupy the lowest trophic level of the marine food chain and, therefore, serve as food for higher trophic organisms. Again, HHCB and AHTN impacts on microalgae growth represent a potential bottom-up effect that might result in structural and functional disruption of the ecosystems [37]. Therefore, more studies are required to fully understand the environmental effects of these contaminants in the marine environments.

5. Conclusions

This study evaluated the potential risk of comparable measured environmental concentrations of HHCB and AHTN to microalgae and early life stages of marine organisms, including *A. franciscana*, *P. lividus*, *M. galloprovincialis*, and *S. aurata*, adding to the dearth of information regarding the adverse effects of HHCB and AHTN in the marine environment. For all the species of microalgae, Artemia, sea urchin, mussels, and fish tested, differential sensitivity was observed. Artemia motility and survival were the least sensitive endpoints affected by both substances and *P. lividus* larvae development was the most sensitive species/endpoint. From our data, the environmental risk of HHCB and AHTN was characterized as high for *P. tricornutum*, *I. galbana* growth, *P. lividus* and *M. galloprovincialis* larvae development. Therefore, more studies are required to understand the sub-lethal effects of these compounds in the marine environment.

Supplementary Materials: The following are available online at https://www.mdpi.com/2227-9717/9/2/371/s1, Table S1: Spearman rank order of correlation (r) values recorded for the effect of galaxolide (HHCB) and tonalide (AHTN) on microalagae exposed for 72 h (n = 3). Asterisk(s) * & ** represent significant levels at $p < 0.05$ and 0.01, respectively, Table S2: Spearman rank order of correlation (r) values recorded for the effect of galaxolide (HHCB) and tonalide (AHTN) on marine organisms exposed for 72 h (n = 3). Asterisk(s) * & ** represent significant levels at $p < 0.05$ and 0.01, respectively; (-) represent values not determined.

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Avoidance behaviour of the shrimp *Palaemon varians* regarding a contaminant gradient of galaxolide and tonalide in seawater

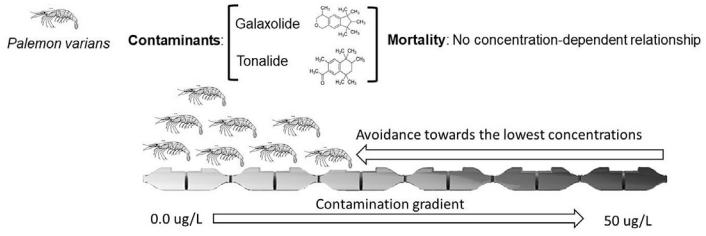
Friday Ojie Ehiguese [a, *], María del Carmen Corada Fernandez [a], Pablo A. Lara-Martín [a], M. Laura Martín-Díaz [a], Cristiano V.M. Araújo [b]

[a] Physical Chemical Department, University Institute of Marine Research (INMAR), International Campus of Excellence of the Sea (CEI.MAR), University of Cadiz, República Saharaui s/n, 11510, Puerto Real, Cádiz, Spain
[b] Department of Ecology and Coastal Management, Institute of Marine Sciences of Andalusia (CSIC), 11510, Puerto Real, Cádiz, Spain

HIGHLIGHTS

- Shrimps were tested for mortality upon acute exposure to galaxolide and tonalide.
- Shrimps were tested for avoidance in a multi-compartmented, non-forced system.
- Avoidance response to a contaminant gradient was concentration-dependent.
- Population immediate decline is expected to be driven by avoidance behaviour.
- We recommend integration of both approaches in environmental risk assessments.

GRAPHICAL ABSTRACT



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ABSTRACT

The musk fragrances galaxolide (HHCB) and tonalide (AHTN) are compounds of emerging concern that have been found in various environmental compartments. The present study addressed the ability of HHCB and AHTN to elicit the avoidance response in the estuarine shrimp *Palaemon varians* and to predict the population immediate decline (PID) of *P. varians* when exposed to HHCB and AHTN by integrating both avoidance (non-forced exposure) and lethality (forced exposure) responses. The avoidance response was tested in a non-forced multi-compartmented static system, in which the shrimps could move freely among the compartments with different concentrations. The shrimps ($n = 3$ shrimps per compartment/concentration; 18 shrimps per system) were exposed to a gradient (0, 0.005, 0.05, 0.5, 5 and 50 μg/L) of both substances and their positions were checked at every 20 min for a 3 h period. The results from 24-h forced exposure showed no dose-response relationship and the highest percentage mortality was 17% for HHCB at 0.005 and 0.5 μg/L. In the 3-h non-forced exposure to a gradient of HHCB and AHTN, significant concentration-dependent spatial avoidance was observed for both substances. The shrimps avoided the lowest concentration of HHCB and AHTN (0.005 μg/L) by 15% and 16%. The avoidance increased significantly ($p < 0.005$) to a 61% and 57%, respectively, for the highest concentration (50 μg/L). The population immediate decline was driven by the avoidance behaviour of the shrimps rather than mortality. These

* Corresponding author.
E-mail address: friday.ehiguese@alum.uca.es (F.O. Ehiguese).

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results indicated that the aversiveness of HHCB and AHTN might have serious consequences for habitat selection processes by organisms.

1. Introduction

Maintenance and restoration of ecological integrity is the primary objective of environmental risk assessment (Ramesh and Kaplana, 2015). Consequently, various regulatory agencies have established guidelines to protect water bodies within their jurisdiction for the sustainable use of aquatic resources (EC, 2000; EPA, 2002) and, as a result, levels of individual contaminants, water quality parameters, or description of changes in the water bodies' conditions have been established in order to protect them. The United States Environmental Protection Agency's (USEEPA) aquatic life criteria is derived from the criterion maximum concentration, with the aim of protecting aquatic ecosystems from severe acute effects, and from the criterion continuous concentration, that has been set to protect against long term effects on survival, growth, reproduction etc. (Beaman et al., 2008). Data from ecotoxicity studies, through which the toxic effects of substances are assessed, are extrapolated from laboratory toxicity tests involving forced exposures, which implies a continuous exposure of the organisms to a specific concentration of chemicals or environmental samples (e.g. water, sediment, soil). The forced exposure approach makes it possible to identify the potential toxicity of contaminants, determine the concentration-response relationship and provide information about their mechanisms of action (Newman, M.C., Unger, M.A., 2001; Martinez-Haro et al., 2015). However, applying the forced exposure approach makes it difficult to check how contaminants affect the spatial distribution of organisms and their habitat selection processes for the cases in which they are able to flee from the contaminated areas, as it may occur (for mobile organisms and heterogeneous contamination scenarios) in natural environments (Åtland and Barlaup, 1995; Hansen et al., 1999; Moreira-Santos et al., 2008).

Spatial avoidance involves the emigration of organisms from a noxious environmental condition to a safer area, thus indicating the aversive character of the area (Jutfelt et al., 2017; Tierney, 2016). Experimentation concerning active avoidance was first performed by Shelford and Allee (1913), but spatial avoidance to assess organisms' behavioural response to contaminants was later performed with fish in tubes containing contaminants at one end and clean water at the other, thus allowing the fish to detect differences in contamination levels and to move to a less contaminated environment (Jones, 1947). Different exposure systems including two compartments, steep gradients, laminar flow chambers, avoidance/preference chambers, fluvarium systems and dilution gradients involving several compartments have been developed and employed to assess contamination-driven avoidance (Folmar, 1976; Gunn and Noakes, 1986; Hartwell et al., 1989; Moreira-Santos et al., 2008; Richardson et al., 2001; Smith and Bailey, 1990; see also review by Jutfelt et al., 2017). The use of the free-choice, non-forced, multi-compartmented exposure system developed by Lopes et al. (2004), in which contamination gradients or patches are simulated, have also proven to be a suitable approach to assess how contaminants can interfere in the spatial distribution of organisms (see review by Araújo et al., 2016b; Araújo and Blasco, 2019). Laboratory exposure of fish, amphibians, decapods, molluscs, dipterans, copepods and annelids to fungicides, contaminated effluents, metals and organic compounds to determine avoidance responses at sublethal concentrations in a free-choice multi-compartmented exposure system have been reported, and have indicated that contaminants potentially can, to some extent, drive the spatial distribution and habitat selection processes by organisms (Araújo et al., 2016a, 2014b; 2014c, 2014a; Dornfeld et al., 2009; Moreira-Santos et al., 2008; Rosa et al., 2012; Silva et al., 2017). This non-forced, multi-compartmented approach simulates a realistic heterogeneous contamination scenario where organisms are not restricted to continuous exposure as they are in forced exposure, thus providing a complementary approach to environmental risk assessment based not exclusively on toxicity, but also on the displacement patterns of organisms.

The polycyclic musk compounds (PMCs) – galaxolide (HHCB) and tonalide (AHTN) – are contaminants of emerging concern (CEC). They are applied in many personal care products including detergents, lotions, deodorants and shampoos, to mention just a few (Reiner and Kannan, 2011). In Europe, they constitute about 95% of the total fragrance materials in the perfumery industry (OSPAR, 2004). The use of these substances has reportedly increased in recent years and Southern European countries are the highest consumers (Cunha et al., 2018; European Commission, 2008a, 2008b). PMCs are lipophilic and possess high octanol/water partition coefficient (K_{OW}) values ranging from 5.4 to 6.3 (Cunha et al., 2018), so they are not readily soluble in water. The presence of these substances in environmental samples has been reported and effluents of wastewater treatment plants have been identified as the primary route of entrance into aquatic ecosystems (Petrie et al., 2014). The concentrations recorded are highest at wastewater treatment effluent pools (Chase et al., 2012; Díaz-Garduño et al., 2017) and decrease along the water course (Sumner et al., 2010). Although most environmental measurements with high concentrations (6–13330 ng/l) were from effluents and rivers (Chase et al., 2012; Dsikowitzky et al., 2002; Fromme et al., 2001; Lee et al., 2010; Reiner and Kannan, 2011; Zhang et al., 2008), up to 2098 ng/l of HHCB and 159 ng/l of AHTN have been measured, respectively, in coastal waters (Sumner et al., 2010).

Contamination by PMCs can be considered ubiquitous as they have been detected in many environments such as: air (Peck and Hornbuckle, 2006), sediments (Fromme et al., 2001; Heberer, 2002; Zhang et al., 2008), particulate suspended matters (Gatermann et al., 2002) and human adipose tissue (Kannan et al., 2005; Moon et al., 2012b). Hence, bioaccumulation of PMCs in a wide diversity of freshwater and marine organisms including cetaceans, sharks, fish and shellfish in Europe, Japan, Korea, China and the USA has been extensively studied (Gatermann et al., 2002; Lee et al., 2014; Moon et al., 2012a; Nakata, 2005; Nakata et al., 2007; Picot Groz et al., 2014; Rüdel et al., 2006; Zhang et al., 2013). Assessment of the potential toxicity of HHCB and AHTN based on lethality from forced exposures, their chemical properties and ability to affect the human pheromone-endocrine system suggest they may alter the structure of a community and impoverish certain ecosystems (Breitholtz et al., 2003; Kallenborn et al., 1991; Rimkus et al., 1997; Wollenberger et al., 2003). However, despite the risks of contamination that PMCs might pose to organisms, ecotoxicological assessments of PMCs in freshwater (Balk and Ford, 1999) and marine environments (Ehiguese et al., unpublished) are still only incipient. Efforts to evaluate the potential of HHCB and

AHTN to trigger avoidance in aquatic organisms has been even less explored. Therefore, the present study first addressed the potential of HHCB and AHTN to elicit avoidance response in the estuarine shrimp *Palaemon varians* causing emigration to areas that are less contaminated. *P varians* is a euryhaline estuarine shrimp found mainly in shallow saltmarsh pools from west Baltic and British Isles southwards to the west Mediterranean. It is ecotoxicologically important for the trophic web as it can accumulate contaminants and acts as vector for the upper trophic levels (Raimbow et al., 2006). Previous studies in our laboratory have indicated that the presence of chemical contaminants may trigger their evasion to favourable areas. To this end, a free-choice, non-forced, multi-compartmented exposure system was used (Araújo et al., 2014c) because it allows a contamination gradient of compounds to be simulated. The second aim was to predict the population immediate decline (PID) (Rosa et al., 2012) of *P. varians* when exposed to HHCB and AHTN by integrating avoidance and lethality in short-term experiments to evaluate whether the shrimps were able to detect toxicity and avoid potentially toxic concentrations before suffering acute effects.

2. Materials and methods

2.1. Test organisms

Shrimps (1.0–1.5 cm length) were obtained from the Salina El Pópulo aquaculture farm in San Fernando (Southwest Spain) and immediately transported to the ecotoxicology laboratory of the Institute of Marine Sciences of Andalusia (ICMAN-CSIC), in Puerto Real (Spain). They were acclimated for 2 weeks in two 250 L aquaria supplied with filtered (0.5 μm) seawater (deep-well seawater: pH of 7.5 and salinity of 35) in a flow-through system with continuous aeration. The organisms were monitored regularly and dead shrimps were removed immediately. No food was provided during laboratory acclimation. Laboratory conditions were: 20 ± 0.5 °C temperature and a 12:12 h light/dark photoperiod.

2.2. Chemicals

Analytical grade HHCB and AHTN (85.0% and 97.0% GC, respectively) were purchased from Sigma Aldrich Spain. Due to their low solubility in water, stock solutions were prepared using dimethyl sulfoxide (DMSO 0.001%v/v). The stock solutions were diluted using nanopure water to reach the desired concentrations (0.005, 0.05, 0.5, 5 and 50 μg/L). The filtered seawater used in the culture was spiked with each concentration for the tests.

2.3. Multi-compartmented exposure system

A non-forced, multi-compartmented static assay system (Fig. 1) was used in the avoidance experiments following the design used by Araújo et al. (2014c). In brief, six compartments made of plastic bottles were constructed (the dimensions are stated below), which were connected at the cut-out bases and mouths of the containers using glue (Sikaflex® construction sealant, Switzerland) to obtain a six-compartmented system. An opening was carefully cut out at the top of each compartment to facilitate the introduction of organisms and contaminant.

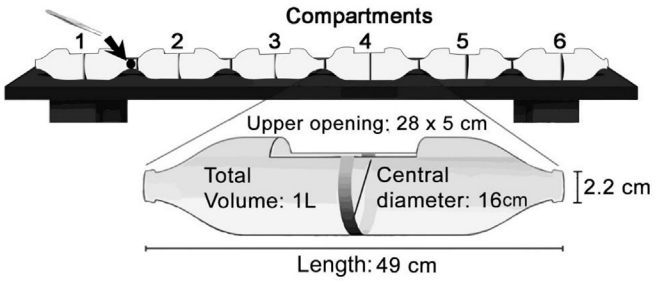

Fig. 1. Non-forced multi-compartmented static exposure system used in the avoidance tests (Islam et al., 2019).

2.4. Avoidance tests

To ascertain the non-interference of external factors in the avoidance tests and to verify random distribution of shrimps (no preference for any compartment), validation (control) tests with no contaminant were performed using seawater and seawater spiked with DMSO (0.001% v/v). The compartments were each filled with 1 L of seawater and then the shrimps ($n = 3$) were added to each compartment (18 shrimps in total per system) and the distributions of the organisms were recorded at each 20 min for 3 h. The tests were performed in quadruplicate.

For each avoidance test with HHCB and AHTN, plasticine plugs were positioned at the junction of each compartment before filling them with seawater spiked with HHCB and AHTN. The gradients of contaminants were in the order of 0 (seawater), 0.005, 0.05, 0.5, 5 and 50 μg/L. Three shrimps were added to each compartment and then the plasticine plugs were removed. The tests were conducted in quadruplicate for each substance with 18 organisms per system (three organisms × six concentrations). The distribution of the organisms in each compartment was recorded at each 20 min for 3 h. The tests were conducted in the dark at 20 °C. At the end of each test, samples of the HHCB and AHTN in each compartment were collected using clean dark amber bottles and stored at −20 °C for analyses.

2.5. Acute toxicity tests using forced exposure

Acute toxicity tests (24 h exposure) were performed with HHCB and AHTN. The concentrations used for each contaminant were 0.005, 0.05, 0.5, 5 and 50 μg/L including seawater and solvent (DMSO) controls. The tests were carried out in quadruplicate. Each aquarium used in the tests was filled with 1 L of seawater spiked with the contaminants. Three shrimps were added per aquarium totalling 12 shrimps per treatment. Mortality was recorded at 1, 3, 7 and 24 h. No aeration was provided. The temperature was 20 ± 0.1 °C. Initial and final dissolved oxygen levels were 5.9 ± 0.1 and 5.2 ± 0.2 mg/L, respectively.

2.6. Sample collection and analyses

Samples taken at the beginning and end of all the tests were analysed for HHCB and AHTN concentrations using stir bar sorptive extraction (SBSE) following a modification of the methodology described by Pintado-Herrera et al. (2014). Prior to use, all polydimethylsiloxane bars (PDMS, 10 mm × 0.5 mm) were preconditioned by soaking them in a mixture of acetonitrile/methanol (80:20, v/v). Subsequently, these bars were placed in amber glass flasks containing the aqueous samples (350 ml). Internal standard (triphenylphosphate d15) was added to determine possible fluctuations during the extraction and analysis procedures and stirred at 900 rpm during 4 h at room temperature. After extraction, the bars were desorbed by liquid desorption (LD); the bars were sonicated for 30 min in vials containing 100 μL of ethyl acetate. Then, gas chromatography (SCION 456-GC, Bruker) and mass spectrometry (SCION TQ from Bruker with CP 8400 Autosampler) were used to identify and quantify the compounds. Capillary gas chromatography analysis was carried out on a HP-5MS column

(30 m × 0.25 mm i.d. × 0.25 μm film thickness of 5% phenyl, 95% polydimethylsiloxane), keeping the helium carrier gas flow at 1 mL/min. The mass detector acquired in multiple-reaction monitoring (MRM) mode. Details of the detection methodology can be found in Pintado-Herrera et al. (2016). Calibration curves were constructed for each compound in the range of 0.005–50 μg/L. Method limits of quantification were calculated using a signal-to-noise ratio 10 to 1, respectively, for water samples that was lower than 0.04 ng/L. The recovery rate of the method was higher than 85% for both analytes. The mean concentration and standard deviation of the internal standard (triphenylphosphate d15) was 46.7 ± 10.6 μg/L. Concentrations of the contaminants measured in all the tests are presented in Table S1 – Supplementary Material.

2.7. Statistical analysis

The percentage of organisms recorded in each compartment at different observation times were arcsine transformed. The random distribution of organisms in the seawater and solvent controls, and avoidance tests with the contaminants were evaluated using mixed-designed (time as a repeated measure, within factor, and compartment as between factor) analysis of variance (ANOVA). Mauchly's test was used to check the sphericity. Where sphericity was violated (the variances of the differences are not equal: $p < 0.05$), Greenhouse-Geisser correction was applied (see Tables S2a, S3a, S4a and S5a – Supplementary Material). Statistical ($p < 0.05$) differences between factors were checked using the Bonferroni test. The avoidance to HHCB and AHTN was determined by calculating the difference between the number of organisms expected (N_E) and observed (N_O) as described in Moreira-Santos et al. (2008). N_E represents the number of organisms initially introduced into each compartment. As three shrimps were inserted into each compartment at the beginning of the test, for the compartment with the highest concentration N_E was 3. As the next compartment had initially 3 organisms plus 3 organisms introduced in the adjacent compartment with the highest concentration, N_E was 6. For the sixth compartment, which contained only control seawater with no contaminant, N_E was 18. N_O was calculated considering the number of organisms recorded per time per compartment including the organisms observed in the compartments with higher concentrations. The avoidance percentage for each compartment was computed as (Avoiders/N_E) *100.

Median lethal concentrations (LC_{50}) and their respective confidence intervals (CI) from the forced exposure experiments and median avoidance concentrations (AC_{50}) from the non-forced exposure ones were calculated using PriProbit 1.63 software (Sakuma, 1998). The population immediate decline (PID) was computed following the method described by Rosa et al. (2012). The avoidance and mortality percentages were integrated to calculate the PID (*x* in percent) induced by each HHCB and AHTN concentration that simultaneously caused a *y* mortality percentage (i.e. the 24 h LC*y*) and a *w* avoidance percentage (i.e. the 3 h AC*w*) as thus:

$$X = [1 - (1 - y/100 * (1 - w/100)] * 100$$

The PID was calculated on the premise that some organisms first emigrate (avoidance %) and that mortality is then calculated based on the remaining organisms that did not emigrate. PID_{50} was calculated following the above procedure for AC_{50} and LC_{50}.

3. Results

3.1. Acute toxicity test

The shrimp mortality recorded during the 24 h acute test with HHCB was not concentration-dependent and was highest (17%) for 0.005 and 0.5 μg/L. For AHTN, no mortality was observed at 0 and 0.5 μg/L, and it was around 8% at 0.005, 5 and 50 μg/L (Table S2 – Supplementary Material).

3.2. Avoidance test

No mortality was recorded in the non-forced experiments. The distribution of shrimps between the compartments over time was not significantly different ($p < 0.005$) for both seawater ($p = 0.64$, $F_{(5,18)} = 0.693$) and DMSO ($p = 0.99$, $F_{(5,18)} = 0.085$) controls (Tables S3b and S4b – Supplementary Material) and the distribution of shrimps between the compartments was not significantly different ($p < 0.005$) for the seawater control and the DMSO control (Tables S3c and S4c – Supplementary Material). In the absence of contaminants, the distribution of shrimps in both seawater and DMSO controls (Fig. 2) was random (showing no preference for any compartment) during the 3 h exposure.

In the tests with each contaminant, the shrimps' distribution did not vary over time (Tables S5b and S6b – Supplementary Material). The statistical analysis of the shrimps' distribution in the contaminant gradients of HHCB ($F_{(5,18)} = 7.388$, $p < 0.001$) and AHTN ($F_{(5,18)} = 6.127$, $p < 0.002$) revealed that the organisms significantly ($p < 0.05$) preferred the uncontaminated compartments (Tables S5c and S6c – Supplementary Material). Significant differences in the distribution of shrimps exposed to each substance are shown in Fig. 3. The mean percentage distribution of *P. varians* exposed to gradient of HHCB and AHTN after 3 h was 28.9% and 30.2%, respectively, in uncontaminated compartments and it was over 4 times higher than the mean percentage distribution of organisms in compartment with the highest concentration (50 μg/L: 6.5% and 7.1% for HHCB and AHTN, respectively).

The avoidance behaviour, mortality and PID data of the shrimps exposed to the contaminant gradients of HHCB and AHTN for 3 h are shown in Fig. 4. For both contaminants, a concentration-dependent avoidance response was observed. For HHCB, the mean percentage avoidance for the lowest concentration (0.005 μg/L) was 14.6% and increased significantly to 60.5% ($p < 0.05$) for the highest concentration (50 μg/L). The same trend was observed for AHTN from 16.2% (at 0.005 μg/L) to 57.1% (at 50 μg/L). The PID curve

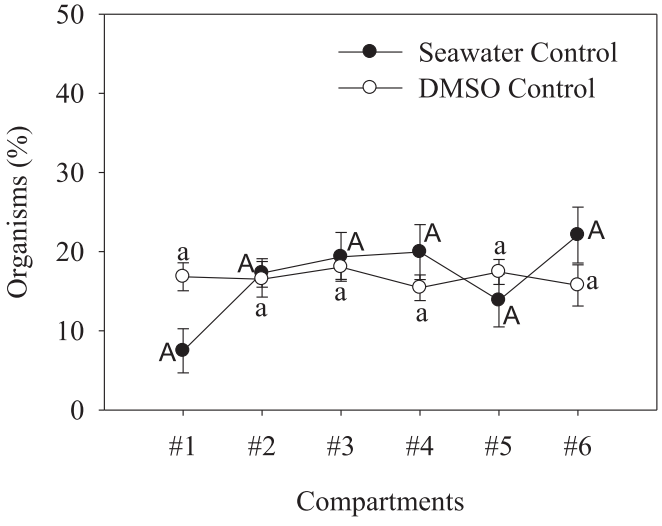

Fig. 2. Mean percentage and standard deviation (n = 9 observation periods) of the number of shrimps *Palaemon varians* in the seawater and solvent (DMSO) control tests recorded in each compartment for 3 h. Different letters (upper case for seawater control and lower case for DMSO control) represent statistically significant difference.

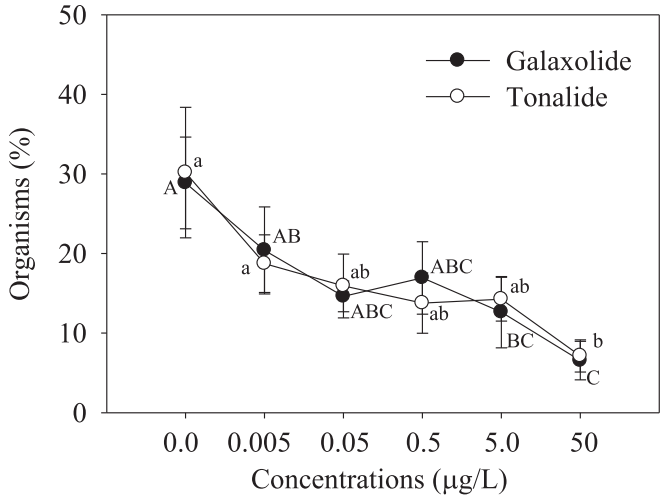

Fig. 3. Mean percentage (n = 9 observation periods) of shrimps *Palaemon varians* exposed to gradient concentrations of galaxolide and tonalide recorded in each compartment for 3 h. Different letters (upper case for galoxide and lower case for tonalide) represent significant difference.

(Fig. 4) from the non-forced exposure data followed the same trend with the avoidance behaviour for both substances, as no mortality was recorded in the non-forced tests (Fig. 4).

3.3. AC_{50}, LC_{50}, and PID_{50}

The values of AC_{50}, LC_{50} and PID_{50} for HHCB and AHTN, based on mortality in the forced system and avoidance in non-forced exposure system, are shown in Table 1. The AC_{50} values (from non-forced exposure tests) obtained for HHCB and AHTN were 14.1 µg/L and 30.8 µg/L, respectively, and their corresponding LC_{50} values (from forced exposure tests) were >50 µg/L (no mortality was recorded). Since there was no mortality in the non-forced tests, the AC_{50} was equal to PID_{50}. However, the AC_{50} values recorded for the non-forced exposures were about twice as high as the PID_{50} values for both substances.

4. Discussion

The present study assessed the potential of the musk fragrances, galaxolide and tonalide, to trigger avoidance response in the estuarine shrimp *P. varians*. The results of the control tests showed that the displacement of the shrimps inside the system in the absence of contaminants was non-preferential. This indicates that the shrimps did not present aggregation behaviour that could condition, to some extent, their movement pattern (Araújo et al., 2016a). Previous studies with shrimp have also shown that the freshwater shrimp *Atyaephyra desmarestii* (Araújo et al., 2018a) and the marine shrimps *Litopenaeus vanamei* (Araújo et al., 2016a) did not aggregate when exposed to uncontaminated water using a similar non-forced, multi-compartmented system. Studies with other organisms such as the crustacean *Daphnia magna* (Rosa; et al., 2008; Rosa et al., 2012), the freshwater fish *Danio rerio* (Araújo et al., 2016b, 2014b) and *Poecilia reticulata* (Silva et al., 2017), exposed to uncontaminated water in non-forced, multi-compartmented systems, also did not display significant differences in the distribution of organisms throughout the different compartments. This random distribution in the absence of contaminants validates the suitability of the experimental system to study the spatial avoidance behaviour of organisms exposed to contaminant gradients.

Regarding the avoidance response, both compounds were detected by the shrimps and the potentially toxic concentrations were avoided. *Palaemonidae* are anatomically and physiologically adapted to detect the presence of chemicals in their environment using both antennule and antennae (Machon et al., 2018), and as such, can select suitable habitats in the instance of chemical perturbation by avoiding contaminated area. Avoidance of both fragrances occurred at concentrations as low as 0.005 µg/L, indicating the high sensitivity of this response to reveal the risk of HHCB and AHTN. The mean avoidance percentages recorded for the lowest concentration (0.005 µg/L) of HHCB and AHTN were about 14 and 16%, respectively, and increased significantly in a concentration-dependent pattern to 60 and 57% at the concentration of 50 µg/L. Assessing the magnitude of this response, regarding how other marine species will react, is difficult due to the lack of studies on the avoidance responses of estuarine/marine organisms elicited by these compounds. The avoidance behaviour of marine shrimp and fish exposed in non-forced, multi-compartmented systems has been described for other compounds and it has revealed how suitable this system is for studying contamination-driven avoidance responses: around 80% of the estuarine shrimp *Litopenaeus vannamei* (whiteleg shrimp) and 60% of the marine fish *Rachycentron canadum* avoided contaminant gradients of copper

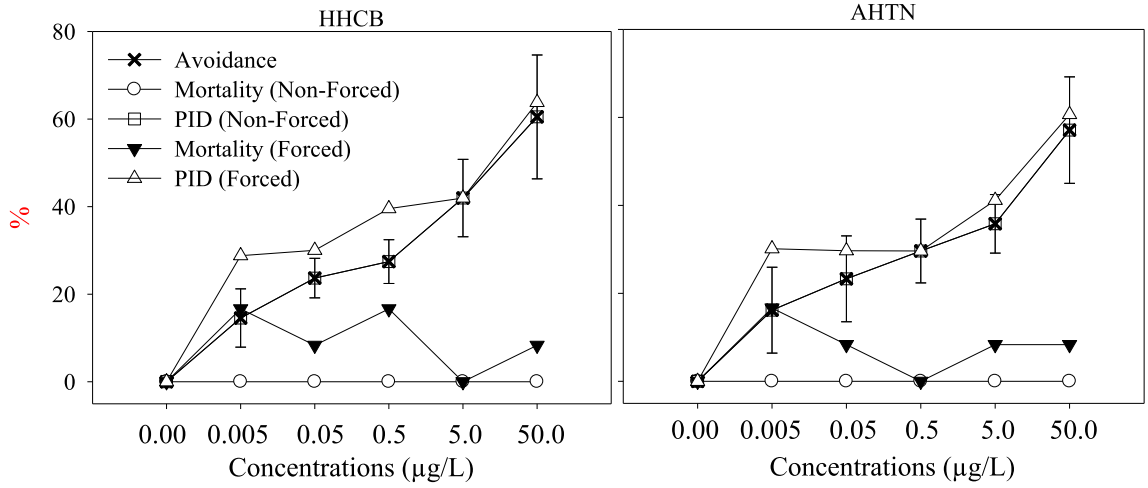

Fig. 4. Concentration-response curves for the avoidance (non-forced exposure) and mortality (forced exposure) responses, and the estimated PID (Population Immediate Decline) for *Palaemon varians* exposed to galaxolide (HHCB) and tonalide (AHTN). Standard deviations were not presented for forced tests, because the mortality was calculated based on the total number of exposed organisms.

Table 1
Values (in μg/L) and their respective confidence intervals of AC_{50}, LC_{50}, and PID_{50} (concentrations that cause 50% of avoidance, mortality and population immediate decline of exposed organisms) for the shrimp *Palaemon varians* exposed to galaxolide (HHCB) and tonalide (AHTN) in non-forced (NFS) and forced (FS) exposure systems.

Substances	AC_{50} (NFS)	LC_{50} (NFS)	PID_{50} (NFS)	LC_{50} (FS)	PID_{50} (FS)
HHCB	14.1 (5.2–61.2)	>50	14.1 (5.2–61.2)	>50	6.3 (1.7–48.7)
AHTN	30.8 (7.7–345.6)	>50	30.8 (7.7–345.6)	>50	18.8 (3.9–381.4)

assayed for 3 h (Araújo et al., 2016a). Regarding contaminants of emerging concern, studies of avoidance using non-forced, multi-compartmented exposure systems have been mainly performed with fish. For instance, around 50% of a population of zebrafish (*Danio rerio*) avoided 1.4 mg/L of the fungicide (pyrimethanil) (Araújo et al., 2014d, 2014b); 22% of the fish *P. reticulata* exposed under the same system for 4 h avoided triclosan concentrations as low as 0.2 μg/L (Silva et al., 2017); the AC_{50} for *P. reticulata* exposed to a bisphenol gradient was 0.15 μg/L, below the values considered safe for aquatic biota (Silva et al., 2018); *P. reticulata* also avoided environmentally relevant atrazine concentrations (0.02 μg/L; Araújo et al., 2018b). Organisms' behavioural response to the presence of a contaminant by avoiding contaminated sites is a protective strategy to prevent toxic impact and lethality (Gunn and Noakes, 1986; Lopes et al., 2004; Oliveira et al., 2013; Silva et al., 2017). The use of a non-forced exposure approach provides an idea of the immediate response that organisms can present due to the aversive characteristic of contaminants and of the possible loss of organisms due to the massive emigration towards less aversive habitats (De Lange et al., 2006; Rosa et al., 2012; Araújo et al., 2016b). Although no effect at the individual level is expected to occur, the influence of a contaminant in the spatial distribution of organisms should be assessed carefully, as the consequences of the spatial avoidance are not restricted to the avoided ecosystem (Moreira-Santos et al., 2019). Whilst emigration from contaminated area is a solution for the avoiders (Moe et al., 2013), the consequences to ecosystem could lead to the loss of abundance and biodiversity (Lopes et al., 2004). The avoidance by the shrimps, that occupy an intermediate trophic level (Walker and Ferreira, 1985), might limit the amount of food available to organisms in the upper trophic strata and reduce the predation pressure on lower trophic levels.

Data of mortality recorded after forced exposure for 24 h suggest that the concentrations used were not acutely toxic, which is in accordance with the argument about the low acute toxicity of HHCB and AHTN (Breitholtz et al., 2003; Wollenberger et al., 2003). The 24 h-LC_{50} value recorded for HHCB (401.7 μg/L) was similar to the 48 h-LC_{50} (470 μg/L) for the marine copepod *Acartia tonsa* (Wollenberger et al., 2003), and to the 96 h-LC_{50} (288 μg/L) for *Chironomus riparus* (Artola-Garicano et al., 2003). However, a higher value (96 h-LC_{50}: >1000 μg/L) for the adult harpaticoid copepod *Nitocra spinipes* has been reported (Breitholtz et al., 2003). The shrimp *P. varians* proved to be more sensitive to AHTN (24 h-LC_{50} value of 88.11 μg/L) compared to *N. spinipes* (96 h-LC_{50}: 610 μg/L; Breitholtz et al., 2003), *C. riparus* (96 h-LC_{50}: >460 μg/L; Artola-Garicano et al., 2003) and *A. tonsa* (48 h-LC_{50}: 2500 μg/L; Wollenberger et al., 2003). Although mortality was the only endpoint considered in the forced exposure systems, symptoms of stupefaction were also observed, which could impair the ability to avoid toxic environments. Subsequently, this and other potential sub-lethal effects under a forced exposure scenario should not be neglected.

To elucidate the immediate impact of contaminant dispersion on aquatic environments at the local level, the PID was estimated by integrating mortality and avoidance responses. Clearly, avoidance played a greater role in the PID than mortality. Although these substances are classified as low risk in terms of lethality and, therefore, are believed not to pose a significant threat at the present environmental concentration levels (European Commission, 2008a, 2008b), it is evident that they may contribute to a significant population decline at a local scale and for a short exposure period by triggering avoidance. For instance, at the lowest concentration (0.005 μg/L) the population of shrimps declined by 28 and 30% for HHCB and AHTN, respectively, as against 16% from mortality data from forced exposures. Several authors have recommended the inclusion of spatial avoidance data measured in non-forced, multi-compartmented exposure systems as a complementary tool for ecological risk assessment. This might help to predict the ecological risk of contaminants more accurately; thus avoiding over-estimation or underestimation of risk prediction by either the forced or non-forced approaches (Araújo et al., 2016b; Moreira-Santos et al., 2008; Rosa et al., 2012; Silva et al., 2017). In other studies where PID was estimated, avoidance also tended to occur at concentrations lower than mortality (Araújo et al., 2014a; Rosa et al., 2012; Silva et al., 2017), indicating that under conditions of gradual and heterogeneous contamination, the contaminated area might lose part of the organism population due to its fleeing from contamination with possible longer-term consequences on local ecosystem structure and functioning. In cases where the contaminant (either due to its mode of action or to the concentrations) was observed to cause stupefaction in the organisms (Gutierrez et al., 2012) and avoidance is prevented, the mortality in the short term might play a more important role for the PID (Araújo et al., 2014a, 2014c).

Particularly in the cases of HHCB and AHTN, the shrimps were not sensitive in the forced exposure tests, but they were able to detect and avoid concentrations of those compounds in the non-forced exposure tests. This might indicate that avoidance helps to prevent even sub-lethal effects that organisms could suffer after a continuous exposure. Under conditions in which an HHCB or AHTN gradient exists, it would be expected that the distribution of the shrimps was, at least to some extent, conditioned by the presence of those contaminants. Since the AC_{50} obtained for HHCB is 28 times lower than the 24 h-LC_{50} for forced exposure, the prediction of the ecological risks of HHCB and AHTN based exclusively on data from forced exposure assays might provide a relevant vision of the potential toxicity of both compounds but fails to assess the complete effects at the community structure level (spatial distribution of the shrimps). Therefore, for contaminants of emerging concern, spatial avoidance using the non-forced, multi-compartmented approach should be considered as an additional line of evidence in environmental risk assessments.

5. Conclusion

Shrimps were not acutely (mortality response) sensitive to the concentrations of both compounds tested. However, under non-forced exposure to HHCB and AHTN contaminant gradients, the shrimps detected different concentrations and avoided those potentially harmful. The population decline for short exposure to environmentally relevant concentrations of HHCB and AHTN was proven to be driven by the avoidance behaviour. It is recommended

that ecological risk assessments of compounds of emerging concern, as well as non-emerging and legacy contaminants, integrate data from both forced and non-forced exposure approaches to avoid underestimation of their full potential risks.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2019.05.196.

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Potential of environmental concentrations of the musks galaxolide and tonalide to induce oxidative stress and genotoxicity in the marine environment

Friday O. Ehiguese [a,*], Md Rushna Alam [a,b], Marina G. Pintado-Herrera [a], Cristiano V. M. Araújo [c], M. Laura Martin-Diaz [a]

[a] Chemical Physics Department, University Institute of Marine Research (INMAR), International Campus of Excellence of the Sea (CEI.MAR), University of Cádiz, República Saharaui s/n, 11510, Puerto Real, Cádiz, Spain
[b] Department of Aquaculture, Faculty of Fisheries, Patuakhali Science and Technology University, Dumki, Patuakhali, 8602, Bangladesh
[c] Department of Ecology and Coastal Management, Institute of Marine Sciences of Andalusia (CSIC), 11510, Puerto Real, Cádiz, Spain

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ABSTRACT

Polycyclic musk compounds have been identified in environmental matrices (water, sediment and air) and in biological tissues in the last decade, yet only minimal attention has been paid to their chronic toxicity in the marine environment. In the present research, the clams *Ruditapes philippinarum* were exposed to 0.005, 0.05, 0.5, 5 and 50 μg/L of the fragrances Galaxolide® (HHCB) and Tonalide® (AHTN) for 21 days. A battery of biomarkers related with xenobiotics biotransformation (EROD and GST), oxidative stress (GPx, GR and LPO) and genotoxicity (DNA damage) were measured in digestive gland tissues. HHCB and AHTN significantly ($p < 0.05$) induced EROD and GST enzymatic activities at environmental concentrations. Both fragrances also induced GPx activity. All concentrations of both compounds induced an increase of LPO and DNA damage on day 21. Although these substances have been reported as not acutely toxic, this study shows that they might induce oxidative stress and genotoxicity in marine organisms.

1. Introduction

The polycyclic musk compounds (PMCs) 1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethyl-cyclopenta-(g)-2-benzopyran (HHCB) branded Galaxolide® and 7-acetyl-1,1,3,4,4,6-hexamethyltetralin (AHTN) branded Tonalide® are bi-cyclic aromatic compounds. They are comprised of acetylated and highly methylated tetralin or indane skeletons (Sumner et al., 2010; Ricking et al., 2003) and are lipophilic with high octanol-water partition coefficients (Kow) of 5.9 and 5.7 for HHCB and AHTN, respectively (Chen et al., 2012; Reiner and Kannan, 2011; Sumner et al., 2010). These substances are applied in a wide range of personal care products such as cosmetics, lotions, deodorants, detergents and more (Reiner and Kannan, 2011) because of their musky scent and fixative properties that enable them to bind to fabrics (Ramskov et al., 2009; Reiner and Kannan, 2011). According to a European Commission risk assessment report, the production of HHCB and AHTN in Europe in the year 2000 was between 1000 and 5000 ton/year (Eschke, 2004; 2008b) and accounted for approximately 95% of fragrance material in the perfumery industry (Balk and Ford, 1999; Pedersen et al., 2009). Reports have shown that, since 1990, all fragrances consumed in the United States have doubled (Roosens et al., 2007) and increased by 25% between 1996 and 2000 (Peck et al., 2006). Because of their high production they have been placed on the "High Production Volume List" by the United States Environmental Protection Agency (US EPA) (Peck et al., 2006).

Although HHCB and AHTN were first detected in aquatic environments (Eschke, 2004), recent studies have reported their presence in sediment and air (Fromme et al., 2001; Peck et al., 2006; Peck and Hornbuckle, 2006). In effluents from sewage treatment plants (STPs), concentrations of HHCB and AHTN have reached levels ranging from 1800 to 9000 ng L^{-1} (Bueno et al., 2012; Díaz-Garduño et al., 2017). Those concentrations were similar to previous studies that reported environmental concentrations between 509 and 2337 ng L^{-1} for AHTN and between 4750 and 13399 ng L^{-1} for HHCB (Chen et al., 2012; Lee et al., 2010; Pintado-Herrera et al., 2014). Research has shown that approximately 50%–90% of the total synthetic musks are eliminated,

* Corresponding author.
E-mail address: friday.ehiguese@alum.uca.es (F.O. Ehiguese).

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especially if tertiary treatments are employed, while the rest enters the receiving rivers and coastal waters (Heberer, 2002; Ricking et al., 2003; Lee et al., 2010). Since legislation requirements for effluents from Wastewater Treatment Plants (WWTPs) do not include tertiary depuration treatments [such as the multi-barrier treatment that can remove chemicals at 97%, and completely eliminate fragrances from wastewaters (Díaz-Garduño et al., 2017)] as mandatory, concentrations that could have been trapped during tertiary depuration escape into aquatic ecosystems.

To the best of our knowledge, only few studies have assessed PMCs' presence in the marine environment (Balk and Ford, 1999; Sumner et al., 2010). However, reports have demonstrated that they are found to bioaccumulate in marine organisms (Kannan et al., 2005; Moon et al., 2011, 2012). HHCB and AHTN have been measured in the tissues of mussels, crustaceans, fish, marine birds and mammals (Kannan et al., 2005; Moon et al., 2011, 2012; Nakata, 2005) coupled with bioaccumulation factors of toxic concern (Ricking et al., 2003). The bioaccumulation factor values based on lipid weight calculated for HHCB in zebra mussels at the Upper Hudson River (USA) was between 2610 and 4890 (Reiner and Kannan, 2011), and in Germany, bioaccumulation factor based on lipid weight calculated for AHTN was between 5100 and 40100 (Gooding et al., 2006). Additionally, bioconcentration factor measured in *Lepomis machrochirus* for HHCB and AHTN in a laboratory study were 1584 and 597, respectively (Balk and Ford, 1999)Yet little is known about PMCs' toxicity in aquatic ecosystems and some of the studies have focused on freshwater ecosystems, while others used concentrations higher than those considered environmentally relevant (Wollenberger et al., 2003; Breitholtz et al., 2003; Chen et al., 2012; Chen et al., 2011; Gooding et al., 2006; Parolini et al., 2015; Pedersen et al., 2009). Environmental concentrations of HHCB and AHTN have been shown to experimentally elicit avoidance behaviour in the estuarine shrimps *Palaemon varians* when exposed to a gradient of both compounds in a free-choice multi-compartmented non-forced exposure system (Ehiguese et al., 2019). However, information about sub-lethal effects to marine organisms resulting from chronic exposure is incipient.

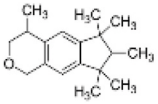
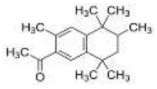
The aim of this study was to determine the potential risk of HHCB and AHTN to the clam *Ruditapes philippinarum* after chronic exposure to environmentally relevant concentrations. The risk to this species was based on a battery of biomarkers of exposure and effect (oxidative stress and genotoxicity), such as ethoxyresorufin-O-deethylase (EROD), glutathione-S-transferase (GST), glutathione peroxidase (GPx), glutathione reductase (GR), lipid peroxidation (LPO) and DNA damage. *R. philippanarum* is an important test organism in ecotoxicology because it is readily available, inexpensive and its biology is well understood. These euryhaline infauna suspension feeders have been used in studies related to bioaccumulation of contaminants (Moschino et al., 2012; Santana et al., 2017), metal toxicity (Aouini et al., 2018; Ji et al., 2019; Liu et al., 2011b), Ocean acidification (De Marchi et al., 2017; Velez et al., 2016; Xu et al., 2016), wastewater effluents as contaminants mixture (Díaz-Garduño et al., 2017; Maranho et al., 2015) and pharmaceutical and personal care products (Aguirre-martin et al., 2013; Aguirre-martínez et al., 2016, 2015; Almeida et al., 2015; Trombini et al., 2019).

2. Materials and methods

2.1. Selection and concentrations of the polycyclic musks

HHCB and AHTN were purchased from Sigma Aldrich, Spain. The characteristics of these substances are presented in Table 1. The concentrations (0.005, 0.05, 0.5, 5.0 and 50.0 μg/L) of HHCB and AHTN used in the experiments were carefully selected based on concentrations measured (maximum of 9 μg/L) in different environmental matrices (Bueno et al., 2012; Díaz-Garduño et al., 2017; Sumner et al., 2010). Initially, stock solutions were prepared and stored in dark bottles at 4 °C. Each substance was dissolved in dimethyl sulfoxide (DMSO) in glass vial.

Table 1
Characteristics of HHCB and AHTN.

Characteristics	Galaxolide® (HHCB)	Tonalide® (AHTN)
Structural Formula		
Chemical Name	1,3,4,6,7,8-hexahydro-4,6,6,8,8-hexamethylcyclopenta-γ-2-benzopyran	6-Acetyl-1,1,2,4,4,7-hexamethyltetralin
CAS No	1222-05-5	21145-77-7
Log K_{ow}	5.9[a]	5.7[a]
Water Solubility	1.75 mg/l	1.25 mg/l

[a] Balk and Ford (1999).

2.2. Organisms: acclimation and maintenance conditions

A total of 360 specimens of *R. philippinarum* (average size: 42 ± 0.9 mm; n = 120) were purchased from an aquaculture farm in the Bay of Cádiz, (Ctaqua, Cadiz, Spain). Organisms were immediately transported to the laboratory for acclimation and kept in a 300 L aquarium for seven days. The aquarium was supplied with constant aeration and the specimens were fed *ad libitum* once every day with the microalgae *Isochrysis galbana*. The physical and chemical parameters (photoperiod of 12 h light/12 h dark; temperature: 15 ± 1 °C; salinity: 34.6 ± 0.3; pH: 7.8–8.2; dissolved oxygen: >5 mg/L) in the aquarium were monitored daily.

2.3. Experimental approach

The bioassay was conducted in duplicates using a 10 L rectangular glass aquarium including sea water control and solvent control (DMSO, v/v 0.001%) to prevent any effect from the solvent (Aguirre-martínez et al., 2016). Sixteen individuals were exposed in each replicate (32 individuals per treatment). Exposure to compounds lasted 21 days and was performed in a semi-static regimen with total renewal of each test substance every 3 days (days 3, 6, 9, 12, 15 and 18). The glass aquariums were filled with 8 L of sea water spiked with the stock solution. Physiochemical parameters were similar to the acclimation conditions reported above. The specimens were sampled on days 3, 7, 14 and 21 (n = 6).

2.4. Water samples collection and analyses

Water samples for each treatment were collected on days 0 and 3 using amber bottles and immediately stored at −20 °C before determination of the concentrations of HHCB and AHTN. The methodology described by Pintado-Herrera et al. (2014) based on the SBSE-LD (stir bar sorptive extraction coupled to liquid desorption) by using polydimethylsiloxane bars (PDMS, 10 mm × 0.5 mm) was employed. The compounds were separated, identified and quantified using gas chromatography (SCION 456-GC, Bruker) coupled to a tandem mass spectrometry (SCION TQ from Bruker with CP 8400 Autosampler). Details of the detection and extraction efficiency of the methodology can be found in Pintado-Herrera et al. (2016) and (2014). The concentrations of HHCB measured in the exposure water ranged from 54% to 80% of the nominal concentration on day 0 and from 21% to 23% on day 3 just before renewal in the semi-static experiment. Regarding AHTN, concentrations on day 0 ranged between 73% and 97% of the nominal concentrations and from 23% to 29% on day 3, just before renewal. The differential between nominal and measured concentrations is consistent with other reported studies due to instability of HHCB and AHTN.

2.5. Biological samples preparation

Three individuals (n = 6 per treatment) were sampled from each replicate after 3, 7, 14 and 21 days of exposure. Organisms were dissected on ice and digestive gland tissues were extracted. Homogenization buffer was prepared with 100 mM NaCl, 25 mM HEPES salt, 0.1 mM EDTA and 0.1 mM DTT. Digestive gland tissue (n = 6) was homogenized following the procedure described by Lafontaine et al. (2000) and centrifuged at 15.000 g for 20 min at 4 °C to obtain the supernatant fraction S_{15} and at 3000 g for 20 min at 4 °C to obtain the supernatant fraction S_3. Supernatant fractions were carefully extracted and stored at −80 °C. The total protein concentration (TP) (expressed as mg TP) was determined in the homogenized fraction and in S_{15} and S_3 fractions following an adaptation of the methodology by Bradford (1976). All biomarkers were measured using a kinetic microplate reader, Infinite® M200.

2.6. Biomarker analyses

2.6.1. Ethoxyresorufin O-deethylase (EROD) activity

EROD activity was measured using the method adapted from rainbow trout fingerling (Gagnè and Blaise, 1993). 50 µl of S_{15} samples (25 µl sample + 25 µl of MilliQ) were added in 96-well flat bottom dark microplates. 160 µl of 7-ethoxyresorufin and 10 µl of reduced NADPH were added with 100 mM K_2HPO4 buffer at pH 7.4. The reaction was activated using NADPH and allowed to proceed for 60 min at 30 °C. 7-hydroxyresorufin was determined fluorometrically using 516 nm excitation and 600 nm emission filters and readings were taken at 15 min intervals. The calibration curve was developed using concentrations of resorufin and results were normalized to their corresponding total protein expressed as pmol/min/mgTP.

2.6.2. Glutathione-S-transferase activity

Determination of GST activity was adopted from Balk and Ford (1999). In a transparent 96-well flat bottom microplate, 15 µl S_{15} samples were added to 200 µl reaction buffer of 10 mM HEPES salt, 125 mM NaCl and 1 mM glutathione reduce (GSH) normalized at pH 6.5. Subsequently, 15 µl of homogenization buffer was added to 2 wells and marked up with 200 µl reaction buffer to check the background reaction rate. Absorbance was measured at 340 nm, every 5 min for 30 min based on the appearance of the glutathione conjugate at 22 °C. Results were expressed as microgram per minute per milligram total protein (µg/min/mgTP).

2.6.3. Glutathione peroxidase activity

Glutathione peroxidase activity was measured using the procedure adapted from Mcfarland et al. (1999). 20 µl S_{15} samples (10 µl + 10 µl homogenization buffer) were added to a transparent 96-well flat bottom microplate. 200 µl daily assay mixture prepared with GPx assay buffer (50 mM potassium phosphate, 0.1 mM EDTA and 0.15 mM sodium azide) and substrates (1 mM cumene hydrogen peroxide in 50 ml GPx assay buffer incubated at 30 °C) was added to the microplate and incubated for 2 min at 30 °C. A volume of 50 µl of substrate was then added to each well. The oxidation of NADPH to NADP was observed. Readings were taken at 340 nm for 3 min, at 10 s intervals. 20 µl of homogenization buffer were added in 2 wells as a background corrected value. Results were expressed as nmol/min/mgTP.

2.6.4. Glutathione reductase (GR) activity

The method adapted by Martín-Díaz et al. (2007) was used to measure the activity of GR. In a transparent 96-well flat bottom microplate, 20 µl S_{15} sample (10 µl sample + 10 µl MilliQ) and 200 µl of incubated daily assay mixture (200 mM NaH_2PO4 and Na_2HPO_4 at pH 7.6, 10 mM oxidized glutathione, 1 mM NADPH) were added. A volume of 20 µl homogenization buffer was added to 2 wells as blank to check the background reaction rate. The reaction was measured spectrophotometrically using 340 nm emission at 30 °C. The loss of NADPH was recorded at 2 min interval for 10 min. Results were expressed as pmol/min/mgTP.

2.6.5. Lipid peroxidation (LPO)

The thiobarbituric acid reactive substance (TBARS) procedure was used for LPO measurement (Wollenberger et al., 2003). 150 µl of diluted homogenate samples (90 µl samples + 60 µl MilliQ) were injected in 1.5 ml tubes. A standard solution of tetramethoxypropane (TMP) was prepared with 0.001% TMP and diluted serially with distilled water (0–15 µM TMP). 300 µl of 10% trichloroacetic acid (TCA), 1 mM $FeSO_4$ and 150 µl of 0.67% thiobarbithuric acid (TBA) were added to the sample and standard solution separately. They were then mixed and incubated for 10 min at 70 °C in a J.P. Selecta® incubator. 200 µl of the precipitates (standard solution for the TMP standard curve and homogenate samples) were pipetted into a dark 96-well flat bottom microplate. Production of malondialdehyde (MDA), which is indicative of oxidative stress from the degradation of initial products of free radical attack on fatty acid (Josephy, 2010), was measured spectrophotometrically at 516 nm (excitation) and 600 nm (emission) filter. Optical density values were converted to µgTBARS/mgTP.

2.6.6. DNA damage

The DNA precipitation assay methodology is based on 2% SDS-KCl precipitation of DNA-protein crosslink, which uses fluorescence to quantify the DNA strands (Oxford, 2001; Gagné et al., 1995). When DNA breaks because of exposure to toxic chemicals, the strands are released from cellular protein into the supernatant when centrifuged at low speed (Oxford, 2001). It becomes possible to quantify the amount of double and single stranded DNA at the end of the assay (Gagné et al., 1995). A volume of 25 µl of homogenate was mixed by inversion with 200 µl of SDS 2% prepared with 10 mM EDTA, Tris-Base and 40 mM NaCl. 200 µl of 0.12 mM KCl was added and mixed by inversion. The mixture was incubated for 10 min at 60 °C, cooled at 4 °C for 30 min and centrifuged 8000×*g* at 4 °C for 5 min. For DNA calibration, Salmon Sperm genomic DNA was dissolved in 1 ml TEIX (Tris-HCl and EDTA at pH 8.0) as a standard. In a dark 96-well flat bottom microplate, 50 µl of the supernatant was added to 150 µl of Hoescht dye 0.1 µg/mL diluted with sodium cholate containing 0.4 M NaCl, 4 mM sodium cholate and 0.1 M Tris-Acetate (pH 8.5). Fluorescence was measured at 360 nm (excitation) and 450 nm (emission) filters against blanks containing similar constituents without homogenate. Optical density values were converted to µg DNA/mgTP.

2.7. Statistical analysis

Data for biomarker responses were analyzed using the SPSS®/PC + statistical package. Prior to parametric tests, the normality and homogeneity of the data were analyzed. Significant differences between controls and organisms exposed to polycyclic musk compound treatments were determined using one-way ANOVAs followed by Dunnett's comparison tests and significance levels were set at $p < 0.05$. In order to evaluate the relationship between biomarker responses and musk concentrations over time, Spearman's rank order of correlation tests were run. Significance levels were set at $p < 0.05$ and $p < 0.01$ to obtain pairwise correlations.

3. Results

3.1. Biomarker responses

During the 21 day exposure, there was no significant mortality (3%) and no significant difference in biomarker responses ($p < 0.05$) analyzed in digestive gland tissues of clams exposed to the seawater control group and DMSO. The results of the biochemical biomarkers from digestive gland extracts are presented in Figs. 1 and 2 and correlations between

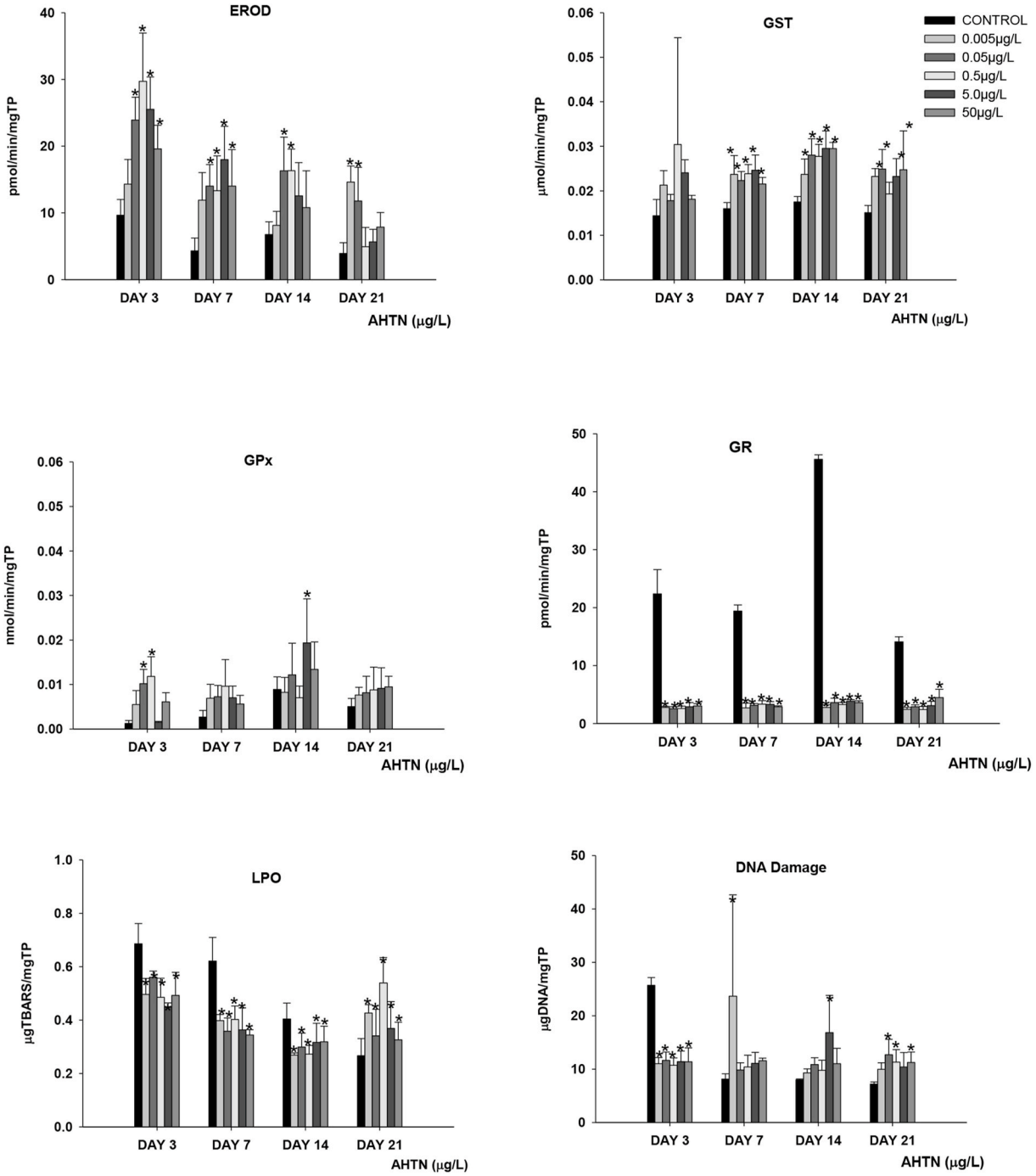


Fig. 1. Biochemical biomarkers including ethoxyresorufin O-deethylase (EROD), glutathione-S-transferase (GST), glutathione peroxidase (GPX), glutathione reductase (GR) activities, lipid peroxidation (LPO) level and DNA damage (strand breaks) measured in the digestive gland tissues of *R. philippinarum* exposed to tonalide (AHTN) for 21 days. Asterisks (*) shows significant differences with control treatment ($p < 0.05$).

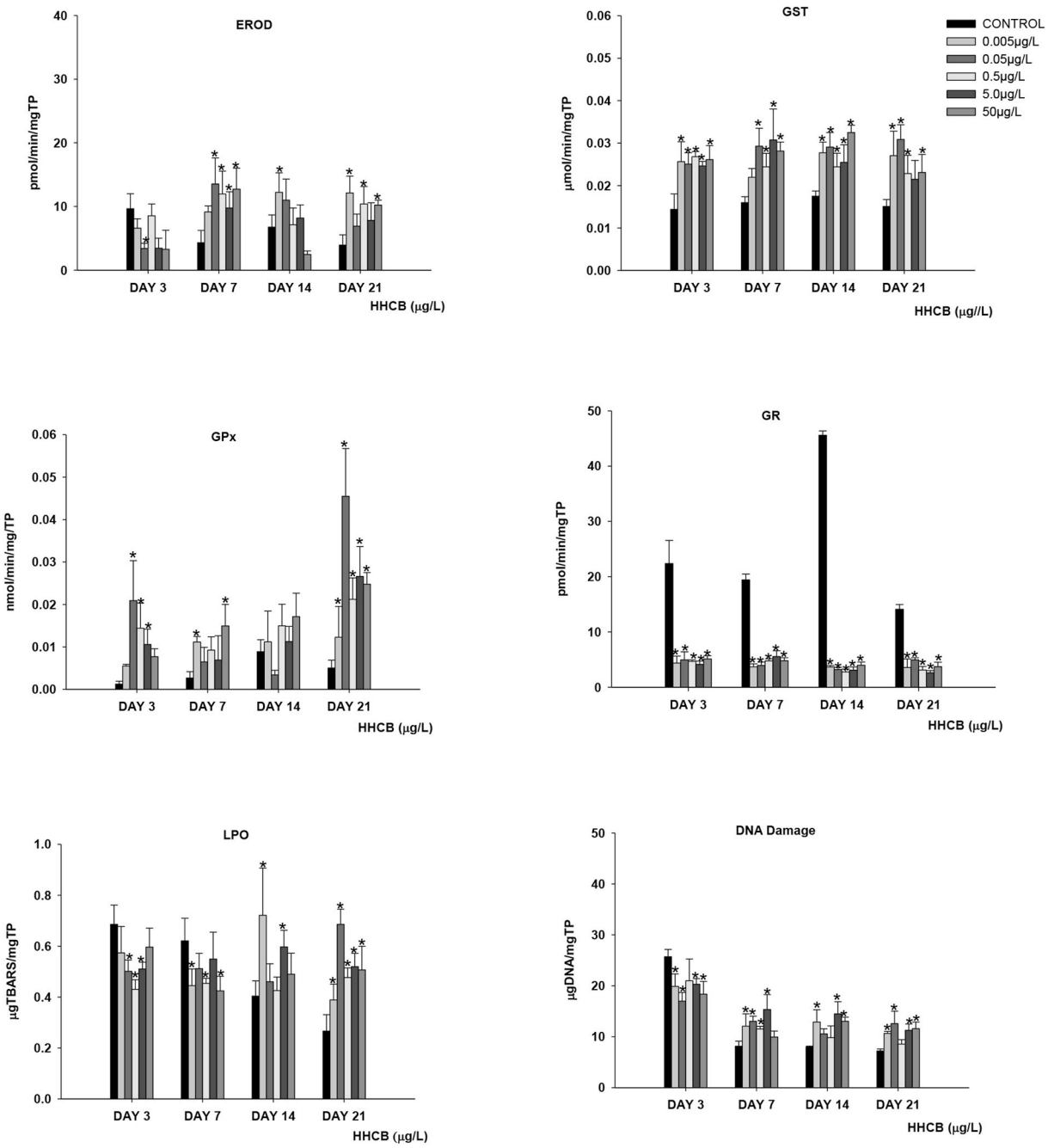


Fig. 2. Biochemical biomarkers including ethoxyresorufin *O*-deethylase (EROD), glutathione-*S*-transferase (GST), glutathione peroxidase (GPX), glutathione reductase (GR) activities, lipid peroxidation (LPO) level and DNA damage (strand breaks) measured in the digestive gland tissues of *R. philippinarum* exposed to galaxolide (HHCB) for 21 days. Asterisks (*) shows significant differences with control treatment ($p < 0.05$).

Table 2
Spearman's rank order of correlation (r_s) test between biomarkers measured in *R. philippinarum* digestive gland tissues after exposure to Galaxolide® (first value in bold) and Tonalide® (second value) concentrations (CON) (n = 3).

Biomarkers	CON	EROD	GST	GPx	GR	LPO	DNA Damage
EROD	0.111/0.333**	1					
GST	0.378**/0.272**	0.207*/0.083	1				
GPx	0.484**/0.273**	0.012/-0.013	0.196*/0.273**	1			
GR	−0.196*/-0.335**	−0.102/-0.362**	0.134/0.268**	−0.255**/-0.022	1		
LPO	−0.018/-0.393**	−0.049/-0.059	0.021/-0.252**	−0.080/-0.250*	0.177*/0.154	1	
DNA Damage	0.352**/0.310**	0.015/0.233**	0.243**/0.136	0.019/-0.088	0.193*/-0.054	0.407**/0.096	1

Asterisks indicate the p values: *$p < 0.05$ and **$p < 0.01$. Ethoxyresorufin O-deethylase (EROD), gluthathione-S-transferase (GST), gluthathione peroxidase (GPX), gluthathione reductase (GR), lipid peroxidation (LPO).

concentrations and biological responses can be found in Table 2.

3.1.1. Biomarkers of exposure

The biotransformation enzymes, which involve EROD enzymatic activity, showed significant induction compared with control in organisms exposed to AHTN and HHCB. Significantly higher values compared with control ($p < 0.05$) were observed on days 3 and 7 (0.05, 0.5, 5 and 50 μg/L) and day 14 (0.05 and 0.5 μg/L) for AHTN (Fig. 1). Regarding HHCB, significantly higher values ($p < 0.05$) were found on day 7 (0.05, 0.5, 5 and 50 μg/L), day 14 (0.005 μg/L) and day 21 (0.005, 0.5, 5 and 50 μg/L) (Fig. 2).

3.1.2. Biomarkers of effect

Induction of GST ($p < 0.05$) was observed in clams exposed to HHCB and AHTN. This induction was significant ($p < 0.05$) on days 7, 14 (0.005, 0.05, 0.5, 5 and 50 μg/L) and 21 (0.005, 0.05, 5 and 50 μg/L) in organisms exposed to AHTN (Fig. 1) and from day 3–21 when clams were exposed to HHCB (Fig. 2). When describing GPx activities, it was observed that significant induction ($p < 0.05$) was found on day 3 for HHCB (0.05, 0.5 and 5 μg/L) (Fig. 2) and AHTN (0.05 and 0.5 μg/L) (Fig. 1). This induction was not concentration-dependent. Significant antioxidant induction was also observed on the last day of exposure to HHCB compared with control ($p < 0.05$) and was positively correlated with the concentrations 0.05, 0.5, 5 and 50 μg/L (Fig. 2). Surprisingly, GR enzymatic activity decreased significantly compared with control ($p < 0.05$) in all treatments for both substances (Figs. 1 and 2). These responses of the antioxidant enzymes showing oxidative stress corresponded with significant ($p < 0.05$) increasing lipid peroxidation in the digestive gland tissues of clams exposed to these polycyclic musk compounds after 21 days of exposure. Moreover, the increased LPO level was positively correlated on day 21 with concentration for HHCB. Significant increase in DNA strand break ($p < 0.05$) was observed on days 7, 14 and 21 (0.005, 0.05, 0.5 5 and 50 μg/L) compared with control ($p < 0.05$) for HHCB (Fig. 2) and only on day 21 (0.05, 0.5, 5 and 50 μg/L) for AHTN (Fig. 1). This induction was positively correlated with the concentration on day 21 ($p < 0.05$).

3.2. Correlations

Taking all biomarker responses into consideration, organisms showed positive and significant ($p < 0.01$) concentration-dependent response correlation regarding detoxification (GST), oxidative stress (GPx) and genotoxicity (DNA damage), and significant ($p < 0.05$) and negative correlation with GR (Table 2) in clams exposed to HHCB. For AHTN, concentration correlated significantly ($p < 0.01$) with induction of EROD, GST, GPx, DNA damage and significant ($p < 0.01$) inhibition of GR (Table 2) in clams exposed to AHTN. Positive correlation between biomarkers of exposure, GST with EROD, GPx ($p < 0.05$), and biomarkers of effects, DNA damage ($p < 0.01$), LPO ($p < 0.05$) in clams exposed to HHCB was determined (Table 2). Induction of GST significantly ($p < 0.01$) correlated with GPx and GR and negatively correlated with LPO, and EROD activation was significantly ($p < 0.01$) correlated with inhibition of GR corresponding to significant ($p < 0.01$) increased DNA damage (Table 2) in clams exposed to AHTN.

4. Discussion

Environmental risk assessments of PMCs have been mainly focused on toxicity for freshwater environments, and information about chronic toxicity in marine environments remains limited. In the present study, a battery of biomarkers related with xenobiotic detoxification (EROD and GST), oxidative stress (LPO, GPx and GR) and genotoxicity (DNA damage) have been tested to provide information regarding the potential toxicity of the fragrances tonalide and galaxolide for the marine clam *R. philippinarum*.

We observed dose-dependent significant induction of EROD activity ($p < 0.05$) in *R. philippinarum* exposed to AHTN and HHCB (Figs. 1 and 2). The cytochrome P450 system has been described to be involved in the metabolism of detoxification of lipophilic compounds, while EROD enzymes catalyze the reactions of degradation of lipophilic compounds (van der Oost et al., 2003). EROD activity, measured in bioindicator species, has been established as a biomarker of exposure to lipophilic chemicals. The activity of P450 enzymes are key in phase I biotransformation of xenobiotics, specifically lipophilic compounds with aromatic backbone. EROD activity studied in aquatic organisms has shown high values after exposure to polycyclic aromatic hydrocarbons, polychlorinated biphenyls and some contaminants of emerging concern (Aguirre-martínez et al., 2016; Luna-Acosta et al., 2015; Maranho et al., 2015; Park et al., 2009; Siebert et al., 2017; Tao et al., 2013). Consequently, HHCB and AHTN may have the capacity to bind to the aryl hydrocarbon (Ah) receptor in CYP450 1A1, because xenobiotics that fail to bind with the Ah receptor showed no induction of EROD activity (Petrulis et al., 2000). The induction of EROD activity recorded in this study is a confirmation of the bioavailability of HHCB and AHTN to marine organisms and thus requires urgent attention to address the potential toxicity in marine ecosystems.

Aromatic xenobiotics are prooxidant chemicals, which increase intracellular generation of reactive oxygen species through the induction of cytochrome P450 pathway (Regoli and Giuliani, 2014) and antioxidant species, such as GST, GPx and GR; furthermore, they are bioindicators of contaminant-mediated oxidative stress that play significant roles in breaking down oxyradicals to less harmful products, consequently preventing oxidative damage (Wu et al., 2011). The significant increase in GST activity in the digestive gland of clams exposed to HHCB and AHTN suggests their capacity for inducing oxidative stress. GST is a phase II metabolic isoenzyme involved in the catalytic conjugation of reduced glutathione to xenobiotic substrates thus encouraging detoxification and preventing interactions with crucial cellular proteins and nucleic acids (Birben et al., 2012; Josephy, 2010). This detoxification process triggers GST induction and the increased GST activity observed in this study suggests that HHCB and AHTN possess electrophilic cores, with which glutathione conjugated (Hampel et al., 2016). The activation of GPx activity in a concentration-dependent pattern as observed in clams exposed to HHCB and AHTN on day 21 indicated an effort to ameliorate oxidative stress, thus protecting the organism from cell damage. GPx activity is an important antioxidant biomarker with involvement in antioxidant metabolism because, in conjunction with GST, it reduces lipid hydroperoxides to alcohol, with some concomitant oxidation of reduced glutathione to oxidized glutathione (Regoli and Giuliani, 2014). GR activity in the present study was significantly inhibited by both HHCB and AHTN (Figs. 1 and 2) and cells need to maintain high levels of GR to function together with other enzymes in defending cells against degenerative attacks (Srikanth et al., 2013). GR acts as a substrate to other glutathione enzyme species to prevent oxidative stress and a balance in the amount of each glutathione enzymes is precursor for optimal cell defence. Therefore, GR inhibition may alter detoxification and antioxidant capacity of GST and GPx (McCay et al., 1976; Oxford Biomedical Roosens et al., 2007; Stojiljković et al., 2007). However, glutathione metabolism enzymes' pattern of responses is not always straightforward and depends on a series of factors, including the species (Antunes et al., 2013). Antioxidant defences can be overwhelmed by some chemical compounds which can depress the antioxidant capacity to remove oxyradicals and prevent cell damage (Regoli and Giuliani, 2014). Recent studies have shown alterations in antioxidant enzymes in aquatic and terrestrial organisms exposed to HHCB and AHTN, indicating the potential for oxidative stress. The goldfish *Carassius auratus* exposed to simulated urban runoff containing HHCB alone and HHCB mixed with cadmium showed a significantly increased antioxidant enzyme activity after 14 days and decreased significantly after 21 days of exposure (Chen et al., 2012). *Eisenia fetida* exposed to HHCB and AHTN upregulated antioxidant defence at a low dose of 0.6 μg cm^{-2} and significantly decreased at a concentration of 6.0

μg cm^{-2} after 48 h of exposure (Chen et al., 2011).

The alterations in the antioxidant enzymes activities could be linked to the significant increase in LPO recorded in this study after 21 days for clams exposed to AHTN and HHCB (Figs. 1 and 2). Similarly, the zebra mussel, *D. polymorpha* exposed to 100 and 500 ng/L of HHCB and 20 and 80 ng/L of AHTN showed significant time-dependent lipid peroxidation after 21 days (Parolini et al., 2015). *E. fetida* exposure to HHCB and AHTN also induced a time-dependent significant increase in LPO due to oxidative stress (Chen et al., 2011; Liu et al., 2011a). LPO is a self-propagating chain reaction, and thus, it is believed that the foremost oxidation of only a few lipid molecules can cause serious tissue damage (Mcfarland et al., 1999).

Concentration-dependent significant DNA strand breaks in clams exposed to environmental concentrations of AHTN and HHCB were observed at the end of the exposure (Figs. 1 and 2 and Table 2). This is expected to occur as a result of significant oxidative stress, because direct reaction between DNA and free radical species can result in DNA damage including damaged bases, structural breaks and/or inter and intra strand crosslinks. It is possible that genetic damage was also as a result of the metabolites of the parent compounds, which in some cases could be more toxic to organisms than the parent compounds tested. Significant correlations between pollutant induced reactive oxygen species and DNA damage in marine invertebrates have been reported (Mamaca et al., 2005). Our result is supported by previous research, which documented significant time-dependent DNA fragmentation in the zebra mussel *D. polymorpha* after exposure to environmentally relevant concentrations (100 and 500 ng/L for HHCB) even after four days. The authors also demonstrated that 80 ng/L of AHTN induced significant time-dependent DNA damage with a value 3.6-fold higher than the control on day 21 (Parolini et al., 2015). These results are indicative of chronic effects of HHCB and AHTN in freshwater and marine ecosystems.

In addition, EROD correlated with GST ($p < 0.05$) and GST positively correlated with GPx ($p < 0.05$) and DNA damage ($p < 0.01$) in clams exposed to HHCB (Table 2). GST positively correlated with GPx and GR ($p < 0.01$) (Table 2). Taking these relationships into account, it is clear that the exposure of clams to these substances exerted significant activation of biomarkers of exposure and effect as observed in the significant correlation of HHCB and AHTN concentrations with DNA damage ($p < 0.01$) assessed in the digestive gland of clams (Table 2) which could be consequential to unsuccessful detoxification of contaminant-generated oxyradicals.

The biomarkers evaluated in *R. philippinarum* to understand the environmental risk of HHCB and AHTN have been found useful and sensitive to the exposure to both substances in the marine environment. Although the studies with HHCB and AHTN are incipient, our findings indicating a potential chronic risk of both compounds are corroborated by previous studies that examined oxidative damage by quantifying the malondialdehyde level for LPO as a biomarker in the earthworm *E. fetida* (Chen et al., 2011) and *Dreissena polymorpha* (Parolini et al., 2015) for terrestrial and aquatic environments, respectively. Additionally, it has also been shown under laboratory conditions that environmental concentrations of HHCB and AHTN might elicit avoidance behaviour in the estuarine shrimps *P. varians*, probably due to their organoleptic characteristics (Ehiguese et al., 2019). Because of the volatile nature of these contaminants under laboratory exposure, the reported biological effects may be underestimated (Tumová et al., 2019). Taking into account the different types of chronic effect that HHCB and AHTN can potentially produce, either in terrestrial (Liu et al., 2011a), freshwater (Parolini et al., 2015) or marine [Ehiguese et al. (2019) and the current study] organisms, it is crucial to review the regulatory status, when the environmental concentrations just indicate a slight risk before irreversible environmental effects are exerted.

5. Conclusions

The current research revealed that environmental concentrations of HHCB and AHTN might have adverse effects (oxidative stress and genotoxicity) on the marine clam *R. philippinarum*. Results from this study showed that, in particular, the activities of EROD, GST, GPX, GR, LPO levels and DNA damage were useful essential biomarkers to evaluate effects of PMCs at environmental concentrations in the marine environment because changes related with the concentrations of AHTN and HHCB were detected.

Declaration of competing interest

The authors declare that there is no conflict of interest for the publication of this present work and are willing to provide any clarification whenever called upon to do so.

CRediT authorship contribution statement

Friday O. Ehiguese: Conceptualization, Investigation, Methodology, Formal analysis, Data curation, Visualization, Validation, Project administration, Writing - review & editing. **Md Rushna Alam:** Methodology, Formal analysis, Data curation, Writing - original draft, Writing - review & editing. **Marina G. Pintado-Herrera:** Methodology, Formal analysis, Data curation, Writing - original draft, Writing - review & editing. **Cristiano V.M. Araújo:** Conceptualization, Methodology, Formal analysis, Validation, Data curation, Supervision, Funding acquisition, Writing - review & editing. **M. Laura Martin-Diaz:** Conceptualization, Visualization, Validation, Methodology, Formal analysis, Data curation, Supervision, Funding acquisition, Project administration, Writing - review & editing.

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Galaxolide and tonalide modulate neuroendocrine activity in marine species from two taxonomic groups

Friday Ojie Ehiguese [a,b,*], Maria L. Rodgers [b], Cristiano V.M. Araújo [c], Robert J. Griffitt [b], M. Laura Martin-Diaz [a]

[a] Physical Chemistry Department, University Institute of Marine Research (INMAR), International Campus of Excellence of the Sea (CEI.MAR), University of Cadiz, República Saharaui S/n, 11510, Puerto Real, Cádiz, Spain
[b] Division of Coastal Sciences, School of Ocean Science and Technology, University of Southern Mississippi, Ocean Springs, MS, 39564, USA
[c] Department of Ecology and Coastal Management, Institute of Marine Sciences of Andalusia (CSIC), 11510, Puerto Real, Cádiz, Spain

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ABSTRACT

Galaxolide (HHCB) and tonalide (AHTN) are polycyclic musk compounds (PMCs) used in household and personal care products that have been included on the list as emerging contaminants of environmental concern due to their ubiquity in aquatic and terrestrial environments. There still exists a dearth of information on the neurotoxicity and endocrine disrupting effects of these contaminants, especially for marine and estuarine species. Here, we assessed the neuroendocrine effects of HHCB and AHTN using adult clams, *Ruditapes philippinarum*, and yolk-sac larvae of sheepshead minnow, *Cyprinodon variegatus*. The clams were treated with concentrations (0.005–50 μg/L) of each compound for 21 days. Meanwhile, sheepshead minnow larvae were exposed to 0.5, 5 and 50 μg/L of HHCB and AHTN for 3 days. Enzyme activities related to neurotoxicity (acetylcholinesterase - AChE), neuroendocrine function (cyclooxygenase - COX), and energy reserves (total lipids - TL) were assessed in *R. philippinarum*. Gene expression levels of *cyp19* and *vtg1* were measured in *C. variegatus* using qPCR. Our results indicated induction of AChE and COX in the clams exposed to HHCB while AHTN exposure significantly inhibited AChE and COX. Gene expression of *cyp19* and *vtg1* in yolk-sac *C. variegatus* larvae exposed to 50 μg/L AHTN was significantly downregulated versus the control. The results of this study demonstrate that HHCB and AHTN might pose neurotoxic and endocrine disrupting effects in coastal ecosystems.

1. Introduction

Galaxolide (HHCB) and tonalide (AHTN) are polycyclic musk compounds (PMCs) used in household and personal care products and are included on the list of emerging contaminants of environmental concern due to their ubiquity in aquatic and terrestrial environments (Schreurs et al., 2004; Zhang et al., 2013). HHCB and AHTN consist of aromatic structures consisting of acetylated and extremely methylated pyran and tetralin bases (Sumner et al., 2010). They possess high *n*-octanol – water partition coefficients (K*ow*) akin to most persistent organic pollutants and may persist in the marine environment. The presence and toxicity of PMCs in transitional and coastal ecosystems is yet to be fully understood. Most research regarding these compounds has been focused on freshwater ecosystems (Balk and Ford, 1999a, 1999b; Parolini et al., 2015; Yamauchi et al., 2008) with only a few reports available on the toxicity of PMCs in the marine environment (Breitholtz et al., 2003; Luckenbach et al., 2004; Wollenberger et al., 2003). Recently, Ehiguese et al. (2019) studied the avoidance behavior of the shrimp *Palaemon varians* and found that HHCB and AHTN potentially elicited avoidance behavior in this shrimp. Chronic exposure to environmentally relevant concentrations of these substances suggests that they may alter antioxidant enzyme activity and potentially trigger oxidative stress in Manila clams (Ehiguese et al., 2020). To the best of our knowledge, the neurotoxicity and endocrine disrupting effects of these contaminants in the marine environment are yet to be addressed.

About 30% of commercially available chemicals are estimated to possess neurotoxic and endocrine disrupting properties (Tilson et al., 1995). These chemicals can target neurotransmitter pathways and their components such as neurotransmitters, receptors, biosynthetic enzymes, catabolic enzymes, and transporters (Basu, 2015). Neuroendocrine

* Corresponding author. Physical Chemical Department, University Institute of Marine Research (INMAR), International Campus of Excellence of the Sea (CEI.MAR), University of Cadiz, República Saharaui S/n, 11510, Puerto Real, Cádiz, Spain.
E-mail address: friday.ehiguese@alum.uca.es (F.O. Ehiguese).

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compounds promote diverse physiological and behavioral effects that alter the capacity of organisms to reach their biotic potential, cope with stress and other environmental challenges, and survive (Waye and Trudeau, 2011). Signals from environmental contaminants can interfere with neurotransmission and disrupt endocrine functions in marine organisms because of their potential to mimic the natural hormone estrogen, and can bind to estrogen receptors and influence estrogen biosynthesis (Waye and Trudeau, 2011). Many persistent organic pollutants have been implicated as neuroendocrine disruptors in the marine environment causing adverse effects related to changes in thyroid morphometry and functions, suppression of ovarian follicle development, altered sex differentiation, and mortality (Berg et al., 2016; Porte et al., 2006; Schnitzler et al., 2008). Furthermore, environmental concentrations of some pharmaceutical products have been shown to inhibit monoamine oxidase activity, increase plasma cortisol levels, and reduce feeding in aquatic organisms (*L. A. Maranho et al., 2015*; Melnyk-Lamont et al., 2014). Importantly, HHCB and AHTN have been demonstrated to disrupt neuroendocrine activity in several *in vitro* studies(Li et al., 2013; Mori et al., 2007; Schreurs et al, 2002, 2004, 2005) and significantly alter gene expression levels in male medaka fish (Yamauchi et al., 2008).

The aim of this study was to investigate the neurotoxic and endocrine disrupting effects of HHCB and AHTN in the marine environment. We assessed biochemical activities in Manila clams (*Ruditapes philippinarum*) using biomarkers of neuroendocrine toxicity (AChE, COX) and energy reserves (total lipids; TL) in a 21 day exposure. We also assessed and gene expression levels of *cyp19* and *vtg1* in yolk-sac larvae of sheepshead minnow (*Cyprinodon variegatus*) after 3 days of exposure to HHCB or AHTN. The suitability of *R. philippinarum* for ecotoxicological studies has been previously stated by Ehiguese et al. (2020). Sheepshead minnow are a suitable marine model used in ecotoxicological studies because they are easy to breed under laboratory conditions and spawn continuously with relatively large demersal eggs (Cripe et al., 2009). They have been used in the assessment of endocrine disrupting chemicals in transitional and coastal waters (Bowman et al., 2000; Folmar et al., 2000; Hemmer et al., 2001) as well as to characterize alteration of immune pathways (Jones et al., 2017; Rodgers et al., 2020) and oxidative stress (Rodgers et al., 2018) after chemical exposures.

2. Materials and methods

2.1. Test chemicals

For the clam experiments, analytical grades of HHCB (85.0%) and AHTN (97.0%) were obtained from Sigma Aldrich Spain. The details of dissolution and preparations can be found in Ehiguese et al. (2020). In brief, stock solutions were prepared using DMSO (0.001%v/v) as the organic solvent to dissolve the test chemicals which were further diluted with distilled water to reach the concentrations needed (0.005, 0.05, 0.5, 5 and 50 μg/L). These concentrations were selected based on reported environmental concentrations measured in marine environments (Díaz-Garduño et al., 2017; Pintado-Herrera et al., 2013). For the fish experiments, 100 g of analytical grade AHTN (97%) was purchased from Sigma Aldrich, USA. 10 g of the product was dissolved in 0.001%v/v DMSO to form the stock solution. 25 g of HHCB dissolved in 50% diethyl phthalate containing 49% pure HHCB was purchased from TCI America, USA and the concentrations required were calculated based on the percentage of the active ingredient of HHCB in the solution. The stock solution was diluted using distilled water to create 0.5, 5.0 and 50.0 μg/L solutions for each compound.

2.2. Test organisms

The clams, *R. philippinarum* (550 specimens), were obtained from an aquaculture farm in the south-west of Spain and were transported to the laboratory of Marine Culture, Faculty of Marine and Environmental Science (University of Cadiz, Spain). The adult clams (average size of 43.2 ± 1.6 mm) were acclimated in a 250 L aquarium and were fed with *Isochrysis galbana* once per day. During acclimation, aeration was provided to improve the oxygen content (dissolved oxygen >5 mg/L) of the medium and other physiochemical parameters in the aquarium were monitored and controlled. The temperature, salinity, pH and photoperiod during the acclimation were 15 ± 1 °C, 34.7 ± 0.4‰, 7.8–8.2 and 12 h light/12 h dark, respectively.

Adult sheepshead minnows (*C. variegatus*) previously purchased and kept in artificial seawater (15‰), between the temperature range of 25–27 °C and photoperiod (12:12 light/dark) in 300 L static recirculating raceways at the Toxicology Building, Gulf Coast Research Laboratory, University of Southern Mississippi (Ocean Springs MS, USA) were used as brood stocks. Before breeding, the brood stocks were fed daily with *Artemia* nauplii and commercial flake food. Four Spawntex® Mats (15–20 cm; Pentair Aquatic Eco-Systems) were placed in the two holding raceways overnight for spawning, two per raceway, containing gravid females with a female:male at a ratio of 2:1. The fertilized eggs were collected by gently tapping the Spawntex® Mats into a clean laboratory dish. The embryos were gently rinsed and transferred into a hatching jar supplied with aeration to aid suspension of the fertilized eggs in the water column and they were incubated in an ISOTEMP 115 (Fisher Scientific) at 30 °C until hatching (Dangre et al., 2010; Griffitt et al., 2012). These yolk-sac larvae were then carefully collected in 100 ml beakers filled with artificial seawater (15‰) prior to exposure.

2.3. Biochemical effects: Experimental approach

R. philippinarum were exposed aqueously to either HHCB or AHTN in 10 L rectangular glass aquariums. The bioassay experiment was carried out in duplicate for all treatments including the controls (seawater and DMSO). Natural, filtered seawater was obtained from the Marine Culture laboratory of the University of Cadiz, and 8 L of the seawater was mixed with each test chemical. 16 clams were added to each aquarium, totaling 32 per treatment except for the seawater and solvent controls with 14 specimens each. The treatments were renewed every three days, during which the water was siphoned out of the holding tanks and carefully cleaned and refilled with seawater spiked with freshly prepared contaminant. Any dead clams were immediately removed and recorded. The physical and chemical properties were adjusted to the same conditions as reported above during the acclimation period. Three clams were randomly collected from each replicate on day 3, 7, 14 and 21, and tissues (digestive gland and gonads) were immediately harvested on ice and stored at −80 °C in the laboratory prior to homogenization.

Buffer was prepared for sample homogenization using 0.1 mM EDTA, 100 mM NaCl, 25 mM HEPES salt, and 0.1 mM DTT. The samples stored in the freezer were thawed on ice and the digestive glands and gonads of three clams from each aquarium were pooled together for homogenization. The pooled samples were homogenized, and a fraction of each homogenate (HF) was centrifuged to obtain supernatant portions at a speed of 15.000×*g* for 20 min at 4 °C (S_{15}) and 3.000×*g* for 20 min at 4 °C (S_3). The Bradford (1976) methodology was adapted to determine the corresponding total protein (TP) concentration with values expressed as mg/mL for different extracts (HF, S_3 and S_{15}). The biochemical analyses of the biomarkers were quantified using a kinetic microplate reader, Infinite® M200.

2.4. Collection and analysis of exposure water

Exposure water for each concentration was sampled using clean amber bottles on day 0 and 3 for analysis of initial and final concentrations and the samples were kept in −20 °C prior to the chemical analysis. Details of the methodology for the chemical analysis and the results of the detection and quantification can be found in Ehiguese et al. (2020).

2.5. Biochemical analyses

2.5.1. Acetyl Cholinesterase (AChE) activity

AChE activity was measured in the post-mitochondria fraction of the digestive glands according to the methodology described in Guilhermino et al. (1996). 20 μL of the centrifuged S_{15} fraction was added to 20 μl of 50 mM potassium phosphate buffer at a pH of 7.5 in 96 transparent, flat bottom wells. 130 μL of 5,5-Dithiobis (2-nitrobenzoic acid) (DTNB) solution was further added with 50 μL of acetylthiocholine iodide solution. The activity of AChE was determined by absorbance measured at 405 nm at every 40–52 s for 5–7 min. The data were expressed as the formation of thiols in pmol DTNB/min/mg TP.

2.5.2. Cyclooxygenase (COX) activity

COX activity was measured according to Gagné et al. (2015), following the oxidation of 2,7-dichlorofluoresceine in the presence of arachidonate (Fujimoto et al., 2002). Briefly, 96 dark, flat bottom well microplates were coated with 50 μL of the S15 sample. 200 μL of the assay buffer containing 50 μM arachidonic acid and 2 μM dichlorofluoresceine was added with 0.1 μg/mL horseradish peroxidase containing 50 mM Tris-HCl, pH 8.0 and 0.05% Tween 20. The reaction was incubated at 30 °C for 0, 5, 10, 15, 20, 25 and 30 min and the fluorescence was measured at 485 nm (excitation) and 520 nm (emission). The data were expressed as μmol fluorescein/min/mg TP.

2.5.3. Total lipids (TL)

TL were measured in gonad and digestive gland tissues following the phosphovanilin method by Frings et al. (1972). Dark microplates with 96 flat-bottom wells were coated with 10 μL of samples diluted with 10 μL of MilliQ. 30 μL of concentrated sulphuric acid and 150 μL of phosphovanilin prepared with vanillin and phosphoric acid in water were added and incubated for 10 min at 80 °C and cooled at 4 °C for 2 min. The absorbance was determined at 540 nm. A standard solution of Triton X-100 was used for calibration and the results were expressed as μg TL/mg TP.

2.6. Molecular effects: Experimental approach

All tests were performed in static renewal bioassays in triplicate; 200 mL of seawater spiked with each treatment (0.5, 5.0 and 50 μg/L) was transferred into laboratory dishes. Then, 10 yolk-sac larvae of *C. variegatus* were randomly selected and transferred into each dish including the controls (seawater and DMSO). They were incubated in a Precision Scientific Incubator (Thermo, MA, USA) at 30 °C and the exposure water in each dish was renewed every 24 h during the 3 days exposure. Upon termination of the experiment, the larvae were inserted into 1.5 mL tubes containing 500 μL RNALater® solution and stored at −80 °C prior to RNA extraction.

2.7. Molecular analysis: Quantitative PCR (qPCR)

For RNA extraction, six larvae per replicate were pooled from each treatment and both controls (seawater and DMSO) for homogenization. Total RNA was extracted using RNeasy kits (Qiagen, Hilden, Germany) as described in the manufacturer's protocol, and the total RNA quantity and quality was assessed spectrophotometrically using a NanoDrop™ 2000 (Thermo Scientific, Wilmington, DE, USA). Reverse transcription of total RNA to single stranded cDNA was performed using RevertAid First Strand cDNA Synthesis Kit following the manufacturer's instructions (Thermo Scientific, Wilmington, DE, USA).

Real-time qPCR was performed using the primers listed in Table 1. 18s was used as an endogenous control, while *cyp19* and *vtg1* were used as biomarkers of endocrine disruption. All qPCR reactions were performed in triplicates using an Applied Biosystems 7500 Fast Cycler with Fast SYBR Green Master Mix (Life Technologies, Carlsbad, CA). Relative quantification values compared to the control samples were determined by applying the ΔΔCT method. Fold changes in *cyp19* and *vtg1* genes were log transformed to normalize the data.

Table 1
Details of forward and reverse primers.

Gene	Primers	Amp. Length	Ref
18s	F: GCTGAACGCCACTTGTCC R: ATTCCGATAACGAACGAGACTC	100	Simning et al. (2019)
cyp19	F: CTGTCCCCTGCAATCCCAAT R: AAAGGGGACCCAAACCCAAG	72	This study
vtg1	F: ATGTCACTGTGAAGGTCAACGAA R: ACCTGTTGGGTGGCGGTAA	68	Knoebl et al., 2004

2.8. Statistical analysis

All data were analyzed using SPSS (16.0) statistical package. Data normality and homogeneity were assessed before statistical tests were performed. One-way analysis of variance (ANOVA) coupled with Dunnett's multiple comparison tests were performed and significant differences between controls and clams treated with HHCB and AHTN were determined at $p < 0.05$. The relationships between effects and concentrations were checked using Spearman's rank order of correlation, and significant points were set at $p < 0.05$ and $p < 0.01$. For gene expression levels, significant differences were determined using a least-square difference test and significant difference was set at $p < 0.05$.

3. Results

3.1. Biochemical effects

No mortality was recorded in control clams, meanwhile 3% mortality was recorded in clams treated with HHCB and ~4% mortality in the clams treated with AHTN during the experiment.

The effects of HHCB and AHTN on AChE activity are presented in Fig. 1. There was significant inhibition ($p < 0.05$) of AChE activity in the clams treated with HHCB at 5.0 μg/L and 50 μg/L after 3 days (Fig. 1a). As the exposure continued, significant differences in AChE activity were further seen in the clams treated with 0.005 and 5 μg/L HHCB on days 14 and 21, respectively (Fig. 1a). Prolonged significant inhibition of AChE ($p < 0.05$) was triggered by all concentrations of AHTN tested until day 7, but only 0.005, 0.05 and 5.0 μg/L concentrations produced significant AChE inhibition by day 14 (Fig. 1b). Interestingly, at the end of the experiment (day 21), the 50 μg/L AHTN exposure showed a significant increase in AChE activity. (Fig. 1b).

The impact of HHCB and AHTN on COX activity measured in *R. philippinarum* after 21 days exposure in a semi-static bioassay is presented in Fig. 2. COX activity was induced in the clams treated with HHCB and this induction was significant in the 0.005 and 50 μg/L exposures on day 3. Continuous exposure to HHCB up to day 14 produced significant effects on COX activity at the highest concentration (50 μg/L) tested, but at day 21 only the 0.05 μg/L concentration significantly increased ($p < 0.05$) COX activity (Fig. 2a). On the other hand, AHTN inhibited COX activity in *R. philippinarum* and the inhibition was significantly different from the control group ($p < 0.05$) on days 7 and 14 in all the concentrations tested except for 0.005 μg/L on day 7 (Fig. 2b).

The energy reserves, measured as total lipids, were determined in the digestive gland (DTL) and gonad (GTL) tissues of clams exposed to HHCB and AHTN. There was no significant difference in DTL in the clams exposed to HHCB (Fig. 3a). For the clams exposed to AHTN, DTL increased significantly ($p < 0.05$) throughout the exposure period with the exception of the 0.05 μg/L treatment group on days 3, 7, and 14, and the 0.5 μg/L treatment on days 14 and 21 (Fig. 3b). There was no clear pattern in GTL of the clams exposed to HHCB, as we observed both

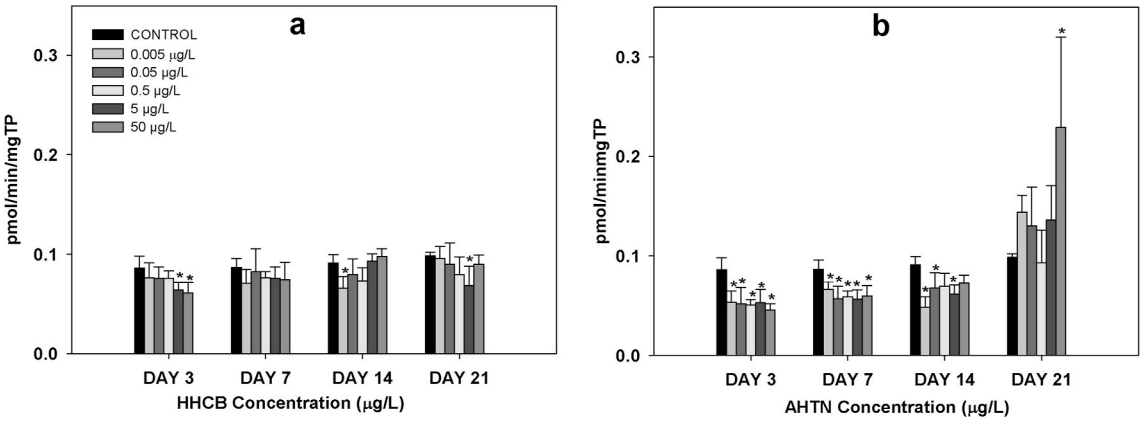

Fig. 1. Acetyl Cholinesterase (AChE) activity measured in the digestive gland tissues of *R. philippinarum* exposed for 21 days to (**a**) galaxolide (HHCB) and (**b**) tonalide (AHTN). Asterisks (*) show significant differences from control ($p<0.05$).

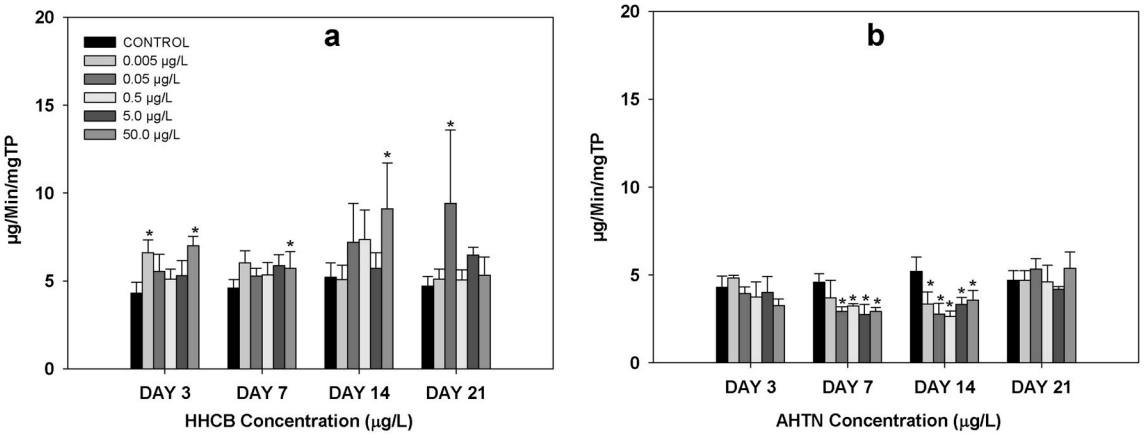

Fig. 2. Cyclooxygenase (COX) activity measured in the digestive gland tissues of *R. philippinarum* exposed for 21 days to (**a**) galaxolide (HHCB) and (**b**) tonalide (AHTN). Asterisks (*) show significant differences from control ($p<0.05$).

significant increases and decreases in GTL depending on the treatment and time point (Fig. 3c). However, GTL decreased significantly ($p < 0.05$) after exposure to AHTN in all treatment groups except at the lowest concentration (0.005 µg/L) measured on day 7 (Fig. 3d).

The activities of neuroendocrine biomarkers (AChE and COX) measured in the clams correlated significantly with the concentrations of HHCB ($p < 0.01$) over time (Table S1 – Supplementary Materials). For AHTN, a significant time and concentration-dependent correlation of AChE and COX was observed. In addition, the inhibition of AChE and COX activities correlated significantly ($p < 0.01$) (Table S2 – Supplementary Materials). Finally, gonad energy reserves (GTL) were significantly depleted over time (Table S2 – Supplementary Materials).

3.2. Molecular effects

There was no mortality in the yolk sac larvae of *C. variegatus* exposed to HHCB; for AHTN exposures, 3% mortality was recorded in the fish exposed to the 50 µg/L treatment.

The expression of *cyp19* in yolk sac larvae of *C. variegatus* exposed to HHCB was slightly upregulated, though not significantly in any of the treatments (Fig. 4a). However, concentration-dependent downregulation of *cyp19* was observed in the larvae exposed to AHTN and was significantly different ($p < 0.05$) at the highest concentration (50 µg/L) with more than a 3-fold change in expression compared to the controls (Fig. 4b).

Expression of *vtg1* measured in the yolk-sac larvae of *C. variegatus* exposed to both substances had a similar pattern to *cyp19* expression (Fig. 5). HHCB slightly induced the expression of *vtg1* and the induction was highest at 5.0 µg/L, though not significant (Fig. 5a). For AHTN, a concentration-dependent downregulation of *vtg1* was observed and decreased significantly ($p < 0.05$) by 3.40-fold versus the controls at the 50 µg/L exposure concentration (Fig. 5b).

4. Discussion

The current study assessed the neuroendocrine effects of environmental concentrations of HHCB and AHTN in the marine environment by assessing enzyme activities and gene expression levels in marine organisms from two taxonomic groups. Biomarkers of endocrine disruption (*cyp19* and *vtg1*) were measured in *C. variegatus* and neurotoxicity (AChE) was measured in *R. philippinarum* together with assessments of neuroendocrine and inflammation responses (COX) and energy reserves (TL).

4.1. Biochemical effects

Significant concentration-dependent AChE inhibition was observed in the clams treated with environmentally comparable concentrations of HHCB and AHTN, at the first time point (3 days of exposure - Fig. 1). AChE is a well-established biomarker in toxicological studies of neurotoxicity and is the enzyme responsible for the deactivation of acetylcholine at the cholinergic synapses, preventing a build-up of acetylcholine, which is necessary for the normal functioning of sensory and neuromuscular systems (van der Oost et al., 2003; Sturm et al.,

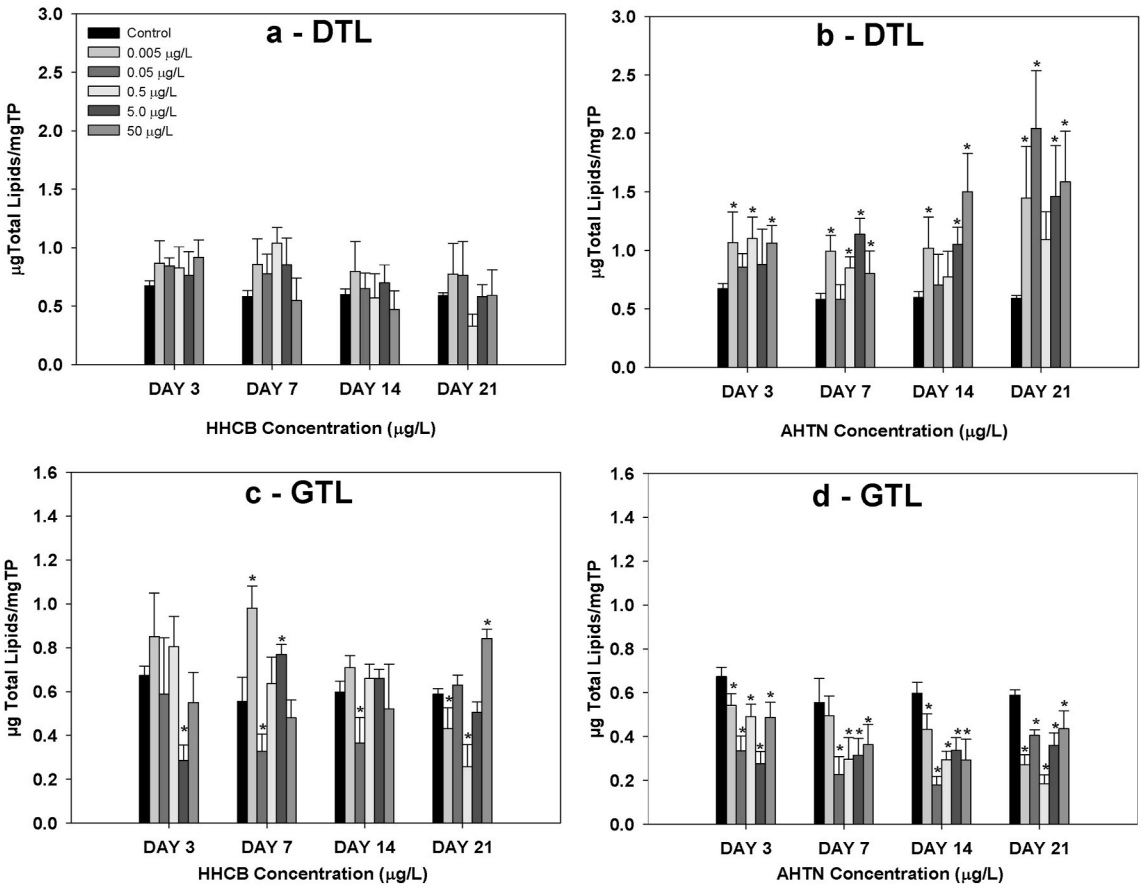

Fig. 3. Energy reserves measured as total lipids in digestive gland (**a** & **b - DTL**) and gonad (**c** & **d - GTL**) tissues of *R. philippinarum* exposed for 21 days to galaxolide (HHCB) and tonalide (AHTN). Asterisks (*) show significant differences from control ($p<0.05$).

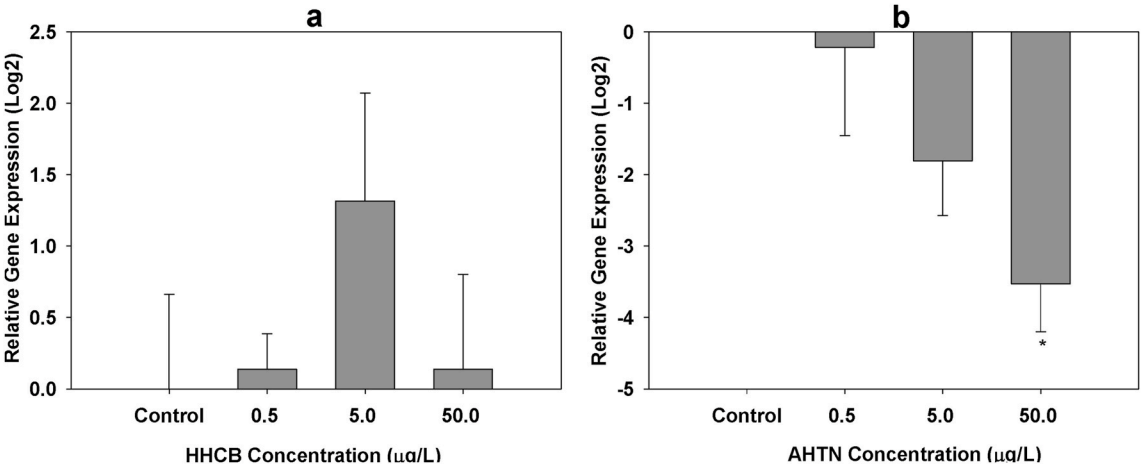

Fig. 4. Relative gene expression s for *cyp19* in *C. variegatus* yolk sac larvae exposed to (**a**) galaxolide (HHCB) and (**b**) tonalide (AHTN) for 96 h. Asterisks (*) show significant differences from control ($p<0.05$).

2000). AChE is also a target of many organic pollutants, toxic metals, human pharmaceuticals and personal care products, which have all been reported to inhibit AChE activity (Aguirre-Martínez et al., 2016; L A Maranho et al., 2015; Matozzo et al., 2005; Stefano et al., 2008). Our observations showed that these substances might possess the ability to bind with cholinesterase, preventing the breakdown of acetylcholine. It appears that HHCB and AHTN can inhibit AChE after a short exposure to environmental concentrations, but this does vary with the concentration and duration of the exposure (Fig. 1). For HHCB, limited AChE inhibition was observed at days 14 to day 21 with no significant inhibition on day 7 (Fig. 1a). This may be because the clams were able to metabolize HHCB to less toxic metabolites during the exposure (Balk and Ford, 1999a). On the other hand, all the AHTN exposure concentrations inhibited AChE activity until day 14 when all but the highest concentration of AHTN significantly ($p < 0.05$) inhibited AChE activity (Fig. 1b). Consequently, chronic inhibition of AChE in clams could lead to high levels of acetylcholine, over-stimulation of cholinergic receptors, alteration of postsynaptic cell function, and signs of cholinergic toxicity

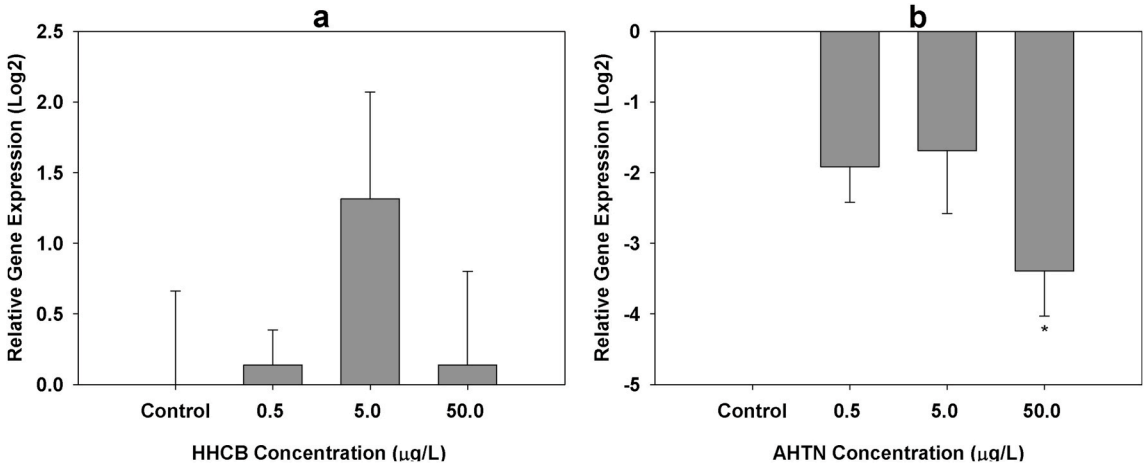

Fig. 5. Relative gene expression for *vtg1* in *C. variegatus* yolk sac larvae exposed to (**a**) galaxolide (HHCB) and (**b**) tonalide (AHTN) for 96 h. Asterisks (*) show significant differences from control ($p<0.05$).

such as morphological and behavioral changes may start to manifest (Nallapaneni et al., 2008; Pope et al., 2005; Song et al., 2004; Waseem et al., 2010). Furthermore, fatality may occur if AChE activity is depressed during exposure to cholinesterase-inhibiting chemicals due to the overstimulation of the target cells (Sancho et al., 2000). The nitro musk compound ambrette was previously reported to possess neurotoxic potential as well as elicit carcinogenesis in organisms, leading to its prohibition (Nair et al., 1986; Spencer et al., 1984). Our results are comparable with other studies reporting inhibition of AChE in bivalves after chronic exposure. Shan et al. (2020) reported significant inhibition of AChE in the digestive gland of Asian clams (*Corbicula fluminea*) exposed to 20–2000 µg/L imidacloprid for 30 days. Similarly, 0.1–1 µg/L carbamazepine, 5 and 50 µg/L caffeine, and 50 µg/L ibuprofen significantly decreased AChE activity assessed in the digestive gland of *C. fluminea* after 21 days exposure (Aguirre-Martínez et al., 2018). In contrast, environmental concentrations (15 µg/L) of ibuprofen and carbamazepine reportedly increased AChE activity assessed in the gills of *R. philippinarum* after 7 days exposure (Trombini et al., 2019). Although both HHCB and AHTN inhibited AChE activity at various points, AHTN appears to be more robust at inhibiting AChE because it exerted prolonged inhibition of AChE activity until day 14, although the clams seem to have recovered by day 21 (Fig. 1b). These results demonstrate the potential of polycyclic musk compounds as neuroinhibitors and provide a baseline upon which neurotoxicity of HHCB and AHTN could be further investigated.

HHCB increased COX activity in clams, though this inhibition was also concentration and time-dependent (Fig. 2a). COX catalyzes arachidonic acid to form prostaglandins which are responsible for several physiological and reproductive functions in aquatic organisms (Di Costanzo et al., 2019). It is an important indicator of inflammation in aquatic organisms exposed to environmental stressors (Gagné et al., 2015). Clams exposed to wastewater effluents in the Bay of Cadiz (Spain) exhibited significantly inhibited COX activity and triggered inflammatory responses in the gonad tissues, which correlated significantly with general stress, measured as lysosomal membrane stability (Díaz-Garduño et al., 2018). Recent studies have demonstrated that both HHCB and AHTN induce oxidative stress in clams (Ehiguese et al., 2020). In addition, COX synthesis of prostaglandins is involved in the control of oogenesis and spermatogenesis in aquatic invertebrates (Di Costanzo et al., 2019) and controls the effect of serotonin in the spawning process of bivalves (Matsutani and Nomura, 1987). Prameswari et al. (2017) demonstrated that arachidonic acid induced a significantly ($p < 0.001$) increased ovarian index, oocyte diameter and ovarian vitellogenin in the freshwater crab (*Oziothelphusa senex senex*). The authors reported that COX inhibitors, including indomethacin and aspirin, significantly ($p < 0.001$) reduced ovarian index, oocyte diameter and ovarian vitellogenin levels, corroborating the involvement of COX in the regulation of female reproduction in crabs. Given the concentration-dependent alteration of COX activity in clams exposed to HHCB and AHTN, reproductive success could be at risk, but additional research is needed to validate this hypothesis. Furthermore, the functions of COX activity in marine bivalves is not fully understood but the significant correlation of COX and AChE activities in clams exposed to AHTN (Table S2 – Supplementary Materials) suggests that COX activity may be involved in neuroendocrine functions in marine bivalves. Consequently, AHTN might be a neuroendocrine disruptor in the marine environment.

To understand toxicity-driven energy deficit in clams, TL in the digestive gland and gonadal tissues were measured. It was hypothesized that chemical stress may trigger significant energy demand due to relatively high enzyme activities. For *R. philippinarum* exposed to HHCB, total lipids measured in DTL were unaffected and we did not observe any clear tendency in energy level measured in the gonads (Fig. 3 a, b). The DTL measured in the clams exposed to AHTN was significantly ($p < 0.05$) higher than the control for most treatments and time points (Fig. 3b), but GTL was significantly reduced in most treatments and time points (Fig. 3d). Total lipids assessed in the gonad of *R. philippinarum* exposed to wastewater effluents was significantly reduced in other studies (Díaz-Garduño et al., 2018; Maranho et al., 2016). The variation in total lipids assessed in this study, especially for HHCB, did not reveal any clear trend.

4.2. Molecular effects

Exposure of fish to endocrine disrupting chemicals in the marine environment has serious consequences concerning survival and reproduction. In yolk-sac *C. variegatus* larvae exposed to HHCB, the expression of *cyp19* showed no concentration-dependent transcriptional effect (Fig. 4a). Meanwhile, we observed concentration-dependent downregulation of *cyp19* expression levels after 3 days of exposure to AHTN (Fig. 4b). Similarly, significant downregulation of *cyp19b* expression levels were measured in juvenile salmon exposed to 0.04–1 mg/L of the organophosphate flame retardant, tris(2-cloroethyl) phosphate for 7 days (Arukwe et al., 2016), and significant dose-dependent downregulation of *cyp19a* and *cyp19b* were recorded in the ovaries of adult marine medaka exposed to 2–5 ng/L 17β-trenbolone for 21 days (Zhang et al., 2020). In contrast, *cyp19a* and *cyp19b* expression levels were upregulated in adult male and female *Danio rerio* exposed to 1 mg/L perfluorodecanoic acid (Jo et al., 2014) and perfluorononanoate (Zhang et al., 2016). *cyp19* is an important biomarker of endocrine disruption in

teleosts because aromatase, the enzyme involved in the conversion of androgen to estrogen, plays an essential role in sexual differentiation, maturation, and reproduction (Cheshenko et al., 2008). *cyp19* is regarded as a major target for endocrine disrupting chemicals because modulation of its expression and function may potentially disrupt estrogen production (Cheshenko et al., 2008; Kazeto et al., 2004). Our results suggest that AHTN may be a more potent modulator of *cyp19* expression than HHCB (Fig. 4). Previous reports in an *in vitro* study using the H295R cell line exposed to 25 μM HHCB demonstrated upregulation of *cyp19*, while AHTN downregulated *cyp19* by 43% of the basal control (Li et al., 2013). The modulation of *cyp19* by HHCB and AHTN in *C. variegatus* larvae may affect estrogen biosynthesis and, as a result, alter the survival, sexual behavior, and sex differentiation in fish. Although most studies of estrogen as an endocrine disruption biomarker in fish are related to reproductive functions or tissues, estrogen alteration may also affect tissue mineralization and mineral homeostasis (Suzuki et al., 2009; Yoshikubo et al., 2005), as well as delay development in early life stage fish (Rawson et al., 2006).

The induction and inhibition of *vtg1* in *R. philippinarum* exposed to HHCB and AHTN, respectively, was similar to *cyp19* (Fig. 5). The basis for this similarity is not well understood but it appears that because vitellogenin is induced by estrogen (which is biosynthesized by the enzyme complex aromatase that converts androgen into estrogen), effects on *cyp19* may trickle down to have an impact on vitellogenin (Andersen et al., 2003). Previous studies have demonstrated correlations between aromatase and vitellogenin in fish exposed to endocrine disrupting chemicals (Andersen et al., 2003; Bizarro et al., 2014). However, further investigation is needed to help understand the relationships between the effects of contaminants on both biomarkers. Similar to our observation for *vtg1* measured in *C. variegatus* yolk-sac larvae exposed to HHCB, there was no statistically different increase in plasma vitellogenin levels in rainbow trout intraperitoneally injected with 1.41×10^{-5} mol/kg of HHCB for 5 days (Simmons et al., 2010). Previous research on the effects of both contaminants have showed that a three day exposure of male medaka to 5, 50 and 500 μg/L of HHCB and AHTN led to a significant induction in the expression of *vtg1* at 500 μg/L (Yamauchi et al., 2008). Meanwhile, the concentration of AHTN that elicited significant inhibition of *vtg1* in our study was much lower (50 μg/L), which may be attributed to age differences as fish larvae tend to be more sensitive to environmental contaminants than adults (Hutchinson et al., 1998). Reports of other chemicals inducing differential *vtg1* expression levels in fish abound. For example, three generations of *Oryzias melastigma* exposed to 20 and 200 μg/L benzo[a]pyrene demonstrated significant downregulation of *vtg1* expression (Sun et al., 2020). In addition, female *Oryzias melastigma* exposed to lower concentrations (2 and 10 ng/L) of 17β-trenbolone (Zhang et al., 2020) and F1 generation male *Oryzias latipes* exposed to metformin (Lee et al., 2019) all significantly downregulated *vtg1* expression levels. Contrarily, exposure of male *Oryzias melastigma* to 10 and 50 ng/L of 17α-ethynylestradiol significantly upregulated *vtg1* expression level (Zhang et al., 2020).

Generally, the concentrations of PMCs causing effects should be interpreted with caution due to their high volatility and potential to adsorb to aquaria walls. From our studies, the concentrations of HHCB and AHTN measured on day 0 were 74.26 ± 18.38% and 88.50 ± 19.09%, respectively and degraded to 22.00 ± 1.41% and 26.37 ± 4.24%, respectively on day 3 (Ehiguese et al., 2020). Tumová et al. (2019) suggested that the toxicity of PMCs for aquatic organisms under semi-static conditions could be underestimated due to the potential volatility in the aquarium, significantly lowering the concentration over time.

5. Conclusion

This study assessed the neuroendocrine effects of HHCB and AHTN using the marine bivalve *R. philippinarum* and yolk-sac larvae of an estuarine species, the sheepshead minnow, *C. variegatus*. Changes in AChE and COX activities as biomarkers of neuroendocrine effects were observed in the clams, though the exact effects varied with concentration and duration of exposure. *cyp19* and *vtg1* gene expression in yolk-sac *C. variegatus* larvae after 3 days of exposure to HHCB revealed no effect for the expression of either gene, but AHTN significantly downregulated the expression of both genes at 50 μg/L. The biomarkers studied provided useful insights to understand the potential neuroendocrine toxicity of both substances in the marine environment. Although significant changes were detected in some of the biomarkers after exposure to each contaminant, AHTN seems to be a more potent inhibitor of neuroendocrine functions in marine organisms than HHCB.

Authors statement

Friday Ojie Ehiguese, Conceptualization, Methodology, Formal analysis, Data curation, project management, Writing – original draft, writing and editing of manuscript. Maria L. Rodgers, Methodology, Formal analysis, Data curation, Writing – original draft and revision of manuscript. Cristiano V.M. Araújo, Conceptualization, Methodology, Formal analysis, Data curation, revision of manuscript. Robert Joe Griffitt, Conceptualization, Methodology, Formal analysis, Data curation, project management and revision of manuscript. M. Laura Martin-Diaz, Conceptualization, Methodology, Formal analysis, Data curation, project management and revision of manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2021.110960.

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Annex II

Supplementary Materials.

Section 3.1

Table S1: Spearman rank order of correlation (r) values recorded for the effect of galaxolide (HHCB) and tonalide (AHTN) on microalagae exposed for 72 h (n = 3). Asterisk(s) * & ** represent significant levels at $p < 0.05$ and 0.01, respectively.

	HHCB	**AHTN**
Phaeodactylum tricornotum	-0.131	-0.360
Tretraselmis chuii	0.284	-0.667**
Isochrysis galbana	0.120	0.098
Raphidocelis subcapitata	-0.349	-0.589*

Table S2: Spearman rank order of correlation (r) values recorded for the effect of galaxolide (HHCB) and tonalide (AHTN) on marine organisms exposed for 72 h (n = 3). Asterisk(s) * & ** represent significant levels at $p < 0.05$ and 0.01, respectively; (-) represent values not determined.

	HHCB	**AHTN**
Artemia franciscana mortality	0.305	-0.118
Artemia franciscana mobility	-0.020	0.300
Paracentrotus lividus fertilization	-0.747**	-0.450
Paracentrotus lividus larvae development	0.787**	0.415
Mytilus galloprovincialis larvae development	0.820**	0.371
Sparus aurata mortality test	0.659**	0.708**

Section 3.2

Table S1: Concentrations of galaxolide (HHCB) and tonalide (AHTN) measured in each compartment at the beginning and end of the test

Compartments	Nominal Concentration		Measured concentration (at the beginning)		Percentage (%)		Measured concentration (at the end)		Percentage (%)	
	HHCB	AHTN	HHCB	AHTN	HHCB	AHTN	HHCB	AHTN	HHCB	AHTN
1	0.00	0.00	0.00	0.00	100	100	0.002	0.001	0.2	0.1
2	0.005	0.005	0.006	0.007	120	140	0.005	0.004	100	80
3	0.05	0.05	0.08	0.051	140	102	0.04	0.063	80	126
4	0.5	0.5	0.452	0.418	95.2	83.6	0.699	1.284	139.8	156.8
5	5	5	3.01	4.004	60.2	80.08	2.418	8.765	48.36	175.3
6	50	50	31.311	55.563	62.62	110.01	21.732	25.258	43.46	50.52

Table S2. Shrimp mortality (in %) recorded during 24 h acute toxicity test with galaxolide (HHCB) and tonalide (AHTN) in a forced exposure system.

Fragrances	Concentrations (µg/L)						
	Control	DMSO	0.005	0.05	0.5	5	50
HHCB	8.33	0.00	16.67	8.33	16.67	0.00	8.33
AHTN	0.00	0.00	16.67	8.33	0.00	8.33	8.33

Table S3a. Mauchly's Test of sphericity for the distribution (%) of the shrimps in seawater control test using the non-forced multi-compartmented system. The nine observation times were treated as a within-subjects factor (repeated measures) and compartments (n = 6) were considered a between-subjects factor.

Within subjects effect	Mauchly W	Approx. chi-square	Degrees of freedom	Significance (p)
Time	0.024	56.131	35	0.017

Table S3b: Tests of within-subjects effects with Greenhouse-Geisser correction (sphericity was violated as the p value in Mauchly's test was lower than 0.05).

Source	Sum of squares	Degrees of freedom	Mean square	F	Significance (p)
Time	38.800	4.732	8.200	0.116	0.986
Time * Compartment	1728.614	23.658	73.066	1.030	0.439
Error (Time)	6038.940	85.170	70.905	-	-

Table S3c: Tests of between-subjects effects.

Source	Sum of squares	Degrees of freedom	Mean square	F	Significance (p)
Compartment	3956.305	5	791.261	0.693	0.636
Error	20564.702	18	1142.482	-	-

Table S4a. Mauchly's Test of sphericity for the distribution (%) of the shrimps in the DMSO control test using the non-forced multi-compartmented system. The nine observation times were treated as a within-subjects factor (repeated measures) and compartments (n = 6) were considered a between-subjects factor.

Within subjects effect	Mauchly W	Approx. chi-square	Degrees of freedom	Significance (p)
Time	0.013	65.88	35	0.002

Table S4b: Tests of within-subjects effects with Greenhouse-Geisser correction (sphericity was violated as the p value in Mauchly's test was lower than 0.05).

Source	Sum of squares	Degrees of freedom	Mean square	F	Significance (p)
Time	19.585	4.423	4.429	0.104	0.986
Time * Compartment	719.235	22.113	32.526	0.762	0.761
Error (Time)	3396.882	79.606	42.671	-	-

Table S4c: Tests of between-subjects effects.

Source	Sum of squares	Degrees of freedom	Mean square	F	Significance (p)
Compartment	199.128	5	39.826	0.085	0.994
Error	8483.467	18	471.304	-	-

Table S5a. Mauchly's Test of sphericity for the distribution (%) of the shrimps exposed to a galaxolide (HHCB) gradient in the non-forced multi-compartmented system. The nine observation times were treated as a within-subjects factor (repeated measures) and compartments (n = 6) were considered a between-subjects factor.

Within subjects effect	Mauchly W	Approx. chi-square	Degrees of freedom	Significance (p)
Time	0.009	70.800	35	< 0.0001

Table S5b: Tests of within-subjects effects with Greenhouse-Geisser correction (sphericity was violated as the p value in Mauchly's test was lower than 0.05).

Source	Sum of squares	Degrees of freedom	Mean square	F	Significance (p)
Time	49.606	3.274	15.149	0.128	0.953
Time * Compartment	3120.075	16.372	190.569	1.616	0.091
Error (Time)	6951.370	58.941	117.938	-	-

Table S5c: Tests of between-subjects effects.

Source	Sum of squares	Degrees of freedom	Mean square	F	Significance (p)
Compartment	8383.203	5	1676.641	7.388	< 0.001
Error	4084.682	18	226.927	-	-

Table S6a. Mauchly's Test of sphericity for the distribution (%) of the shrimps exposed to a tonalide (AHTN) gradient in the non-forced multi-compartmented system. The nine observation times were treated as a within-subjects factor (repeated measures) and compartments (n = 6) were considered a between-subjects factor.

Within subjects effect	Mauchly W	Approx. chi-square	Degrees of freedom	Significance (p)
Time	0.009	71.512	35	< 0.0001

Table S6b: Tests of within-subjects effects with Greenhouse-Geisser correction (sphericity was violated as the p value in Mauchly's test was lower than 0.05).

Source	Sum of squares	Degrees of freedom	Mean square	F	Significance (p)
Time	13.040	3.721	3.504	0.0.043	0.995
Time * Compartment	2465.772	18.606	132.528	1.641	0.073
Error (Time)	5407.876	66.981	80.738	-	-

Table S6c: Tests of between-subjects effects.

Source	Sum of squares	Degrees of freedom	Mean square	F	Significance (p)
Compartment	8027.938	5	1605.588	6.127	< 0.002
Error	4717.219	18	262.068	-	-

Section 3.4

Table S1: Spearman's rank order of correlation (r_s) of time, concentration and biomarkers measured in *R. philippanarum* exposed to environmental concentrations of HHCB for 21 days.

HHCB	AChE	COX	DTL	GTL
Day	0.345**	0.025	-0.411**	-0.143
Concentration	-0.168*	0.416**	0.028	0.006
AChE	1			
Cox	-0.025	1		
DTL	-0.246**	0.085	1	
GTL	-0.095	-0.008	0.280**	1

Asterisks (*) and (**) represent significant difference at $p<0.05$ and $p<0.01$, respectively.

Table S2: Spearman's rank order of correlation (r_s) of time, concentration and biomarkers measured in *R. philippanarum* exposed to environmental concentrations of AHTN for 21 days.

AHTN	AChE	COX	DTL	GTL
Day	0.645**	0.264**	0.270**	-0.191*
Concentration	-0.222*	-0.354**	0.455**	-0.476**
AChE	1			
Cox	0.578**	1		
DTL	-0.093	0.129	1	
GTL	0.083	0.382**	-0.081	1

Asterisks (*) and (**) represent significant difference at $p<0.05$ and $p<0.01$, respectively.

Annex III

Certificates of conference attendance and presentations

SETAC SciCon
SETAC Europe 30th Annual Meeting

We certify that

Friday Ojie Ehiguese

(University of Cadiz, Spain)

attended the

SETAC Europe 30th Annual Meeting
SETAC SciCon from 03 – 07 May 2020

Bart Bosveld
SETAC Europe Executive Director

SETAC EUROPE
Society of Environmental Toxicology and Chemistry
VZW- Non-Profit Organisation
Kunstlaan 53
1000 BRUSSELS
Tel +32 2 772 72 81
Fax +32 2 770 53 86
www.setac.org
setaceu@setac.org

SOCIETY OF ENVIRONMENTAL TOXICOLOGY AND CHEMISTRY

ENVIRONMENTAL QUALITY THROUGH SCIENCE®

We certify that

Friday Ojie Ehiguese

(INMAR - University of Cadiz / Physical Chemical, Puerto Real, Spain)

attended
SETAC SciCon,
the SETAC Europe 30th Annual Meeting,
from 3-7 May 2020

And presented a **Platform presentation**

Entitled:
"Spatial avoidance of the shrimp Palaemon varians exposed to a contaminant gradient of galaxolide and tonalide in seawater"

In Session: From Lab to Field: Relevance of Effects Observed in Lab Studies for Non-target Local Populations and Communities and their Habitat Selection

authors:
Friday Ojie Ehiguese, Carmen Corada-Fernández, Pablo Lara-Martin, Maria Laura Martin-Diaz, Cristiano Araujo

Bart Bosveld
SETAC Europe Executive Director

SETAC Europe vzw | Avenue des Arts 53 | 1000 Brussels, Belgium
T +32 2 772 72 81 | F +32 2 770 53 86 | setaceu@setac.org | setac.org

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ENVIRONMENTAL QUALITY THROUGH SCIENCE®

We certify that

Friday Ojie Ehiguese

(INMAR - University of Cadiz / Physical Chemistry, Puerto Real, Spain)

attended
SETAC YES 2021,
the SETAC Europe 10th Young Environmental Scientists Meeting,
from 22-26 February 2021

And presented a **Poster presentation**

Entitled:
"Polycyclic Musk Compounds Affect Neuroendocrine Activity in Marine Organisms"

In Session: Marine Ecotoxicology

authors:
Friday Ojie Ehiguese, Maria L Rodgers, Cristiano Araujo, Robert Griffitt, Maria Laura Martin-Diaz

Bart Bosveld
SETAC Europe Executive Director

SETAC Europe vzw | Avenue des Arts 53 | 1000 Brussels, Belgium
T +32 2 772 72 81 | F +32 2 770 53 86 | setaceu@setac.org | setac.org

Annex IV

Certificates of Research Stay.

UCA Universidad de Cádiz

Erasmus+

CERTIFICATE OF ATTENDANCE ERASMUS+

(OMSout2B)

Name of the host Institution: _University of Southern Mississippi_____

IT IS HEREBY CERTIFIED THAT:

Mr./Ms.(name of student) Friday Ojie Ehiguese_____ (NIF: Y4630176S)

from the University of Cadiz, Spain (E CADIZ01)

has been a ERASMUS+ student at our institution (Name of the institution): University of Southern Mississippi, Gulf Coast Research Laboratory, Ocean Springs

---

between ___17_ , January____, 2019 _and _06_, June__, __2019 ___(*)

day month year day month year

The language of instruction was ____ English_____, official language of the country of the Host institution (Yes/No) __Yes__ ____ and the student followed a preparatory language course in the host institution (Yes/No) __No__ If "yes", which language? _____

in the Department(s)/ Faculty of: ___Department of Coastal Sciences_____

____6th June 2019_____

Date (*)

Briana Klotz

Stamp and Signature

Office of Study Abroad
The University of Southern Mississippi
118 College Drive #5069
Hattiesburg, MS 39406-0001

(*) The date of signature should not be before the date of departure.

Name of the signatory: _____Briana Klotz_____

Function: _____Exchange Program Coordinator_____

A signed original of this document must be submitted to the International Office of the UCA by the student after his arrival in Spain

Oficina de Relaciones Internacionales. Universidad de Cádiz

Annex V

Thesis compendium documents

UCA Universidad de Cádiz

Tesis por compendio de publicaciones
Documento de conformidad y renuncia de coautores

Dña.: **María Judit González Delgado** con DNI o Pasaporte: [redacted] coautora de la publicación que se identifica a continuación:

Effects and risk assessment of the polycyclic musk compounds galaxolide® and tonalide® on marine microalgae, invertebrates, and fish. Ehiguese, F.O., González-Delgado, M.J., Garrido-Perez, C., Araújo, C.V.M., Martin-Diaz, M.L. Processes, 2021, 9(2), pp. 1–15, 371

de acuerdo con lo establecido en el **Artículo 23.4** del **Reglamento UCA/CG06/2012, de 27 de junio de 2012, por el que se regula la ordenación de los estudios de doctorado en la Universidad de Cádiz (BUOCA nº 208).**

Manifiesta su conformidad para la presentación de la citada publicación como parte de la tesis doctoral de D. **Friday Ojie Ehiguese**, titulada ***"Ecotoxicological assessment of galaxolide and tonalide as contaminants of emerging concern in marine ecosystems"***

"Evaluación ecotoxicológica de la galaxolida y la tonalida como contaminantes de interés emergente en ecosistemas marinos"

Y expresa su renuncia a presentar la citada publicación como parte de otra tesis doctoral en cualquier otra universidad.

En Cádiz, a 27 de abril de 2021

Fdo.: Mª Judit Gonzalez Delgado

UCA Universidad de Cádiz

Tesis por compendio de publicaciones
Documento de conformidad y renuncia de coautores

D.: ***Alam, M.R.,*** con DNI o Pasaporte: [redacted] coautor de la publicación que se identifica a continuación:

Potential of environmental concentrations of the musks galaxolide and tonalide to induce oxidative stress and genotoxicity in the marine environment. Ehiguese, F.O., Alam, M.R., Pintado-Herrera, M.G., Araújo, C.V.M., Martin-Diaz, M.L. Marine Environmental Research, 2020, 160, 105019

de acuerdo con lo establecido en el **Artículo 23.4** del **Reglamento UCA/CG06/2012, de 27 de junio de 2012, por el que se regula la ordenación de los estudios de doctorado en la Universidad de Cádiz (BUOCA nº 208).**

Manifiesta su conformidad para la presentación de la citada publicación como parte de la tesis doctoral de D. **Friday Ojie Ehiguese**, titulada ***"Ecotoxicological assessment of galaxolide and tonalide as contaminants of emerging concern in marine ecosystems"***

"Evaluación ecotoxicológica de la galaxolida y la tonalida como contaminantes de interés emergente en ecosistemas marinos"

Y expresa su renuncia a presentar la citada publicación como parte de otra tesis doctoral en cualquier otra universidad.

En Cádiz, a de de

Fdo.: __27.04.2021___

UCA Universidad de Cádiz

Tesis por compendio de publicaciones
Documento de conformidad y renuncia de coautores

Dña.: **Rodgers, M.L.** con DNI o Pasaporte: [redacted] coautora de la publicación que se identifica a continuación:

Galaxolide and tonalide modulate neuroendocrine activity in marine species from two taxonomic groups. Ehiguese, F.O., Rodgers, M.L., Araújo, C.V.M., Griffitt, R.J., Martin-Diaz, M.L. Environmental Research, 2021, 196, 110960

de acuerdo con lo establecido en el **Artículo 23.4** del **Reglamento UCA/CG06/2012, de 27 de junio de 2012, por el que se regula la ordenación de los estudios de doctorado en la Universidad de Cádiz (BUOCA nº 208).**

Manifiesta su conformidad para la presentación de la citada publicación como parte de la tesis doctoral de D. **Friday Ojie Ehiguese**, titulada ***"Ecotoxicological assessment of galaxolide and tonalide as contaminants of emerging concern in marine ecosystems"***

"Evaluación ecotoxicológica de la galaxolida y la tonalida como contaminantes de interés emergente en ecosistemas marinos"

Y expresa su renuncia a presentar la citada publicación como parte de otra tesis doctoral en cualquier otra universidad.

En Cádiz, a de de

Fdo.: _29 April 2021

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Documento de conformidad y renuncia de coautores

Dña.: **Marina Pintado** con DNI o Pasaporte: [redacted] coautora de la publicación que se identifica a continuación:

Potential of environmental concentrations of the musks galaxolide and tonalide to induce oxidative stress and genotoxicity in the marine environment. Ehiguese, F.O., Alam, M.R., Pintado-Herrera, M.G., Araújo, C.V.M., Martin-Diaz, M.L. Marine Environmental Research, 2020, 160, 105019

de acuerdo con lo establecido en el **Artículo 23.4** del **Reglamento UCA/CG06/2012, de 27 de junio de 2012, por el que se regula la ordenación de los estudios de doctorado en la Universidad de Cádiz (BUOCA nº 208).**

Manifiesta su conformidad para la presentación de la citada publicación como parte de la tesis doctoral de D. **Friday Ojie Ehiguese**, titulada ***"Ecotoxicological assessment of galaxolide and tonalide as contaminants of emerging concern in marine ecosystems"***

"Evaluación ecotoxicológica de la galaxolida y la tonalida como contaminantes de interés emergente en ecosistemas marinos"

Y expresa su renuncia a presentar la citada publicación como parte de otra tesis doctoral en cualquier otra universidad.

En Cádiz, a 5 de Mayo de 2021

Fdo.: Marina G. Pintado Herrera

UCA Universidad de Cádiz

Tesis por compendio de publicaciones
Documento de conformidad y renuncia de coautores

Dña.: ***Mª del Carmen Corada Fernández*** con DNI o Pasaporte: [redacted] coautor de la publicación que se identifica a continuación:

Avoidance behaviour of the shrimp Palaemon varians regarding a contaminant gradient of galaxolide and tonalide in seawater. Ehiguese, F.O., Fernandez, M.D.C.C., Lara-Martín, P.A., Martín-Díaz, M.L., Araújo, C.V.M. Chemosphere, 2019, 232, pp. 113–120

de acuerdo con lo establecido en el **Artículo 23.4** del **Reglamento UCA/CG06/2012, de 27 de junio de 2012, por el que se regula la ordenación de los estudios de doctorado en la Universidad de Cádiz (BUOCA nº 208).**

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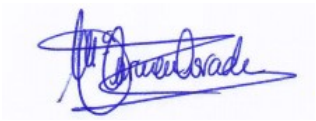
En Cádiz, a 26 de Abril de 2021



Fdo.: Mª del Carmen Corada Fernández

UCA Universidad de Cádiz

Tesis por compendio de publicaciones
Documento de conformidad y renuncia de coautores

Dña.: **Carmen Garrido Pérez** con [redacted] coautora de la publicación que se identifica a continuación:

Effects and risk assessment of the polycyclic musk compounds galaxolide® and tonalide® on marine microalgae, invertebrates, and fish. Ehiguese, F.O., González-Delgado, M.J., Garrido-Perez, C., Araújo, C.V.M., Martin-Diaz, M.L. Processes, 2021, 9(2), pp. 1–15, 371

de acuerdo con lo establecido en el **Artículo 23.4** del **Reglamento UCA/CG06/2012, de 27 de junio de 2012, por el que se regula la ordenación de los estudios de doctorado en la Universidad de Cádiz (BUOCA nº 208).**

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"Evaluación ecotoxicológica de la galaxolida y la tonalida como contaminantes de interés emergente en ecosistemas marinos"

Y expresa su renuncia a presentar la citada publicación como parte de otra tesis doctoral en cualquier otra universidad.

En Cádiz, a 27 de abril de 2021

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UCA Universidad de Cádiz

Tesis por compendio de publicaciones
Documento de conformidad y renuncia de coautores

D. **Pablo Lara** con [redacted], coautor de la publicación que se identifica a continuación:

Avoidance behaviour of the shrimp Palaemon varians regarding a contaminant gradient of galaxolide and tonalide in seawater.
Ehiguese, F.O., Fernandez, M.D.C.C., Lara-Martín, P.A., Martín-Díaz, M.L., Araújo, C.V.M.
Chemosphere, 2019, 232, pp. 113–120

de acuerdo con lo establecido en el **Artículo 23.4** del **Reglamento UCA/CG06/2012, de 27 de junio de 2012, por el que se regula la ordenación de los estudios de doctorado en la Universidad de Cádiz (BUOCA nº 208).**

Manifiesta su conformidad para la presentación de la citada publicación como parte de la tesis doctoral de D. **Friday Ojie Ehiguese**, titulada ***"Ecotoxicological assessment of galaxolide and tonalide as contaminants of emerging concern in marine ecosystems"***

"Evaluación ecotoxicológica de la galaxolida y la tonalida como contaminantes de interés emergente en ecosistemas marinos"

Y expresa su renuncia a presentar la citada publicación como parte de otra tesis doctoral en cualquier otra universidad.

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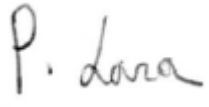
En Cádiz, a 28 de abril de 2021



Fdo.: Pablo A. Lara Martín

UCA Universidad de Cádiz

Tesis por compendio de publicaciones
Documento de conformidad y renuncia de coautores

D.: ***Cristiano V. de Matos Araújo*** con NIE: [redacted] coautor de las publicaciones que se identifican a continuación:

Galaxolide and tonalide modulate neuroendocrine activity in marine species from two taxonomic groups. Ehiguese, F.O., Rodgers, M.L., Araújo, C.V.M., Griffitt, R.J., Martin-Diaz, M.L. Environmental Research, 2021, 196, 110960.

Effects and risk assessment of the polycyclic musk compounds galaxolide® and tonalide® on marine microalgae, invertebrates, and fish. Ehiguese, F.O., González-Delgado, M.J., Garrido-Perez, C., Araújo, C.V.M., Martin-Diaz, M.L. Processes, 2021, 9(2), pp. 1–15, 371.

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Y expresa su renuncia a presentar la citada publicación como parte de otra tesis doctoral en cualquier otra universidad.

En Cádiz, a 26 de abril de 2021

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UCA Universidad de Cádiz

Tesis por compendio de publicaciones
Documento de conformidad y renuncia de coautores

D.: ***María Laura Martín*** con DNI [redacted] coautora de las publicaciones que se identifican a continuación:

Galaxolide and tonalide modulate neuroendocrine activity in marine species from two taxonomic groups. Ehiguese, F.O., Rodgers, M.L., Araújo, C.V.M., Griffitt, R.J., Martin-Diaz, M.L. Environmental Research, 2021, 196, 110960.

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Tesis por compendio de publicaciones
Documento de conformidad y renuncia de coautores

D.: **Griffitt, R.J.** con DNI o Pasaporte: [redacted] coautor de la publicación que se identifica a continuación:

Galaxolide and tonalide modulate neuroendocrine activity in marine species from two taxonomic groups. Ehiguese, F.O., Rodgers, M.L., Araújo, C.V.M., Griffitt, R.J., Martin-Diaz, M.L. Environmental Research, 2021, 196, 110960

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