



An overview of the ecotoxicological effects of cigarette butts on *Artemia franciscana* in a climate change scenario

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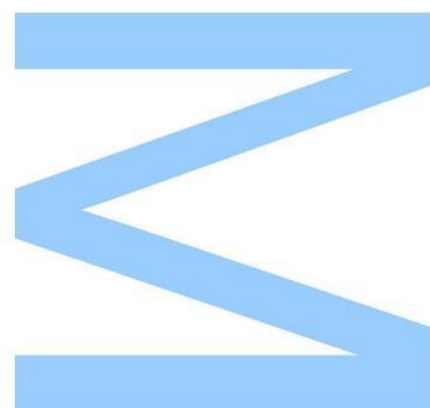
2022/2023

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Acknowledgements

I would like to start by thanking Prof. Doctor Natividade Vieira for giving me the opportunity to intern at a time when I already seemed lost.

Thank you to Sara Rodrigues for agreeing to supervise my dissertation and always being available for anything I needed. Your advice and suggestions helped me a lot and I'm sure they were crucial to the success of this work. A thank you is not enough.

To Carlos, who was tireless and made the entire internship easier, and proved to be an incredible mentor and friend, both inside and outside the laboratory. Thank you for all your help, all your suggestions and all your reminders, and for the contagious good mood you brought every day.

To Sara Couto, who did this internship with me and made this whole experience lighter. A lab colleague who became an incredible friend and supported me all the way until the end. Thank you for everything.

To the researchers at laboratory 1.14, including Prof. Doctor Sara Antunes, who were always available to help and motivate us on the most difficult days.

And finally, to my parents, and my friends, who didn't let me give up. For understanding my absence and for supporting all my frustrations, and always having calm and supportive words. I hope to make you proud.

Abstract

Cigarette butts are one of the most pervasive pollutants worldwide, which inevitably infiltrate in aquatic ecosystems through improper disposal, posing various contamination risks. In addition, aquatic ecosystems are threatened by factors related to climate change, leading to alterations in the physical and chemical water properties. As a consequence of global warming and extreme weather events, aquatic ecosystems are facing fluctuations in the temperature of water. In this sense, it is vital to address pollution by cigarette butts in aquatic environments and understand the influence of temperature on the effects promoted, through evaluating the biological responses of non-target organisms. Thus, this study aimed to investigate the ecotoxicological effects of leachate cigarette butts (CBs) on *Artemia franciscana*. Considering the influence of climate change and global warming on organism health, two different temperatures (25 and 30 °C) were tested to mimic a worst-case scenario. The concentrations evaluated were 0.03125, 0.0625, 0.125, 0.25 and 0.5 CBs/L, for smoked (SCBs) and non-smoked cigarette butts (nSCBs). Individuals were exposed for seven days, and mortality was assessed daily. At the end of this period, growth (mm) and a battery of biochemical biomarkers [neurotransmission (acetylcholinesterase - AChE), antioxidant defense (catalase - CAT and glutathione S-transferases - GSTs), lipid peroxidation (thiobarbituric acid reactive substances - TBARS), and anaerobic metabolism (lactate dehydrogenase - LDH)] were evaluated. Mortality rates were found to be higher at 30°C, reaching 15% for 0.0625 SCBs/L and 11.67% for 0.5 nSCBs/L. Regarding growth, a significant interaction was observed between temperatures and concentration of cigarette butts (SCBs and nSCBs). Furthermore, in nSCBs conditions, the organisms showed growth changes, at both tested temperatures. The results from biochemical biomarkers showed interactions between temperature and CBs concentrations in all parameters tested, except for AChE activity and TBARS levels, for nSCBs. There was a significant increase in AChE activity at 0.25 SCBs/L at 25°C, and for nSCBs, temperature variations led to significant differences in AChE activity, but without interaction with concentrations. Regarding CAT activity, under SCBs conditions, it decreased at 25°C in all concentrations, but increased at 30°C, with the exception of 0.0625 SCBs/L. For nSCBs, CAT activity increased at both temperatures tested (except at 0.03125 nSCBs/L at 25°C and 0.0625 nSCBs/L at 30°C). With regard to GSTs activity, under SCBs conditions, there was an increase only at 25°C to 0.25 and 0.5 SCBs/L, while for nSCBs, a significant increase in GSTs activity was observed at all concentrations at 25°C, but a decrease in 0.25 nSCBs/L at 30°C. Lipid peroxidation, measured by TBARS levels, showed elevated results at 0.25 and 0.5 SCBs/L at 25°C, as well as at all concentrations at 30°C (except 0.0625 SCBs/L). For nSCBs, significant

effects were primarily attributable to temperature, with no interaction between the two factors or concentration effects. Finally, at 25°C, LDH activity showed a decrease in organisms exposed to SCBs (0.03125, 0.0625 and 0.5 SCBs/L) and nSCBs (0.0625 and 0.5 nSCBs/L), while at 30 °C the opposite occurred, with a significant increase in 0.5 SCBs/L and 0.25 nSCBs/L.

The results obtained showed that an increase in temperature can modulate the biological responses of *A. franciscana* to CBs. These findings contribute to the understanding of the ecotoxicological implications of CBs pollution in a warming world. This could help raise awareness about the importance of reducing inappropriate cigarette disposal and direct efforts to minimize this source of pollution, as well as encouraging the search for more sustainable alternatives, such as biodegradable filters or cigarette recycling strategies. As a study that recognizes the interaction between CBs pollution and global warming, it highlights the need for mitigation strategies that consider evolving climate conditions.

Keywords: Pollution; Global warming; Cigarette butts; Ecotoxicology; *Artemia franciscana*; Mortality; Growth; Biomarkers.

Resumo

As beatas de cigarro são um dos poluentes mais persistentes em todo o mundo, que inevitavelmente se infiltram nos ecossistemas aquáticos através do seu descarte inadequado, apresentando diversos riscos de contaminação. Além disso, os ecossistemas aquáticos estão ameaçados por fatores relacionados com as alterações climáticas, levando a alterações nas propriedades físicas e químicas da água. Como consequência do aquecimento global e de eventos climáticos extremos, os ecossistemas aquáticos enfrentam flutuações na temperatura da água. Nesse sentido, é vital abordar a poluição por beatas de cigarro em ambientes aquáticos e compreender a influência da temperatura nos efeitos promovidos, através da avaliação das respostas biológicas de organismos não-alvo. Assim, este estudo teve como objetivo investigar os efeitos ecotoxicológicos de lixiviados de beatas de cigarros (CBs) em *Artemia franciscana*. Considerando a influência das alterações climáticas e do aquecimento global na saúde do organismo, foram testadas duas temperaturas diferentes (25 e 30 °C) para imitar o pior cenário possível. As concentrações avaliadas foram 0.03125, 0.0625, 0.125, 0.25 e 0.5 CBs/L, para beatas de cigarro fumadas (SCBs) e não fumadas (nSCBs). Os indivíduos foram expostos por sete dias e a mortalidade foi avaliada diariamente. No final deste período, foram avaliados o crescimento (mm) e um conjunto de biomarcadores bioquímicos [neurotransmissão (acetilcolinesterase - AChE), defesa antioxidante (catalase - CAT e glutathione S-transferases - GSTs), peroxidação lipídica (substâncias reativas ao ácido tiobarbitúrico - TBARS) e metabolismo anaeróbio (lactato desidrogenase - LDH)]. As taxas de mortalidade foram maiores a 30 °C, atingindo 15% para 0.0625 SCBs/L e 11.67% para 0.5 nSCBs/L. Em relação ao crescimento, foi observada interação significativa entre temperaturas e concentração de beatas de cigarro (SCBs e nSCBs). Além disso, nas condições de nSCBs, os organismos apresentaram alterações de crescimento, em ambas as temperaturas testadas. Os resultados dos biomarcadores bioquímicos mostraram interações entre temperatura e concentrações de CBs em todos os parâmetros testados, exceto na atividade de AChE e níveis de TBARS, para nSCBs. Houve um aumento significativo na atividade da AChE a 0.25 SCBs/L a 25 °C, e para nSCBs, as variações de temperatura levaram a diferenças significativas na atividade da AChE, mas sem interação com as concentrações. Em relação à atividade da CAT, nas condições de SCBs, diminuiu a 25 °C em todas as concentrações, mas aumentou a 30 °C, com exceção de 0.0625 SCBs/L. Para nSCBs, a atividade de CAT aumentou em ambas as temperaturas testadas (exceto 0.03125 nSCBs/L a 25 °C e 0.0625 nSCBs/L a 30 °C). No que diz respeito à atividade de GSTs, sob condições de SCBs, houve um aumento apenas a 25 °C para 0.25 e 0.5 SCBs/L, enquanto para nSCBs

foi observado um aumento significativo na atividade de GSTs em todas as concentrações a 25 °C, mas uma diminuição em 0.25 nSCBs/L a 30 °C. A peroxidação lipídica, medida pelos níveis de TBARS, apresentou resultados elevados em 0.25 e 0.5 SCBs/L a 25 °C, bem como em todas as concentrações a 30 °C (exceto 0.0625 SCBs/L). Para nSCBs, os efeitos significativos foram atribuídos principalmente à temperatura, sem interação entre os dois fatores ou efeitos de concentração. Finalmente, a 25 °C, a atividade da LDH mostrou uma diminuição nos organismos expostos a SCBs (0.03125, 0.0625 e 0.5 SCBs/L) e nSCBs (0.0625 e 0.5 nSCBs/L), enquanto a 30 °C ocorreu o oposto, com um significativo aumento a 0.5 SCBs/L e 0.25 nSCBs/L.

Os resultados obtidos mostraram que o aumento da temperatura pode modular as respostas biológicas de *A. franciscana* a CBs. Estas descobertas contribuem para a compreensão das implicações ecotoxicológicas da poluição por CBs num mundo em aquecimento. Isto poderia ajudar a aumentar a conscientização sobre a importância de reduzir o descarte inadequado de cigarros e direcionar esforços para minimizar esta fonte de poluição, bem como incentivar a busca por alternativas mais sustentáveis, como filtros biodegradáveis ou estratégias de reciclagem de cigarros. Sendo um estudo que reconhece a interação entre a poluição por CB e o aquecimento global, destaca a necessidade de estratégias de mitigação que considerem a evolução das condições climáticas.

Palavras-chave: Poluição; Aquecimento Global; Beatas de cigarro; Ecotoxicologia; *Artemia franciscana*; Mortalidade; Crescimento; Biomarcadores.

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List of abbreviations

Ach	Acetylcholine
AChE	Acetylcholinesterase
ASW	Artificial Seawater
CAT	Catalase
CBs	Cigarette Butts
CDNB	1-chloro-2,4-dinitrobenzene
DNA	Deoxyribonucleic Acid
DTNB	5-50-dithio-bis-2-nitrobenzoate
ECT	Ectoine
EEA	European Environment Agency
GHG	Greenhouse Gases
GSH	Glutathione
GSTs	Glutathione S-transferase
HUFA	highly Unsaturated Fatty Acids
IPCC	Intergovernmental Panel on Climate Change
LC ₅₀	Lethal Concentration 50%
LDH	Lactate Dehydrogenase
MDA	Malondialdehyde
NADH	Nicotinamide Adenine Dinucleotide
NS	Non-significant
nSCBs	Non-smoked Cigarette Butts
OECD	Organization for Economic Co-operation and Development
OS	Oxidative Stress
PAHs	Polycyclic Aromatic Hydrocarbons
PSU	Practical Salinity Units
ROS	Reactive Oxygen Species
SCBs	Smoked Cigarette Butts

TBA	2-Thiobarbituric Acid
TBARS	Thiobarbituric Acid Reactive Substances
WHO	World Health Organization

1. Introduction

The increase of the world's population associated with the economic and technological development has led to an intensification of the production of a wide variety of products to meet the needs of modern population. Among them are plastic-based products, which are the most commonly littered items found (Novotny *et al.*, 2015). Indeed, this development recorded over the years generated a large quantity of waste with diversified origins, without considering the pollution that they cause, especially in water ecosystems, which has become a major concern (Rosas *et al.*, 2021). Nevertheless, the economy and technological development still play a vital role in our lives by providing access to employment, products and services that help humans thrive.

Increasing concentrations of greenhouse gasses (GHG) in the atmosphere are the main drivers of global warming and climate change. These changes consist of an increase in average global temperature, sea level rise and water melt from ice caps and glaciers, etc., (Ledley *et al.*, 1999). Although pollution from cigarette butts (CBs) doesn't have a direct impact on climate change, it is important to consider that the production, consumption, and improper disposal of cigarettes have a significant carbon footprint throughout their life cycle. From growing tobacco, making cigarettes to disposing of waste, the entire process consumes energy and emits GHG, contributing to climate change (WHO, 2022). Thus, since the improper disposal of waste is a prevalent practice, cigarette butts, often perceived as harmless by many, pose a significant issue.

Anthropogenic waste, including the improper disposal of cigarette butts (CBs), regularly finds its way into aquatic environments through various sources, such as runoffs, wind, rain, tourists, and bathers. It is then carried to the oceans by the forces of waves and wind and rivers, ultimately depositing on the seabed or coastal areas (Rosas *et al.*, 2021).

1.1 Climate change

The continuous increase of global temperatures is one of the clearest and worrying consequences of climate change caused by anthropogenic activity. The burning of fossil fuels, deforestation and other human activities release GHG into the atmosphere, creating a "barrier" that retains more heat and increases the average temperature of the planet (Hertzberg *et al.*, 2017). This has widespread effects on climate, such as, increased mean sea levels, polar ice caps melting, higher frequency and intensity of extreme weather events, species migration and loss of biodiversity (Rahman, 2013).

Recently, the Intergovernmental Panel on Climate Change (IPCC, 2022) highlighted that climate change and global warming are happening at a very high rate. The results were based on a multidisciplinary approach where hundreds of experts from different parts of the world collect data from published scientific studies, technical reports, observational data, climate modeling and other types of relevant information. Among the numerous points addressed, the following stood out:

- The average global temperature has already increased approximately 1.1°C since 1880 from pre-industrial levels, and GHG concentrations in the atmosphere continue to increase. If fast and significant action is not taken, the global temperature could increase by 1.5°C by 2040.
- Climate change is affecting natural systems around the world, including oceans, glaciers, forests, and terrestrial ecosystems, which in turn has impacts on water availability, food production, human health, and biodiversity.
- GHG emissions need to be reduced quickly and significantly to limit global warming to 1.5°C. This will require profound changes in energy systems, transportation, land use and construction, among others.

The global surface temperature alterations were projected for different global warming scenarios (Figure 1), with the worst-case scenario indicating a potential 5°C increase by the end of the century (IPCC, 2022).

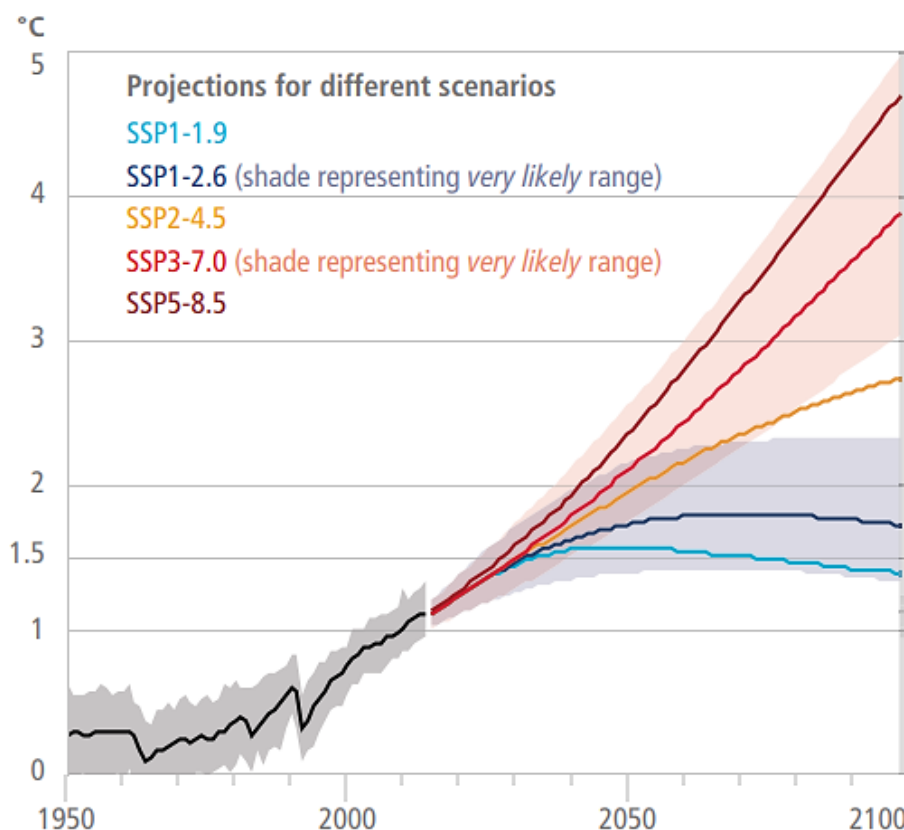


Figure 1 - Projections for different scenarios of global surface temperature changes (°C) by 2100. Increase relative to the period 1850-1900 (Source: IPCC, 2022).

1.2 Cigarette butts

The cigarette consumption peaked worldwide in the middle of the 20th century. Nowadays, numerous studies and reports have documented a generalized drop in the smoking rate. For instance, according to the World Health Organization (WHO), the global prevalence of smoking has declined from 27% in 2000 to 20% in 2016, approximately. This decline results of a combination of tobacco control efforts and measures in many countries (WHO, 2021). However, it is important to note that smoking rates can vary widely between countries and regions (Figure 2) – while some countries show a significant reduction in the number of smokers, others have higher or stagnating rates (OECD, 2021).

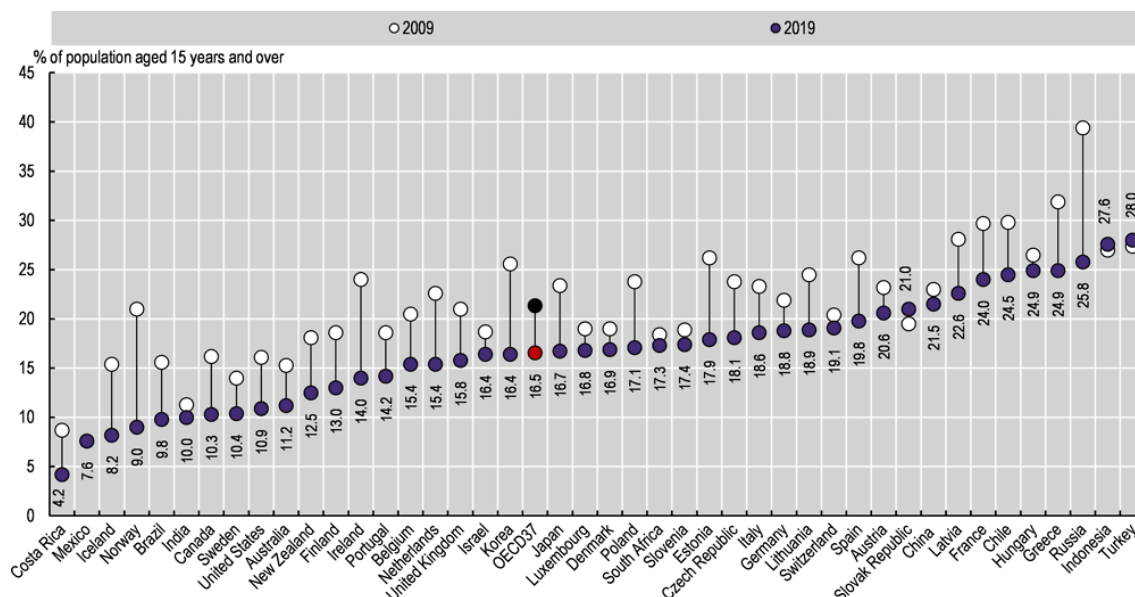


Figure 2 - Population aged 15 and over smoking daily, 2009 and 2019 (Source: OECD Health Statistics 2021) – the black and red dot represent the mean % of population across OECD member countries.

Despite this decreased tendency over the years, smoking rate remains high. As such, CBs are one of the most abundant forms of litter on the planet, with an estimated 4.5 trillion butts deposited in the environment each year, which can enter into marine ecosystems directly or through rain and river runoffs (Slaughter *et al.*, 2011). Cigarettes are made of about 15,000 microfibers of cellulose acetate, a type of non-biodegradable plastic, and their production involves the use of chemicals such as pesticides, herbicides, insecticides, fungicides, and others, whose residues remain in the butts (Belzagui *et al.*, 2021).

1.2.1 Composition and fate of cigarette butts

The act of smoking is responsible for the production of approximately 400 chemical substances including carbon monoxide, hydrogen cyanide, nitrogen oxides, polycyclic aromatic hydrocarbons, ammonia, benzene, acetone, and others, with more than a hundred being proven to be carcinogenic to humans (Slaughter *et al.*, 2011). According to Food and Drug Administration (FDA) there are 93 listed Harmful and Potentially Harmful Constituents in tobacco products, including acetone, benzene, ethyl carbamate, mercury, nicotine, phenol, a series of metals, such as cadmium, chromium, lead, etc. (FDA, 2012). The burning of cigarettes can influence the chemical nature of tobacco through the release of volatile components from the combusting solid and the carbonization of degrading tobacco, and inorganic residues are left usually in the filters (Liu *et al.*, 2012). Moreover, non-smoked cigarettes can contain chemical substances, such as additives and coatings, which are released into aquatic environments upon their contact with water. This

way, the comparison between smoked and non-smoked cigarette butts is also important, since the process of combustion can release chemicals that are not originally present in non-smoked cigarettes.

The use of filters in cigarettes was intended to trap the dangerous residues of oil tar and nicotine, but it has never been shown to work as expected, as many of the substances can still escape and enter the respiratory system of smokers. Furthermore, when released into the environment, the cigarette filters end up in rivers and in the ocean and become bioavailable to aquatic organisms with negative effects (e.g., obstruction of the digestive tract, accumulation of toxic waste in the digestive systems of organisms and biomagnification progressively along the food chain) on their health (Novotny *et al.*, 2011).

Another major concern regarding cigarette butts is their degradation in the environment (Bonanomi *et al.*, 2015). This is a very relevant issue since cigarette filters are the most common waste found in the environment (Novotny *et al.*, 2009). The European Environment Agency (EEA) has an initiative that aims to combat the plastic litter problem, Marine Litter Watch, where citizens have collected 693259 items from 2014 to 2017 and data shows that cigarette butts/filters are on the top of litter items found in beaches (Figure 3), representing 18% of marine litter (EEA, 2018).

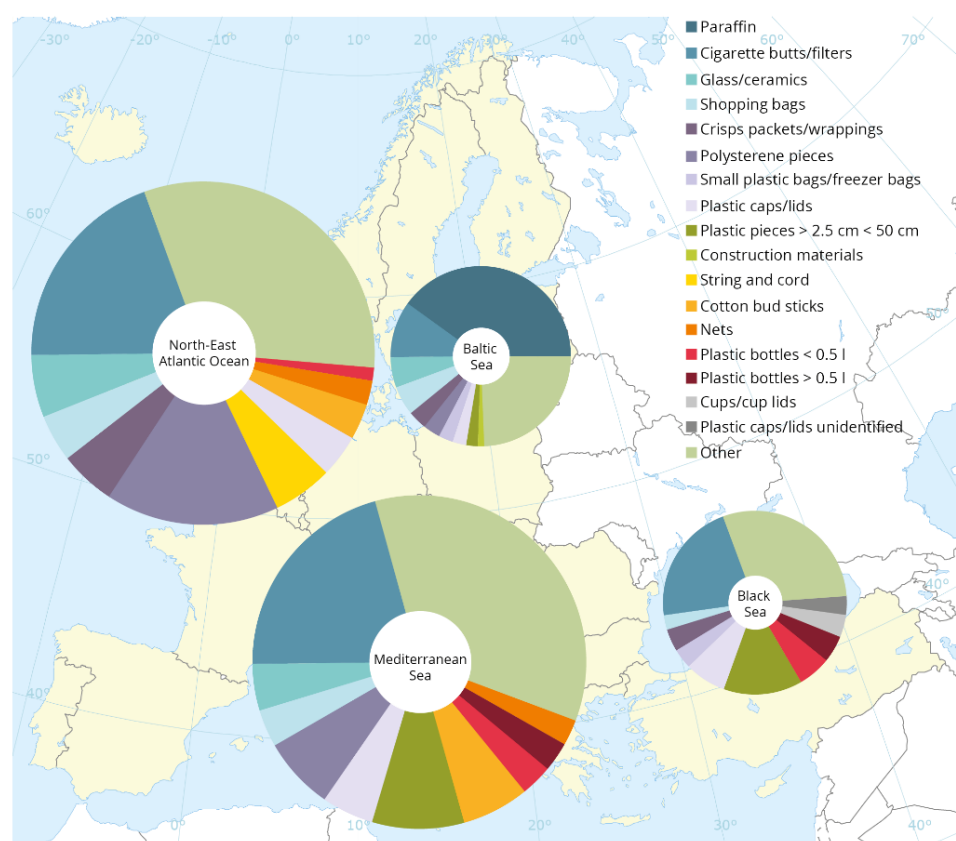


Figure 3 - Top ten items of marine litter reported by the public and where they were found (Source: EEA, 2018).

The process of environmental degradation of cigarette filters involves deacetylation through chemical hydrolysis and acetyl esterases, where cellulose is easily biodegraded by microorganisms (Puls *et al.*, 2011). Even though cellulose acetate is photodegradable, it is a plastic material with high levels of acetate, dense fibrous structure, and includes plasticizers that are beneficial to filter efficiency, but make the biodegradation a slow and difficult process (Novotny *et al.*, 2009), *i.e.*, decomposition lasts more than a decade.

1.2.2 Legislation and social and political awareness

Since cigarette butts are one of the biggest sources of environmental pollution, measures have been taken worldwide regarding cigarettes and their filters, including in Portugal.

In Portugal, on 3rd September 2019, the law no. 88/2019 was published, aiming at "reducing the impact of cigarette, cigar or other tobacco products' ends on the environment" (Decreto Lei No 88/2019, 2019). Among the various law articles, the

following stand out: the prohibition of inappropriate discard of these products; commercial establishments and others must have ashtrays and equipment for the proper disposal of these types of waste; promoting the use of biodegradable materials in the manufacture of tobacco filters; fines ranging from 25 to 250€ for individuals and between 250€ and 1500€ for entities that do not comply with the mentioned articles, etc.

In other countries there are also strategies to reduce the pollution caused by CBs. For instance, in several U.S.A. states, fines are imposed for the improper disposal of cigarette butts (CBs) as litter, and individuals who report littering incidents can receive 50% of the fine as a reward (Barnes, 2011). Also, New Zealand has passed a law banning anyone born after January 1, 2009, from being able to purchase tobacco products, with the purpose to create a “smoke-free” New Zealand by 2025 (CNN *et al.*, 2022).

These laws and regulations raised the consciousness of citizens and led to more campaigns and projects to promote awareness and protect the environment against this type of pollution. There are already several campaigns encouraging the collection and cleaning of cigarette butts on streets and beaches, conducted by municipalities, eco-schools, extracurricular organizations, among many others. For example, portable ashtrays have been provided in beaches of Figueira da Foz (Portugal) for smokers to use them (Figure 4) (Município da Figueira da Foz, 2019).



Figure 4 - Examples of portable ashtrays on Figueira da Foz beach (Source: NOCTULA Channel).

1.2.3 Environmental concern vs. Ecotoxicological studies

In recent decades, the assessment of marine pollution for scientific purposes in coastal areas has been increasing. However, regarding the quantification of specific data for ecotoxicological evaluation on CBs, research is still scarce and limited to few regions (Figure 5). Araújo & Costa (2019) collected information on different studies involving CBs and its quantification at beaches and coastal urban centers, main sources and impacts

generated. This review showed that the quantification of specific data related to CBs is still very scarce, and gathered information of samplings made on ten islands, twenty-two continental coastal areas, one estuary, and four coastal urban centers, by multiple authors. Each of the samplings consisted in establishing transects in the shoreline, collecting the residues and classifying it. The materials found ranged from plastics, paper, and manufactured items to glass fragments, with cigarette butts being one of the most frequent (Araújo & Costa, 2019). For example, in Maui island, in Hawaii, CBs corresponded to about 45% of the total accumulated residues on one of the beaches (Blickley *et al.*, 2016). In the island of Rugen, Germany, CBs were amongst the most abundant items found on four different beaches, with a percentage of 15% (Hengstmann *et al.*, 2017).

Recently more studies have been carried out. Da Silva *et al.* (2023), collected data from 116 articles about CBs and their impacts on the environment, from 2006 to 2022, that showed the country with the highest number of publications on this subject was the United States (n = 21), followed by Iran (n = 18), Brazil (n = 16), Italy (n = 15), France (n = 10), Spain and Malaysia (n = 5) (Da Silva *et al.*, 2023). Figure 5 shows a map with the distribution of locations where studies were carried out on the quantification of pollution from CBs based on publications by Araújo & Costa, 2019, Kungskulniti *et al.*, 2018, Da Silva *et al.*, 2023, Nasab *et al.*, 2022 and Da Silva *et al.*, 2023.

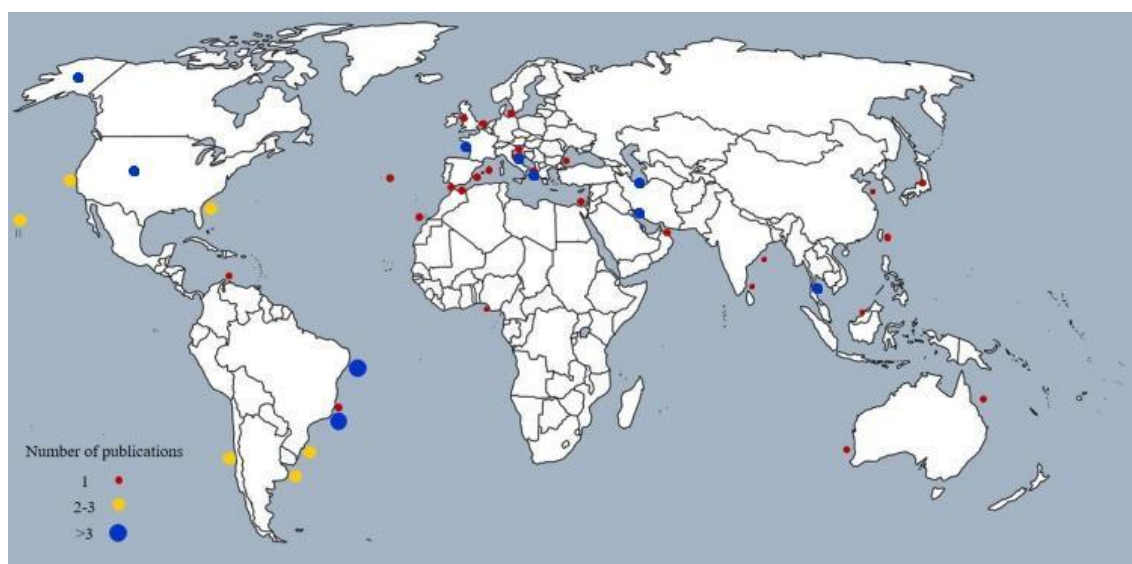


Figure 5 - Distribution of studies regarding the quantification of cigarette butts (1998-2022) Adapted from: Araújo & Costa, 2019).

The literature is scarce in terms of ecotoxicological studies of CBs to aquatic organisms, mainly marine organisms. According to Slaughter *et al.* (2011), two fish

species (*Atherinops affinis* and *Pimephales promelas*) showed acute toxicity to leachates from smoked (SCBs) and non-smoked cigarette butts (nSCBs). For smoked leachates at 48 hours, LC₅₀ was approximately 1.8 SCBs/L for *P. promelas* and 4.4 SCBs/L for *A. affinis*; for non-smoked leachates, the LC₅₀ was approximately 13.5 nSCBs/L for *P. promelas* and 5.1 nSCBs/L for *A. affinis*, which demonstrated that smoked CBs leachate have a higher lethality to these two fish species (Slaughter *et al.*, 2011). In addition, CBs have other lethal effects on aquatic organisms and can cause important sublethal effects, such as changes in behavior and changes in reproductive output in a variety of aquatic organisms (Green *et al.*, 2022).

Other ecotoxicological studies were conducted and revealed acute toxic effects of cigarette butts on some species (Table 1). Although this environmental problem has been recognized for quite some time, the consequences of cigarette butts leaching continue poorly studied, especially in marine species, since the listed species in Table 1 are mostly freshwater. Despite physiological differences, a study observed three snail species that showed 100% mortality after 8 days of exposure to leachate with 5 CBs/L (Booth *et al.*, 2015). Abessa *et al.*, 2021 exposed nauplii of *Artemia* spp. to smoked CBs, where test solutions of 5 SCBs in 500 mL of ASW were diluted to final concentrations of 6.25%, 12.5%, 25%, 50% and 100% of the CBs leachate. Results showed morphological deformities, in body size and shape, and mortality, where the LC₅₀ at 48 h was 45.3% (Abessa *et al.*, 2021).

Table 1 - Different species studied and respective LC50: SCB/L – smoked cigarette butts per liter; nSCB/L – non-smoked cigarette butts per liter

Species	Exposure time	SCB/L (LC ₅₀)	nSCB/L (LC ₅₀)	Reference
<i>Ceriodaphnia dubia</i>	48 h	0.16	1.7	Warner, 2002
<i>Atherinops affinis</i>	96 h	1.8	5.1	Slaughter <i>et al.</i> , 2011
<i>Pimephales promelas</i>	96 h	4.3	13.5	Slaughter <i>et al.</i> , 2011
<i>Daphnia magna</i>	48 h	1.0 – 2.0	>16.0	Mccoy <i>et al.</i> , 2013
<i>Xenopus laevis</i>	96 h	1.2	-	Parker & Rayburn, 2017
<i>Periophtalmus waltoni</i>	96 h	1.36	-	Soleimani <i>et al.</i> , 2023

1.3 *Artemia franciscana* as a model organism in ecotoxicology

1.3.1 Taxonomy

Traditionally, the responses of organisms to different types of stress (threatened homeostasis) have been used to evaluate the quality of the environment where they live. Several invertebrates have been used in these investigations, with *Artemia* spp. being one of the most frequent in toxicity tests (Asem *et al.*, 2010). The commonly known brine shrimp is a small filter-feeding marine crustacean belonging to the phylum Arthropoda, class Branchiopoda, and order Anostraca (Asem *et al.*, 2010).

Throughout time, several species have been identified with seven species currently recognized, namely: *A. salina* (or *A. tunisiana*) (Europe and North Africa); *A. franciscana* (America and Oceania); *A. monica* (Mono Lake, USA); *A. persimilis* (Argentina); *A. urmiana* (Lake Urmia, Iran); *A. sinica* (Asia); *A. parthenogenetica* (Africa, Asia and Europe) (Browne & Wanigasekera, 2000).

1.3.2 Life cycle and morphology

Artemia franciscana is capable of producing larvae (nauplii) by ovoviviparity and eggs (cysts - Figure 6A) whose embryos, under certain environmental conditions, enter diapause (temporary growth arrest), becoming dormant (IPMA, 2023). The cysts usually float in waters with high salinity and are carried to beaches, where they accumulate and dry out, leading to inactivation of the diapause mechanism (Dhont, 2019). When conditions are favorable (temperature between 25 and 28 °C, illumination of at least 2000 lux for a minimum of 6 hours, strong aeration, and salinity at 27 PSU), they hatch into nauplii (Figure 6B), with a length of about 430 µm (IPMA, 2023). The first larval stage (instar I, developmental phase - Figure 6C) appears at 3 hours with a fixed nutritional profile since its digestive system is not yet developed. After 8 hours, it reaches the second larval stage (instar II, metanauplius phase - Figure 6D), in which small food particles (such as algae cells, bacteria, detritus) are filtered and ingested (FAO, 2015).

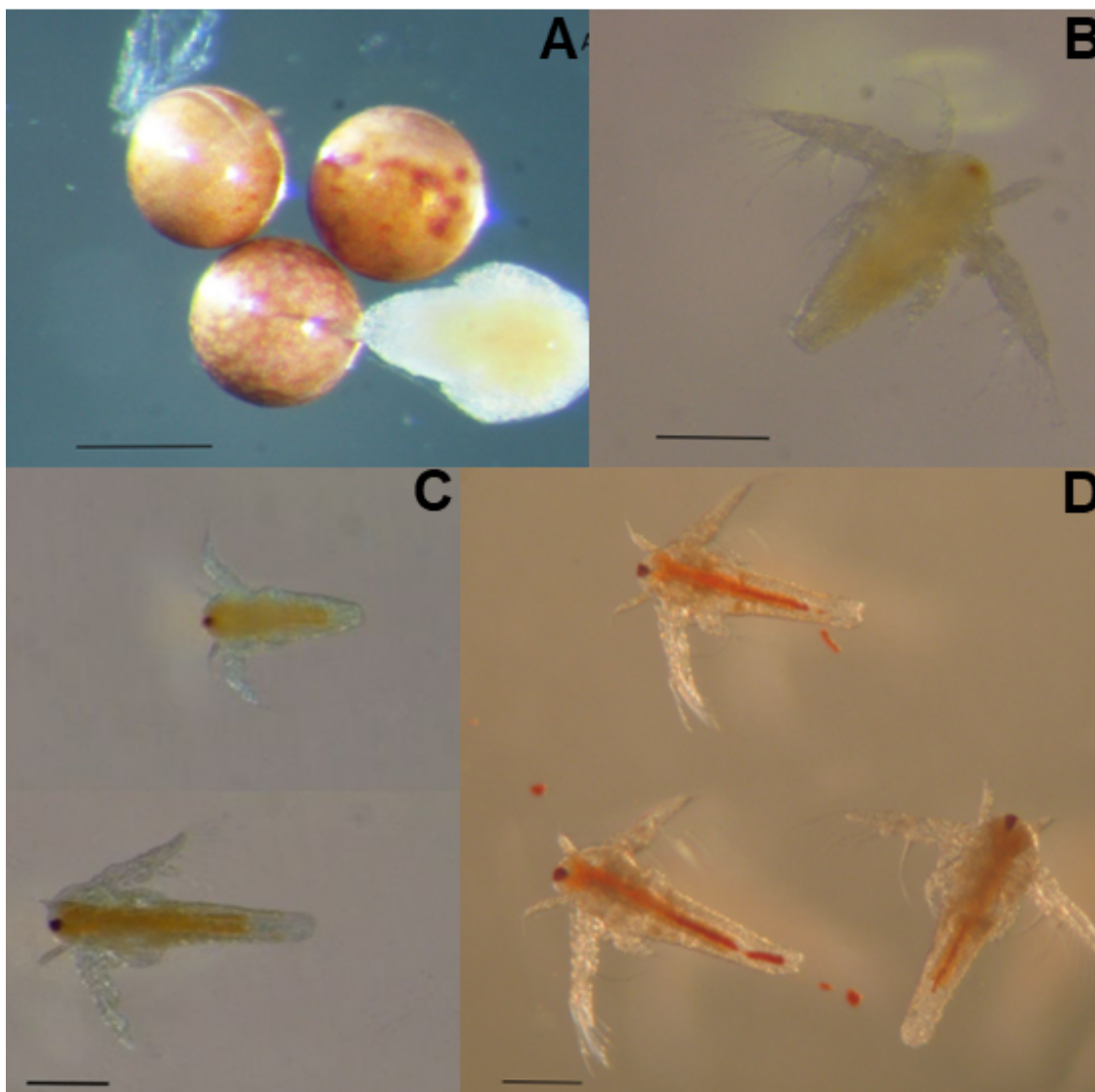


Figure 6 - Stages of the life cycle of *Artemia franciscana*: A – cysts; B – artemia nauplii; C – instar I artemias; D – instar II artemias; black bar = 200 micrometers (Source: <https://www.ipma.pt/pt/pescas/eppo/alimentovivo/index.jsp>).

Throughout the next 10 days, primitive thoracopods and bilateral eyes develop (Piper, 2018). At the end of the third week, the larvae grow from 6 to 8 mm and the shape becomes similar to the adults (pre-adult stage). Maturation ends at 4/5 weeks, when individuals reach 10 mm and become capable of reproducing (adult phase) and may even reach 15-20 mm in length (Piper, 2018).

The external morphology (Figure 7) of an adult *Artemia* includes (Piper, 2018):

- Head: the head is composed of a pair of antennae, which are used to detect food and other substances in the environment. *Artemia* also has compound eyes that can detect light and shadow.

- Thorax: the thorax is composed of 11 segments, each with a pair of thoracopods.
- Abdomen: the abdomen is composed of several segments as well and ends in a bifurcated tail. The tail is used for propulsion and balance in the water.
- Antennae: the antennae are sensory organs that help detect food and other stimuli in the environment.
- Compound eyes: the compound eyes of *Artemia* are sensitive to light and shadow, allowing the animal to detect the presence of predators and other dangers in the environment.

This morphology can vary slightly between different species of *Artemia* and between individuals within the same species (Piper, 2018).

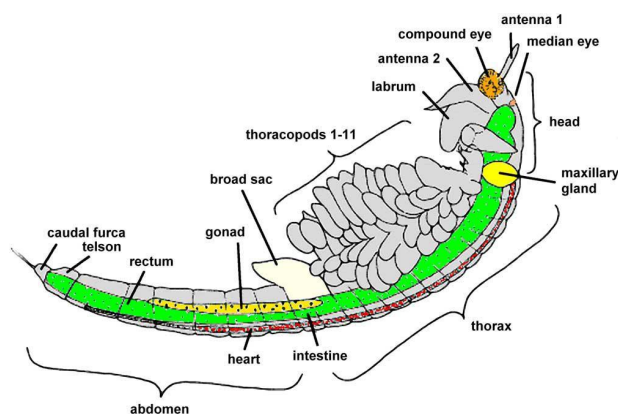


Figure 7 - External morphology of an adult *Artemia* spp. individual (Piper, 2018).

1.3.3 Ecological and ecotoxicological importance of *Artemia* spp.

Not all *Artemia* spp. are considered model species. *A. salina* is commonly used in fields like physiology, genetics, and evolutionary research. Likewise, *A. franciscana* has a high tolerance to extreme conditions that make it a valuable species for scientific research, which is why it has been widely used in areas such as ecotoxicology (Lenormand *et al.*, 2018). In addition, it is a small organism and easy to cultivate, with a constant commercial availability all year and cost efficiency (Kokkali *et al.*, 2011), has a short life cycle which allows experiments to be carried out in short periods of time, has a transparency that enables the study of its internal structures and biological processes and has a rapid response capacity to environmental stimuli (Albarano *et al.*, 2022). Although *A. franciscana* is the best-known model species in this genus, other species can also be used in scientific studies, but their application is less common. Therefore, *A. franciscana* was the species selected for this study.

From an ecological point of view, all *Artemia* species are fundamental in the hypersaline food web, where they constitute the dominant zooplankton. They are the main prey of aquatic birds (Sánchez *et al.*, 2006) and the primary consumer of phytoplankton (Lenormand *et al.*, 2018). *Artemia* spp. is also one of the main sources of live food used in aquaculture and plays a fundamental role in the flow of energy in the food chain of marine ecosystems. This species have a wide geographical distribution and an extraordinary ability to adapt to different hypersaline environments, such as salt lakes, coastal lagoons, and artificial salt pans with high concentrations of sodium chloride (Nunes *et al.*, 2006). In these environments, the absence of predators favors the abundance of *Artemia*, which feeds on bacteria, protozoa, and algae.

According to Terwilliger and Ryan, 2001, *Artemia* spp. are also capable of efficiently synthesizing respiratory pigments (hemocyanin) to cope with low levels of dissolved oxygen that are usually characteristic of hypersaline environments (Terwilliger & Ryan, 2001). *A. franciscana* shows a higher concentration of hemocyanin (Terwilliger & Ryan, 2001), which is another important characteristic that allows this species to successfully overcome environmental adversities (Mangum, 1980). *A. franciscana* inhabits hypersaline environments such as salt lakes, coastal lagoons, and solar salterns, which makes it able to tolerate large changes in salinity, ionic composition, temperature, and oxygen saturation (Lenormand *et al.*, 2018). Since there are few animals capable of tolerating osmotic stress, the absence of predators and competitors allows the formation of monocultures (Lenormand *et al.*, 2018).

An important characteristic to consider when selecting test organisms is the type of reproduction among different strains of *Artemia*. Since different strains with different sexual behaviors often coexist in the same habitat, it is essential to control reproductive patterns to meet the requirements of cost-effectiveness, homogeneity, reliability, viability and predictability of responses among tests (Nunes *et al.*, 2006). Therefore, it is widely used in ecotoxicology as a biological model. The referred genetic homogeneity is important because by minimizing the genetic differences between the individuals used in tests, it's possible to better control other variables and factors that may influence the results and it also facilitates the standardization of the experiments and the comparison of the results between different studies and laboratories (Costa *et al.*, 2008).

1.4 Biomarkers

In 1998, the National Institutes of Health Biomarkers Definitions Working Group defined biomarker as “a characteristic that is objectively measured and evaluated as an

indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention” (Atkinson *et al.*, 2001). In the report on the validity of biomarkers in environmental risk assessment, the WHO has stated that a true definition of biomarkers includes “almost any measurement reflecting an interaction between a biological system and a potential hazard, which may be chemical, physical, or biological. The measured response may be functional and physiological, biochemical at the cellular level, or a molecular interaction” (International Programme on Chemical Safety, 1993).

The use of biomarkers in ecotoxicology is important since they can be used to assess the exposure of living organisms to toxic substances in the environment (López-Barea, 1995). Regular monitoring of biomarkers in sensitive species allows detecting changes in environmental health over time (Hagger *et al.*, 2006). They can also be used as ecological risk indicators, helping as early warning of possible contamination and to assess the impact of toxic substances at population and ecosystem level. For this, biomarkers should be determinable by a specific, sensitive, reproducible, and robust assay; being measurable within the detection limits of a reliable analytical procedure (Griffiths *et al.*, 2002).

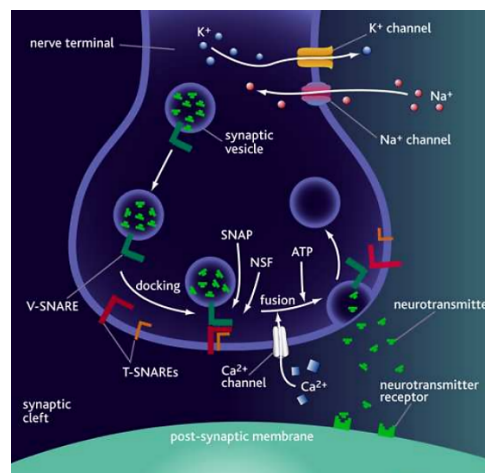
In general, biomarkers can be classified into three types: exposure, effect and susceptibility (Hagger *et al.*, 2006). Those of exposure indicate the exposure of an organism to chemical substances, environmental pollutants, or toxic agents, providing important information about the amount and duration of exposure. Effect biomarkers are used to evaluate the biological effects caused by the exposure to a certain substance or condition. And the susceptibility biomarkers indicate an individual's predisposition to respond in a particular way to a specific exposure (Hagger *et al.*, 2006).

1.4.1 Biochemical biomarkers

Biochemical biomarkers play a key role in ecotoxicology, as they reflect biochemical or physiological changes in organisms exposed to toxic substances or environmental stressors. These biochemical biomarkers fall under the effect biomarkers (Lionetto *et al.*, 2013; Niki, 2014; Jurisic *et al.*, 2015) and make it possible to monitor the exposure of organisms to toxic substances over time, providing information on the effects caused by a given contamination; help assess environmental risk and identify areas of concern regarding a given contamination. In addition, they are very important for the determination of safe limits of exposure to chemical substances, helping to establish regulatory standards to protect the environment and the health of living beings (Oost *et al.*, 2003; Walker, 1995).

For this work, the biomarkers in focus were acetylcholinesterase activity (AChE), activity of catalase (CAT) and glutathione S-transferases (GSTs), lipid peroxidation, and lactate dehydrogenase activity (LDH), widely used in ecotoxicological studies (Brandão *et al.*, 2013; Prokić *et al.*, 2019).

Acetylcholinesterase is one of the oldest biomarkers and has been initially tested for pesticide detection and is now used for various types of pollutants. It is an enzyme responsible for hydrolyzing the neurotransmitter acetylcholine (ACh) in cholinergic synapses, which acts in the transmission of the message from one neuron to another (Brunton & Brunton, 2012). The acetylcholine cycle is shown in Figure 8, in which AChE deactivates the excess



of this neurotransmitter. These synapses are important in maintaining a variety of physiological functions (e.g. nerve transmission in the central nervous system (H. Thany & Tricoire-Leignel, 2011)), as they are distributed throughout the central and peripheral nervous systems (Brunton & Brunton, 2012), most commonly in vertebrates and humans, while other organisms may use different neurotransmitters (Wiesner *et al.*, 2007).

The quantification of acetylcholinesterase is an important biomarker in studies evaluating different types of pollutants, since it is an indicator that an organism may have been exposed to neurotoxic pollutants (Lionetto *et al.*, 2013; Fu *et al.*, 2018). These pollutants can have the ability to inhibit AChE activity, leading to an excessive accumulation of acetylcholine in the nervous system (Phyu & Tangpong, 2014), affecting neurotransmission.

For survival and balance between free radicals and antioxidant defenses to be maintained, the existence of complex defense mechanisms, such as antioxidant enzymatic and non-enzymatic defenses (Table 2) is crucial (Lobo *et al.*, 2010). These defensive agents are antioxidants that delay or inhibit cellular damage mainly through their free radical scavenging property. They can safely interact with free radicals and terminate the chain reaction before vital molecules are damaged (Halliwell *et al.*, 1995). Some of these antioxidants, such as glutathione, ubiquinol and uric acid, are naturally produced during normal metabolism in the body (Shi *et al.*, 1999).

Table 2 - Identification of Enzymatic and Nonenzymatic antioxidant defenses (Adapted from: Lobo *et al.*, 2010).

Antioxidants		
Enzymatic	Nonenzymatic	
Superoxide dismutase (SOD)	Vitamin E (α -tocopherol)	Ubiquinol
Catalase (CAT)	Vitamin C (Ascorbic Acid)	Uric acid
Glutathione system (Glutathione, Glutathione reductase, Glutathione peroxidase, Glutathione S-transferases)	Vitamin A (Retinol)	Bilirubin
	Carotenoids (β -carotene)	Thiol group (Glutathione, Thioredoxin)
	Melatonin	Polyphenols (Flavonoids)

Catalase (CAT) is an antioxidant enzyme that is present in almost all aerobic organisms, and works by breaking two molecules of hydrogen peroxide into one molecule of oxygen and two molecules of water (Ossowski *et al.*, 1993). This enzyme plays an important role in protecting cells against free radicals (resulting from oxidative metabolism). Since, oxidative stress (OS) can occur when there is an imbalance between the production of reactive oxygen species (ROS) and the antioxidant defense mechanisms of cells. By converting hydrogen peroxide into water and oxygen, CAT lowers the concentration of ROS and protects cells against their harmful effects (Nandi *et al.*, 2019).

The effect of this enzyme is evaluated as follows: if catalase activity is increased, it may be a sign that the body is under increased production of ROS and is defending itself by activating its antioxidant enzymes; on the other hand, a decrease in catalase activity may indicate an overload of oxidative stress and the inability of cells to deal with the excessive production of ROS (Nandi *et al.*, 2019), and show the presence of certain compounds which inhibit its activity (Xianyong *et al.*, 2016).

Another way to measure oxidative stress is through the activity of glutathione S-transferases (GSTs), which are a class of detoxification enzymes present in phase II reactions of catalyzing xenobiotics (Xu *et al.*, 2018), that have the ability to neutralize toxic compounds by conjugating glutathione as a substrate to xenobiotics (Pour *et al.*, 2014). These enzymes are involved in the detoxification and removal of a wide range of compounds, including some exogenous chemicals and xenobiotics, reactive metabolic products and ROS (Sheehan *et al.*, 2013; Varó *et al.*, 2007). Therefore, it can be said that GSTs have a dual role - in detoxification and in the regulation of oxidative stress - which is essential for the homeostasis of organisms.

Lipid peroxidation results from a disturbance of the physiological balance between the oxidant and antioxidant system, where the oxidant action is favored, leading to cell damage (oxidative damage) (Lobo *et al.*, 2010). Biomarkers of oxidative damage (as lipid

peroxidation) are part of the group of biological effect markers, that include effects in three types of molecules - lipids, proteins, and DNA. When free radicals attack these three structures, they can interfere with lipids, causing lipid peroxidation (Repetto *et al.*, 2012). The high production of ROS can be based on sources such as chemicals, tobacco, cigarette smoke, ozone, radiation, infectious agents, industrial solvents, pesticides, pharmaceuticals etc. (Phaniendra *et al.*, 2015).

When the production rate of free radicals is higher than the elimination rate, antioxidants cannot respond effectively, which causes an imbalance and can trigger cellular damage at the level of lipids (e.g., lipid peroxidation), proteins, and DNA (Barbosa *et al.*, 2008). Thus, the existence of biomarkers capable of measuring and evaluating the mechanisms and biological implications associated with oxidative damage, such as thiobarbituric acid reactive substances (TBARS) that are formed as a byproduct of lipid peroxidation (i.e. as degradation products of fats), is crucial for effective ROS control and to understand the potential impact of pollutants in the organism homeostasis (Barbosa *et al.*, 2008).

Lactate dehydrogenase (LDH) is an isoenzyme present in many tissues and cells of various living systems, showing a great variation between animal species (Klein *et al.*, 2020). This enzyme catalyzes the production of lactate during the anaerobic process of glucose, which led to an increase in the lactic acid content in the organisms (Ambili *et al.* 2013). This response might also serve to face the chemical stress caused by the toxicity of pollutants when the expression of this enzyme's activity increases in relation to baseline levels, it may be an indication of tissue damage (Rehak *et al.*, 2000; Miao *et al.*, 2013). This finding is related to the fact that with the increase and decrease in exposure concentrations organisms can alter energy reserve levels and pathways to obtain energy, to fight against oxidative stress caused by of stressor agents. Thus, LDH activity can be used as a biomarker to diagnose and monitor various health conditions, in particular, hepatocyte damage, muscle damage (skeletal or cardiac muscle) and hemolysis (or prolonged serum contact with erythrocytes) (Klein *et al.*, 2020).

1.5 Objectives

Although there are studies that show the interactions between climate change and chemical pollution (e.g., United Nations Environment Programme, 2021; Naidu *et al.*, 2021), there are no published studies that address the influence of temperature on the impacts caused by cigarette butts in organisms, such as marine species. Hence, this study aimed to evaluate the potential toxic effects of cigarette butts (smoked and

non-smoked), under different temperature conditions (25 and 30 °C) on individuals of *Artemia franciscana*. The temperatures tested were based on the optimal conditions for *A. franciscana* – 25° C (Manfra *et al.*, 2015) – and the projected increase of 5 °C by the end of the century – 30° C (IPCC, 2022). On the other hand, comparing smoked and non-smoked conditions, allows the evaluation of the toxicity of various chemicals stemming from the combustion of tobacco that are not originally present in non-smoked cigarette butts.

In order to understand the ecotoxicological effects that different CBs can have on non-target organisms and the influence of temperature on the sensitivity of the organisms to this environmental pollutant, several ecotoxicological endpoints were evaluated. For this, daily mortality was monitored, and growth and a battery of biomarkers were assessed, after 7 days of exposure. Several biomarkers involved in different physiological functions and metabolic pathways were evaluated, namely: i) neurotoxicity - determination of the acetylcholinesterase activity; ii) oxidative stress - determination of the activity of the catalase and glutathione s-transferases; iii) lipid peroxidation (thiobarbituric acid reactive substances levels), and energy production – lactate dehydrogenase activity.

2. Material and Methods

2.1 Preparation of artificial seawater

The artificial seawater (ASW) was obtained by mixing deionized water with sea salt (TropicMarin® Sea Salt, Germany), with salinity set to 35 PSU. Next, the ASW was filtered through Whatman® cellulose nitrate membrane filters (1.0 µm pore size and 47 mm diameter) using a vacuum pump (VWR® - VCP 80) and aerated for 48 h prior to use. The final ASW was used to hatch *Artemia franciscana* cysts and to prepare the different stock and test solutions.

2.2 Preparation of test solutions

The CBs used in this study were purchased from the same brand and smoked by the same person to reduce variability and minimize the risk of contamination. Two stock-solutions were initially prepared: one with smoked cigarette butts (SCBs) and other with non-smoked cigarette butts (nSCBs), at a final concentration of 1 CB/L. These were stirred for 24 hours at room temperature (23 ± 1 °C) and covered with aluminum foil to avoid chemical degradation caused by light. Afterwards, the solutions were filtered through Whatman® cellulose nitrate membrane filters (1.0 µm pore size and 47 mm

diameter) using a vacuum pump (VWR® - VCP 80) and stored at 4 °C until used (in less than a week) (Lee & Lee, 2015). A dilution factor of 2x was applied to the stock-solutions in the preparation of the five test solutions, *i.e.*, the final concentrations tested were 0.03125, 0.0625, 0.125, 0.25 and 0.5 CBs/L, for both smoked and non-smoked cigarette butts.

The tested concentrations were chosen based on preliminary results where concentrations of 0.187, 0.375, 0.75, 1.5 and 3.0 nSCBs/L and SCBs/L were evaluated. At 48h, an LC₅₀ of 0.94 and 0.98 nSCBs/L at 25 and 30 °C, respectively, and 1.26 SCBs/L at 30 °C were obtained for *Artemia franciscana*.

2.3 Culture and growth of *Artemia franciscana*

Artemia franciscana cysts from Great Salt Lake (Utah, USA) (Ocean Nutrition™, Belgium) were hatched in a 2 L Erlenmeyer flask containing ASW, with continuous aeration under 24 h light photoperiod and temperature 28 ± 1 °C (Varó *et al.*, 2015). Continuous lighting is required to trigger the hatching mechanism in embryos, and studies have shown that a 24 h photoperiod had 95% hatching percentage for *Artemia* spp. embryos (Arun *et al.*, 2017). After the first 24 hours, under a magnifying glass, the phototrophic nauplii were transferred using a light source and a Pasteur pipette to glass flasks with ASW.

2.4 *Tetraselmis suecica* culture

The green microalgae *Tetraselmis suecica* was used as a food source for *Artemia franciscana* (Varó *et al.*, 2015) during the sub-chronic assay. Algae cultures were established under continuous light and aeration at room temperature (23 ± 2 °C) using ASW, under the same salinity conditions mentioned above, and 1% Fabregas medium for growth (Fabregas *et al.*, 1984). Moreover, algae count was carried out in a Neubauer chamber to adjust the final volume of algae to 200 000 cells/mL of *T. suecica*.

2.5 Sub-chronic exposure

The sub-chronic assay was conducted in glass test tubes for 7 days (168 h). A total of 10 replicates with six *A. franciscana* individuals each were used per concentration of SCBs and nSCBs (0.03125, 0.0625, 0.125, 0.25 and 0.5 CBs/L) at 25 °C and 30 °C, in a final volume of 30 mL of test solution. The control group was performed at both temperatures, and the organisms were exposed in non-contaminated ASW (0.0 CBs/L).

Test tubes were covered with parafilm to avoid external contamination allowing, nevertheless, gas exchange. The tubes were maintained in a water bath at 25 °C and 30 °C, with a photoperiod of 14h:10h (light:dark) (Loredana Manfra *et al.*, 2012), and the medium was renewed at the 2nd and 5th days for feeding purposes, at a final concentration of 200.000 cells/mL in each tube. Daily observations were made regarding the mortality, and on the last day of exposure, one individual per replicate was photographed to assess the growth (10 replicates per concentration and condition), using ImageJ program (Schneider *et al.*, 2012). Mortality was defined as total absence of movement, i.e., cessation of swimming activity and/or movement of the appendages, after resuspension of the medium and observation for 10 seconds. Subsequently, all living individuals were collected and stored at -80 °C until the performance of biochemical biomarkers determinations.

2.7 Biochemical biomarkers

After exposure, organisms from different replicates were randomly separated to different biochemical analysis: 3 replicates with 5 individuals for AChE determination; 3 replicates with 9 individuals for oxidative stress and lipid peroxidation biomarkers, such as CAT, GSTs activities and TBARS levels; and 3 replicates with 5 individuals for LDH activity.

2.7.1 Total protein

Total protein concentration of samples was determined in all samples according to the methodology described by Bradford (1976), adapted to microplate, in order to express all biochemical biomarkers per mg of protein. The Bradford method (Kruger, 2002; Fanglian, 2011) involves the binding of a dye to the total protein, originating a colored and stable complex that can be quantified at 595 nm. γ -globulin (1 mg/mL) was used as standard.

2.7.2 Acetylcholinesterase

For the quantification of AChE, the samples were first homogenized in 1 mL of phosphate buffer solution pH 7.2 at 4 °C, using a sonicator (Misonix Microson Ultrasonic

Cell Disruptor XL), for about 10 seconds. Subsequently, the samples were centrifuged at 4 °C, for a period of 3 minutes at 6000 rpm. AChE activity was performed according to the protocol described by (Ellman *et al.*, 1961), using acetylthiocholine as substrate, and was determined by the increase of absorbance at a wavelength of 412 nm, which occurs when AChE hydrolyses the substrate acetylthiocholine forming thiocholine. This compound reacts with 5-50-dithio-bis-2-nitrobenzoate (DTNB) forming a complex that absorbs radiation in the above-mentioned wavelength. The enzymatic activity was expressed as nmol of the complex formed, per minute, per milligrams of total soluble protein.

2.7.3 Oxidative stress

For biomarkers of stress and oxidative damage, catalase, glutathione-s-transferases activities and thiobarbituric acid reactive species levels were quantified. Firstly, samples were sonicated in 500 µL of ice-cold phosphate buffer (50 mM, pH = 7.0 with 0.1 % of Triton X-100) using a sonicator (Misonix Microson Ultrasonic Cell Disruptor XL). After that, the homogenates were centrifuged at 14,000 rpm for 10 min at 4 °C. The quantifications were adapted to microplates and determined in quadruplicates, except for TBARS levels which were measured in triplicates.

CAT activity was assayed by the procedure described by Aebi (1984), in which the degradation rate of the substrate H₂O₂ for 5 min and at 240 nm was monitored. The results were expressed by considering that one unit of activity equals the number of moles of H₂O₂ degraded per minute, per milligram of protein (Aebi, 1984).

Glutathione transferases are a group of enzymes that catalyze the conjugation reaction of the 1-chloro-2,4-dinitrobenzene (CDNB) substrate with glutathione (GSH), forming a thioether that can be monitored by the increase in absorbance at 340 nm. GSTs activity was determined according to Habig *et al.* (1974), and expressed as millimoles of thioether produced per minute, per milligram of protein.

Finally, TBARS levels, which are indicators of lipid peroxidation or oxidative damage, were measured by the quantification of thiobarbituric acid reactive substances, according to Buege & Aust (1978). Absorbance readings of each sample were performed at 535 nm. This methodology is based on the reaction of compounds such as malondialdehyde (MDA) formed by degradation of initial products of free radical attack, with 2-thiobarbituric acid (TBA). TBARS levels were expressed as malondialdehyde (MDA) equivalents per milligram of protein.

2.7.4 Lactate dehydrogenase activity

Samples for the quantification of LDH activity were homogenized in 1 mL of TRIS buffer (1 M, pH 7.2) cold, for about 10 seconds. Then, 3 consecutive cycles of freezing/melting at -20 °C and room temperature were performed, followed by centrifugation of the samples at 4 °C, for a period of 3 minutes at 6000 rpm. LDH activity was determined following the method of Vassault (1983). Determination of its activity was performed by measuring the reduction of absorbance caused by the oxidation of the reduced form of nicotinamide adenine dinucleotide (NADH) in the presence of pyruvate, at a wavelength of 340 nm. Activity was expressed in millimoles of β -NADH oxidized per minute, per milligram of protein.

2.8 Data analysis

After performing the normality and homogeneity tests with the Shapiro-Wilk Test and the Levene's Test, respectively, the sub-chronic effects of SCB or nSCB concentrations and temperatures were analyzed for all biochemical parameters (AChE, CAT, GSTs, TBARS and LDH) and growth. For growth parameter, and to assess the ANOVA assumptions the data were transformed using $\log(x+1)$ (x = growth value in mm) to validate the normality test. Data processing was performed in IBM SPSS Statistics 26 software, using a two-way analysis of variance (ANOVA) to assess the ecotoxicological effects of SCB or nSCB in *A. franciscana*, and the influence of temperature, followed by a Dunnett test to discriminate differences between CB concentration and the respective control treatment ($p \leq 0.05$). A significance level (α) of 0.05 was used in all analysis.

3. Results

3.1 Mortality

3.1.1. Smoked cigarette butts

The mortality rate results observed throughout the 7 days (168 h) in the organisms exposed to SCBs concentration at 25 and 30°C are presented in Table 3. LC_{50} values were not determined due to the low mortality rates registered (< 15%).

As seen in Table 3, the toxicity of SCBs was not linear, *i.e.*, an increase in the mortality rate was not observed as the concentration increased at both temperatures.

Briefly, the highest mortality rates were all observed at 30 °C at 0.0625 (15%), 0.125 (11.67%) and 0.500 (10%) SCBs/L.

Moreover, no significant differences were found between the control group and the tested concentrations, at each temperature.

Table 3 - Cumulative mortality rate (%) of the individuals exposed to smoked CBs (SCBs) at 25 and 30 °C.

Concentration SCBs/L	25 °C						
	24 h	48 h	72 h	96 h	120 h	144 h	168 h
0.000	0%	0%	3.33%	3.33%	3.33%	3.33%	3.33%
0.03125	0%	1.67%	1.67%	1.67%	1.67%	3.33%	3.33%
0.0625	0%	0%	0%	0%	0%	1.67%	1.67%
0.125	0%	0%	1.67%	1.67%	1.67%	1.67%	1.67%
0.250	0%	0%	1.67%	3.33%	3.33%	3.33%	3.33%
0.500	0%	0%	0%	0%	0%	1.67%	1.67%
	30 °C						
	24 h	48 h	72 h	96 h	120 h	144 h	168 h
0.000	0%	0%	0%	0%	0%	0%	3.33%
0.03125	0%	0%	0%	0%	1.67%	6.66%	8.33%
0.0625	0%	1.67%	1.67%	1.67%	8.33%	15%	15%
0.125	0%	1.67%	1.67%	1.67%	8.33%	11.67%	11.67%
0.250	0%	0%	0%	1.67%	1.67%	3.33%	3.33%
0.500	1.67%	3.33%	3.33%	6.66%	6.66%	8.33%	10%

3.1.2. Non-smoked cigarette butts

The mortality rate of the *A. franciscana* individuals exposed to nSCBs at 25 and 30 °C is presented in Table 4. Similar to SCBs, LC₅₀ values were not determined due to low mortality rates (< 12%) and toxicity was not linear at both temperatures.

Here, the concentrations with the highest mortality rates were 0.500 nSCBs/L, at 30 °C, and 0.0625 nSCBs/L, at 25 °C, with a 11.67% and 10% mortality rate, respectively.

Furthermore, no significant differences were found between the control group and the tested concentrations, at each temperature.

Table 4 - Cumulative mortality rate (%) of the individuals exposed to non-smoked CBs (nSCBs) at 25 and 30 °C.

Concentration nSCBs/L	25 °C						
	24 h	48 h	72 h	96 h	120 h	144 h	168 h
0.000	0%	0%	3.33%	3.33%	3.33%	3.33%	3.33%
0.03125	0%	0%	0%	0%	1.67%	1.67%	1.67%
0.0625	0%	8.33%	10%	10%	10%	10%	10%

0.125	0%	1.67%	1.67%	1.67%	1.67%	1.67%	1.67%
0.250	1.67%	1.67%	1.67%	1.67%	1.67%	3.33%	3.33%
0.500	0%	1.67%	1.67%	1.67%	1.67%	1.67%	1.67%
	30 °C						
0.000	0%	0%	0%	0%	0%	0%	3.33%
0.03125	0%	3.33%	3.33%	3.33%	5%	5%	6.66%
0.0625	0%	0%	0%	0%	1.67%	3.33%	3.33%
0.125	0%	0%	0%	0%	0%	0%	0%
0.250	0%	0%	0%	0%	0%	0%	0%
0.500	3.33%	3.33%	3.33%	3.33%	8.33%	11.67%	11.67%

3.2. Growth

Figure 9 shows the results of *Artemia franciscana* growth (mm) after 7 days of exposure to both smoked and non-smoked CBs at 25 and 30 °C. A significant interaction with SCBs concentrations and temperature was observed (Figure 9; Table 5). The temperature vs SCBs seem to have an effect on growth. Overall, in the organisms exposed to SCBs, higher growth results were found at highest temperature (30 °C) when compared to 25 °C, with the exception of 0.25 SCBs/L. Although the 2-way ANOVA results (Table 5) demonstrate significant differences, the Dunnet test did not detect these differences between SCBs concentrations and the respective control, for each temperature.

Regarding individuals exposed to nSCBs (Figure 9), a significant interaction between temperature and concentration was also observed in *A. franciscana* after sub-chronic exposure (Figure 9; Table 5). Indeed, at 25 °C, a significant decrease in growth was recorded at concentration of 0.500 nSCBs/L (Figure 9). At 30 °C, a significant decrease in growth at 0.0625 nSCBs/L and a significant increase at 0.500 nSCBs/L were registered (Figure 9), when compared to the control group.

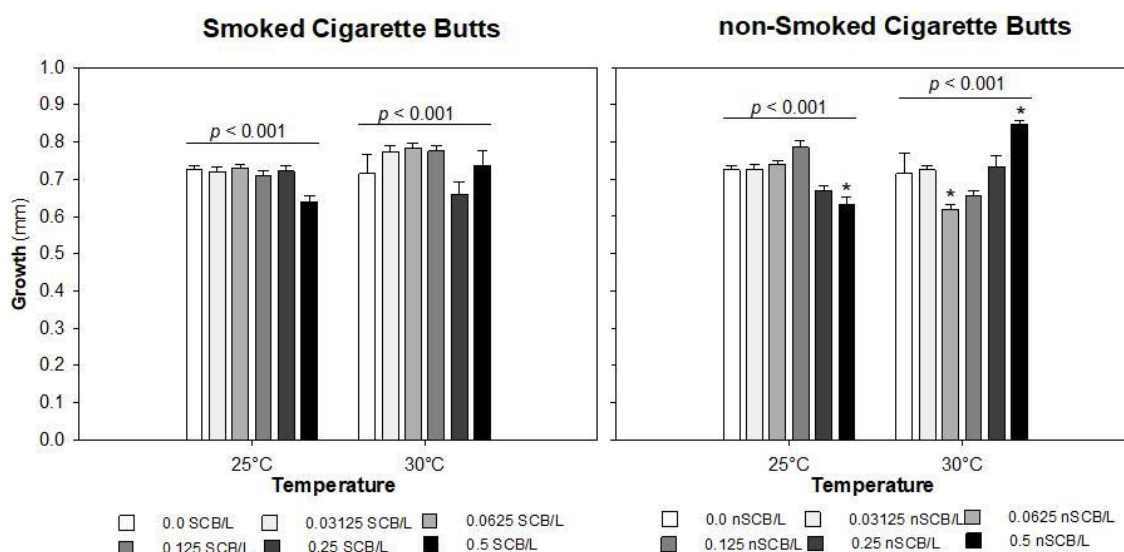


Figure 9 - Results of effects on growth in *Artemia franciscana*, after sub-chronic exposure to smoked cigarette butts (SCB) and temperatures (left) and non-smoked cigarette butts (nSCB) and temperatures (right). Data are expressed as mean $\pm$ standard error bars. Significant (p level) effects of CBs for each temperature are shown, with asterisks (*) discriminating significant differences between the control group and CBs concentrations (Dunnett test; $p < 0.05$).

3.3 Biochemical biomarkers

Regarding AChE activity (Figure 10), significant interactions between SCBs and temperature were recorded (Table 5). A significant increase in AChE activity was verified in *A. franciscana* exposed to 0.25 SCBs/L at 25 °C (Figure 10). Non-significant effects of SCBs for 30 °C were observed. In nSCBs, no interaction between the two factors was observed, however statistically significant differences between temperatures were present (Table 5).

In oxidative stress biomarkers, such as CAT activity (Figure 10; Table 5), a significant interaction between factors (temperature vs smoked or non-smoked CBs) was observed (Table 5). Furthermore, for SCBs, a decrease in CAT activity at 25 °C for all concentrations tested, and an increase in CAT activity at 30 °C in all concentrations, except for 0.0625 SCBs/L were observed. In nSCBs, an increase of CAT activity was observed in two temperatures, in all concentrations except one (0.03125 nSCBs/L for 25 °C and 0.0625 nSCBs/L for 30 °C).

Regarding the activity of GSTs (Figure 10), the response was significant in both CBs exposures, with a significant interaction between factors (Table 5). In the case of SCBs, and at 25 °C, an increase of GSTs activity was only observed in 0.25 and 0.5 SCBs/L. Significant effects on GSTs activity were not observed, in organisms exposed SCBs for 30 °C. On the other hand, the concentrations of nSCBs caused significant

increase of GSTs activity in all concentrations at 25 °C and a significant decrease in organisms exposed to 0.25 nSCBs/L, at 30 °C (Figure 10).

For lipid peroxidation results (TBARS levels; Figure 10), exposure to SCBs conditions had significant interaction in both temperatures (Table 5). However, for both temperatures (25 and 30 °C), we observed an increase of TBARS levels in exposed organisms to 0.25 and 0.5 SCBs/L, at 25 °C, and in all concentrations SCBs (except 0.0625), at 30 °C. Significant effects of TBARS levels, were recorded in nSCBs due only to the effect of temperature (no interaction between the two factors was observed and no effect of the concentration of nSCBs; Table 5).

For the LDH activity (Figure 10; Table 5), temperature and CBs concentration had a significant interaction on both smoked and non-smoked CBs (Table 5). At 25 °C, a significant decrease was observed in both smoked and non-smoked CBs, at some concentrations (0.03125, 0.0625 and 0.5 SCB/L and 0.5 nSCBs/L). At 30 °C, the opposite occurred, that is, a significant increase was observed in both smoked and non-smoked CBs, in only one concentration of each condition (0.5 SCB/L and 0.25 nSCBs/L).

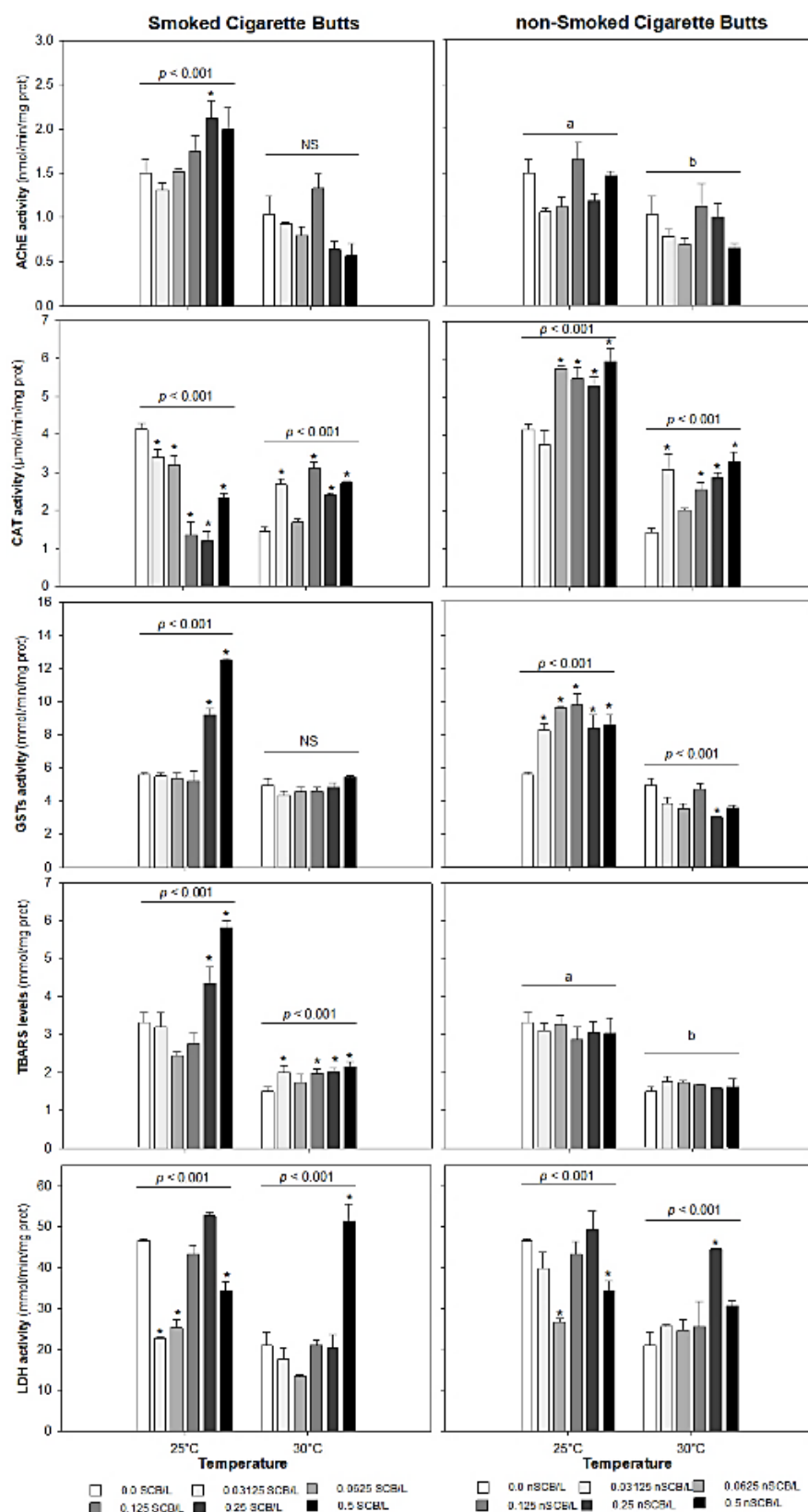


Figure 10 – Biochemical results of sub-chronic exposure to smoked (left) and non-smoked (right) CBs and different temperatures: effects on acetylcholinesterase activity (AChE), catalase activity (CAT), glutathione S-transferases activity (GSTs), lipid peroxidation (TBARS levels) and lactate dehydrogenase (LDH) activity in *Artemia franciscana*. Data are expressed as mean $\pm$ standard error bars. Non-significant (NS) or significant (p level) effects of CBs for each temperature are shown, with asterisks (*) discriminating significant differences between the control group and CBs concentrations (Dunnett test; $p < 0.05$); different letters (a, b) stand for significant differences between conditions.

Table 5 - Summary table of the two-way ANOVA applied to temperature variation and smoked (SCB) and non-smoked (nSCB) cigarette butts' concentrations during sub-chronic exposure. Bold values stand for significant differences between tested factors (cigarette butts vs temperature) ($p < 0.05$).

	Endpoint	Source variation	F	d.f.	p
SCB	Growth	Temp	5.545	1	0.002
		SCB	2.845	5	0.030
		Temp x SCB	2.821	5	0.021
		Residual		108	
	AChE	Temp	87.438	1	<0.001
		SCB	2.025	5	0.111
		Temp x SCB	5.694	5	0.001
		Residual		24	
	CAT	Temp	5.989	1	0.022
		SCB	1.819	5	<0.001
		Temp x SCB	42.228	5	<0.001
		Residual		24	
	GSTs	Temp	197.515	1	<0.001
		SCB	60.303	5	<0.001
		Temp x SCB	39.367	5	<0.001
		Residual		24	
	TBARS	Temp	133.335	1	<0.001
		SCB	16.587	5	<0.001
		Temp x SCB	15.812	5	<0.001
		Residual		24	
	LDH	Temp	97.634	1	<0.001
		SCB	31.472	5	<0.001
		Temp x SCB	29.093	5	<0.001
		Residual		24	
nSCB	Growth	Temp	0.0508	1	0.338
		nSCB	1.993	5	0.010
		Temp x nSCB	18.388	5	<0.001
		Residual		108	
	AChE	Temp	32.225	1	<0.001
		nSCB	3.806	5	0.011
		Temp x nSCB	1.267	5	0.310
		Residual		24	
	CAT	Temp	312.379	1	<0.001
		nSCB	13.077	5	<0.001
		Temp x nSCB	8.382	5	<0.001
		Residual		24	
	GSTs	Temp	319.762	1	<0.001
		nSCB	5.207	5	0.002
		Temp x nSCB	10.145	5	<0.001
		Residual		24	
	TBARS	Temp	110.652	1	<0.001
		nSCB	1.107	5	0.383
		Temp x nSCB	0.279	5	0.920
		Residual		24	
	LDH	Temp	41.730	1	<0.001
		nSCB	10.337	5	<0.001
		Temp x nSCB	4.717	5	0.004
		Residual		24	

4. Discussion

4.1 Mortality

The evaluation of effects of cigarette butts' leachates to aquatic organisms, although scarcely studied, is very important, since CBs are one of the most common types of litter found on beaches, rivers, lakes, and oceans (Araújo & Costa, 2019). Additionally, *Artemia franciscana* plays an important role in the evaluation of CBs' effects as a sensitive organism. In this study, the assessment of mortality is crucial as it is one of the most responsive and immediate indicators of the toxic effects of chemical substances – when an organism dies in response to exposure to a contaminant, this immediately indicates that the substance is very toxic.

The mortality rates registered in the control groups were lower than 10% at all tested conditions. Although there is no standardized protocol for *Artemia franciscana* ecotoxicological assays, the sub-chronic exposure tests performed with this species were validated according to the same threshold values (e.g., validation criteria such as mortality in control group < 10%) defined for other organisms, such as *Daphnia magna* (OECD, 2004); an aquatic branchiopod like *A. franciscana*.

Hence, the results obtained showed that both leachates from smoked and non-smoked CBs had effects on *Artemia franciscana* individuals. Mortality rates, although present, were not significant in either tested condition (maximum of 15% in SCBs and 11.67% in nSCBs). Furthermore, the highest temperature tested (30 °C) presented a greater mortality rate in both treatments, although these differences were not significant, at the end of the study.

Lawal & Ologundudu (2014) conducted a study with the anuran *Hymenochirus curtipes* and the catfish *Clarias gariepinus*, where organisms were exposed up to 10 g/L of leachate of SCBs and nSCBs. Briefly, the results indicated a higher toxicity of SCBs compared to nSCBs: higher mortality rates were observed within 96 hours of exposure, where at the concentration of 0.625 g/L (which is the one closer to the highest concentration tested in our study), for both species, the mortality for the group exposed to SCBs was 20%, while for the group exposed to nSCBs was 10%. This toxicity levels can be explained by the higher concentration of metals (lead, chromium, iron, cadmium, copper, and zinc) in SCBs compared to nSCBs (Lawal & Ologundudu, 2014). The higher concentration of metals in SCBs may be due to the fact that those compounds can be released during the combustion of tobacco, remaining deposited in the smoked CBs. In our work, although we observed a slight difference between SCB and nSCBs, this was not significant, which did not allow us to conclude under the conditions tested here that SCBs

were more toxic than nSCBs, to *A. franciscana*. This may be due to the fact that we are comparing completely different animals that may have different sensitivities (Case et al., 2015), and also because the conditions tested between the two works differ.

However, a study by Slaughter *et al.* (2011) showed that SCBs were more toxic than nSCBs. They evaluated two fish species (*Atherinops affinis* and *Pimephales promelas*) and showed acute toxicity to leachates from smoked (SCBs) and non-smoked cigarette butts (nSCBs). For smoked leachates at 48 hours, LC₅₀ was approximately 1.8 SCBs/L for *P. promelas* and 4.4 SCBs/L for *A. affinis*; for non-smoked leachates, the LC₅₀ was approximately 13.5 nSCBs/L for *P. promelas* and 5.1 nSCBs/L for *A. affinis*, which demonstrated that SCBs leachate had a higher lethality to these two fish species compared to nSCBs (Slaughter *et al.*, 2011). This difference between the conclusions of the presented studies and ours, shows how important it is the need to continue research to expand ecotoxicological information on the effects of cigarette butts' compounds on different organisms.

Abdullah *et al.* (2021) tested the effect of temperature and salinity on survival and growth of *Artemia franciscana*, using five different temperatures (10, 20, 25, 30 and 35 °C) for 14 days. Their results showed that the increase of temperature led to an increase in mortality – 6.7% at 20 °C, 21.7% at 25 °C, 51.7% at 30 °C and 78.4% at 35 °C. Comparing with our results, we obtained lower mortality values, possibly due to the difference in the exposure period and due to the effect of exposure to CBs, but in both cases it is confirmed that the increase in temperature led to an increase in mortality, even if slightly. Furthermore, Velki & Ečimović (2015) assessed the lethal concentrations and effects of temperature (15 and 25 °C) on pesticide toxicity, in *Eisenia fetida* (an invertebrate like *A. franciscana*). The pesticides evaluated were mixed and suspended in distilled water, including chlorpyrifos, cypermethrin, lambda-cyhalothrin and azoxystrobin, which are compounds present in tobacco (Apostolou *et al.*, 2019; Bessin & Entomologist, 2022; Sun *et al.*, 2023; Varó *et al.*, 1998). Their results showed that exposure to pesticides at different temperatures can promote different mortality patterns in exposed organisms (Velki & Ečimović 2015). In most cases, an increase in exposure temperature caused an increase in the toxicity of pesticides and an increase in mortality of *E. fetida*, and a decrease in temperature caused a decrease in toxicity. The mortality obtained by Velki & Ečimović (2015) may have been more significant in relation to our results in *Artemia franciscana* due to the fact that different organisms have different sensitivities (Case et al., 2015). Furthermore, although *Eisenia fetida* lives in lower temperature conditions (Sherman, 2021) than *Artemia franciscana* (Kumar & Babu, 2015), the temperatures tested on the previous study were different from those in our study, therefore this

comparison cannot be direct. However, as mentioned by Velki, the effects of climate change include changes in temperature, and this is a very important factor in ecotoxicology, as it acts as a stressor and influences both the physiology of organisms and the characteristics of pollutants present in the environment. In this way, the results obtained show how the predicted increase in global temperature will affect the toxicity of pollutants, in this case of pesticides and compounds present in discarded CBs.

According to De Silva *et al.* (2009) an increase in temperature increases the activity of the organisms, thus increasing their uptake of pesticides. De Silva *et al.* (2009) compared the toxicity of three pesticides (chlorpyrifos, carbofuran and carbendazim) to *Eisenia andrei* at two different temperatures (20 ± 2 °C and 26 ± 2 °C), using natural and artificial soil. Regarding chlorpyrifos, which is the only one of the tested pesticides that is present in tobacco (Varó *et al.*, 1998), the LC_{50} obtained were lower at higher temperatures (481 mg a.i./kg at 20 °C and 106 mg a.i./kg at 26 °C, in natural soil). For chlorpyrifos and carbofuran, but not for carbendazim, the survival of the organisms tested was more affected at higher temperatures, which was explained by the fact that tropical conditions, with higher temperatures, can increase earthworm activity resulting in greater absorption of pesticides (De Silva *et al.*, 2009). Considering the predicted global warming, this aspect certainly needs to be taken into account in ecotoxicological investigations and predictions of the effects of CBs on non-target organisms.

4.2 Growth

In general, individuals exposed to smoked cigarette butts, at 30 °C exhibited a higher growth. Indeed, according to Wei (2015), higher temperatures accelerate animal metabolism and, therefore, can increase their growth rate. Although an increase in temperature can favor growth, a rapid increase can also lead to a higher mortality rate (albeit slightly under the conditions tested here), as shown in previous studies (Abdullah *et al.*, 2021; Saygı & Demirkalp, 2002). Abdullah *et al.* (2021) tested the effect of temperature and salinity on survival and growth of *A. franciscana*, as previously mentioned. Their results showed that the increase of temperature did not have a linear effect on growth, that is, higher temperatures did not necessarily meant higher growth. In our study and considering control individuals, we did not observe changes in growth, when comparing temperatures, after 7 days of exposure. Abdullah *et al.* (2021) concluded that the highest growth occurred at the temperatures of 20 and 35 °C, which confirmed that temperature did not have a linear effect on growth. They explained that higher temperature may have had effects on food quality and dissolved oxygen that are limiting

factors for growth on *Artemia* spp., which is why at 35 °C there was a decrease in growth – the effect of temperature on the quality of food and the reduction in dissolved oxygen means that individuals have more difficulty developing. This could explain the fact that in our study, *A. franciscana* also presented a higher growth at 30 °C.

For individuals exposed to the highest concentration of nSCBs, at 25 °C, there was a significant decrease in growth, while at 30 °C a significant increase in the same concentration was observed, when compared to the control. Hence, temperature vs nSCBs had an apparent effect on growth, but without an evident and defined toxic effect scenario, under the conditions tested here.

Overall, it was not possible to define whether or not smoked cigarette butts were more toxic than non-smoked cigarette butts, since the results observed did not show linear effects – growth varied without a direct relationship with the increase of concentration and temperatures. For nSCB the effects were more pronounced compared to SCBs, since significant differences between concentrations of nSCBs and respective controls were observed, but not significant enough to establish that nSCB was more toxic than SCBs.

4.3 Neurotoxicity

The results of sub-chronic exposure showed that in *Artemia franciscana* individuals, AChE activity was affected by the toxicity of cigarette butts, both smoked and non-smoked, and these effects also varied with influence of temperature. Pfeifer *et al.* (2005) tested the impact of temperature and salinity in AChE activity with blue mussels from the Baltic Sea (*Mytilus* sp.). Results showed AChE activity to be significantly and positively correlated with the temperature, that is, higher enzyme activities at higher temperatures (Pfeifer *et al.*, 2005). They also explained that during April to August, minimum activities of AChE were recorded, which was not anticipated since those months presented higher temperatures. However, during those months the pesticide load (such as organophosphorus and carbamates) was higher (agriculture and industries), causing the effect of temperature to be neutralized. The previous work corroborates our results, since at higher temperature (30 °C), AChE activity was lower, possibly because of the presence of chemical compounds such as pesticides (such as organophosphorus, carbamates and herbicides), which are present in CBs. Escartín & Porte (1997) explained that the presence of pesticides, including fenitrothion, fenitrooxon and ethyl-acetate, which are compounds present in tobacco production (Morris, 2005; National Registration Authority, 1999) may influence AChE activity, showing that these neurotoxic substances have a more pronounced effect than temperature. In this study, an inhibition of AChE activity

indicates neurotoxicity promoted by the presence of toxic compounds in cigarettes, such as pesticides (Kapeleka *et al.*, 2019) or other neurotoxic components, such as nicotine and polycyclic aromatic hydrocarbons (PAHs) (Oropesa *et al.*, 2017; Slotkin *et al.*, 2019). Furthermore, since the increase in temperature favors the release and volatilization of toxic substances present in CBs, for example, nicotine and heavy metals (Schwanz *et al.*, 2020; Z. Xu *et al.*, 2022), as well as absorption by organisms, the effect of their toxicity would be more accentuated on AChE activity through its inhibition (Balbus *et al.*, 2013), when exposed to higher temperatures.

Lucia *et al.* (2023) conducted a study where *Mytilus galloprovincialis* was used to assess the toxicity of CBs in several biological effects including neurotoxicity. Results showed neurotoxic effects, with a decrease of AChE activity at 5 CBs/L, which could be related with the competitive binding of nicotine to nicotinic acetylcholine receptors, that modified the conformation and physiology of the cholinergic system (Lucia *et al.*, 2023). The inhibition of this enzyme is concerning since it leads to accumulation of ACh in the synaptic cleft resulting in over-stimulating nicotinic receptors and stopping the neurotransmission (Colovic *et al.*, 2013). In turn, this leads to muscle contraction, cholinergic hyperactivity, lack of muscle coordination, respiratory depression, sensory behavioral changes and mortality (Russom *et al.*, 2014).

4.4 Oxidative stress parameters

Regarding antioxidant defenses, significant interactions were observed between the two stressors, *i.e.*, temperature and CBs. Although non-contaminated organisms showed similar biochemical responses regardless of the temperature, this factor is an agent that can change the sensitivity of organisms to contaminants present in aquatic ecosystems (e.g., in terms of oxidative metabolism). It is already documented that, temperature affects the activity of the CAT enzyme, that is, it accelerates the activity of this enzymatic antioxidant defense (Eyster, 1967). The study by Adam *et al.* (2014) used *Daphnia magna* to determine the influence of ectoine (ECT) in response to heat stress in two temperature gradients (1 and 0.1 °C/min from 23 to 42 °C). ECT is an amino acid produced to prevent the loss of water from the cell, and in this case was used to evaluate the effect of temperature in the organisms. Results showed that CAT activity increased in response to the increase in temperature (with the gradient of 0.1 °C/min), thus thermal stress increased the production of oxygen free radicals, and in the case of hydrogen peroxide it can be eliminated by CAT (Adam *et al.*, 2014). Our results show that the higher CAT activity was found at the lowest temperature tested (25 °C), in nSCBs conditions,

which can be associated with the different compounds present in SCBs, that induced the organisms' antioxidant system.

However, regarding the effect of toxicity of SCBs concentrations, it was found that the increase of concentrations caused the inhibition of CAT activity at 25 °C, while at 30 °C, it's activity was increased. CAT is responsible for regulating high concentrations of H₂O₂, therefore, a decrease in the activity of the catalase enzyme may indicate that the organisms' ability to break down H₂O₂ has decreased (Restrepo et al., 2020), which happens when there's an imbalance between the production of ROS and the antioxidant defense mechanism of cells. On the other hand, an increase in CAT activity means that there is an increase of the concentration of H₂O₂, due to altered oxidative metabolism. For nSCBs in both temperatures, and SCB at 30 °C, an increase in concentration favored CAT activity. These findings could mean that organisms enhanced antioxidant defense mechanisms against reactive oxygen species (ROS) formation, due to CBs exposure (Rodrigues et al., 2019).

GSTs have a key role in phase II metabolism (biotransformation), but also as antioxidant defense (Sheehan et al., 2013). In the case of *A. franciscana* individuals, it was found that the activity of GSTs was significantly affected by exposure to cigarette residues, both SCBs and nSCBs, mainly at 25 °C. This indicates that the presence of CBs components can have an impact on the activity of these detoxification enzymes in exposed organisms. Zhang et al. (2020) determined the function of GSTs in *Eriocheir sinensis* in order to investigate the metabolic detoxification of GSTs under the exposure to three pesticides. They divided 450 crabs in aquariums with 0.002 µg/L of avermectin, 0.05 g/L of trichlorfon and 0.001 µg/L of β-cypermethrin (Zhang et al., 2020), the latter being present in tobacco (Apostolou et al., 2019). Their results showed that under the three pesticides, expression of GSTs increased significantly. Since exogenous substances induce GSTs activity, this promotes the combination of those substances with GSH, showing that GSTs might have been involved in the detoxification of pesticides (Zhang et al., 2020), or other CB residues, in this study. Sule et al. (2022) stated that organophosphorus pesticides (such as chlorpyrifos, also present in tobacco (Varó et al., 1998) can induce oxidative stress due to the generation of free radicals and activate antioxidant defenses, such as the depletion of endogenous antioxidants like glutathione, or the inhibition of antioxidant enzymes.

Regarding the effect of temperature, Cerezer et al. (2020) analyzed, among other parameters, biochemical biomarkers in freshwater crustacean *Aegla longirostri* at different temperatures (18, 21, 24 and 26 °C) for 48 hours. Results showed that GSTs activity decreased with the elevation of temperature, which would cause the decrease in its

functions, such as lowering the metabolism of xenobiotics (Cerezer *et al.*, 2020). In this study, for both conditions SCBs and nSCBs, individuals presented a lower GSTs activity at 30 °C compared to 25 °C, which demonstrates that temperature can modulate the effects promoted by CBs leachates, namely the activity of GSTs.

Free radicals are produced when there is an imbalance between antioxidants and oxidants, leading to cellular damage and inducing lipid peroxidation (Phaniendra *et al.*, 2015). Exposure to SCBs promoted an increase in TBARS levels, related to temperature and concentration, indicating the occurrence of lipid peroxidation. Cerezer *et al.* (2020) analyzed biochemical biomarkers in freshwater crustacean *Aegla longirostri* at different temperatures (18, 21, 24 and 26 °C) for 48 hours. Results regarding lipid peroxidation showed that the increase in water temperature had a direct relation to an increase in TBARS levels, which could lead to irreversible cell damage and cellular functions (Cerezer *et al.*, 2020). However, the opposite was observed in our study, since the higher TBARS levels were obtained at the lowest temperature tested, which can be related to the effect that CBs compounds had on lipid peroxidation, which overcame the effect of temperature. Nambi *et al.* (2017) conducted tests on zebrafish *Danio rerio*, exposing them to five different concentrations of nicotine (3, 4, 5, 6 and 7 mg/L) for 96 hours, and it significantly increased the elevation of lipid peroxidation compared to control groups, where significant differences were observed from 3 mg/L. These results prove that CBs compounds have significant effects on lipid peroxidation, which is in line with our results.

Exposure to nSCBs seemed to have less toxic effect on lipid peroxidation, due to SCBs being more toxic than nSCBs. According to Abdollahi *et al.* (2004), there are free radicals such as those from the hydroxyl group that are highly toxic and react with lipids, causing lipid peroxidation. This group of compounds normally forms during the combustion of tobacco, where chemical reactions occur that can produce a variety of compounds, such as methyl alcohol (methanol), which shows how SCBs can be more toxic than nSCBs. This can be confirmed as well by the study of Lee & Lee (2015), where they exposed Medaka embryos to explore the toxicity of leachates of smoked and non-smoked tobacco (ST and UST) and filters (SF and USF) at 0.2, 2, 5 and 10 pieces of filter per liter. The results, based on heart rate and head development, originated a rank of toxicity of ST > UST > SF > USF, showing that low concentrations accelerated the development and high concentrations suppressed it, which means that SCBs were more toxic than nSCBs (Lee & Lee, 2015). It was also confirmed by Slaughter *et al.* (2011) who found increase in toxicity from unsmoked filters to smoked filters to smoked cigarette butts, to *A. affinis* and *P. promelas*. This finding shows why SCBs had a more significant effect on lipid peroxidation. In our study, temperature appeared to be the main factor

influencing TBARS levels, that is, there were no significant variations between concentrations of nSCBs. Madeira *et al.* (2014) evaluated the effect of temperature (24 to 35.7 °C) on lipid peroxidation of three species of crabs: *Pachygrapsus marmoratus*, *Carcinus maenas* and *Liocarcinus marmoreus*, and results demonstrated that both species displayed a high increase in lipid peroxidation at temperatures equal or higher than 34 °C. Each species has its optimal temperature, and it may be different between organisms of different species, however, the increase of temperature can disturb changes in fluidity and permeability of the membrane, alterations of ions transport and inhibit metabolic processes, and injury organs and tissue in organisms (Ayala *et al.*, 2014; Catalá & Díaz, 2016). Given the predictions of an increase in global temperature, this evidences this environmental problem to be important, as it brings so many changes and disturbances to organisms. On the other hand, as previously reported by John *et al.* (2018), the amount of nicotine in cigarettes can increase with temperature, which relates to the combustion temperature of the compounds in the act of smoking. This proves that the composition of CBs is a crucial factor in the response of the organisms tested. These results indicate the complexity of biological responses to CBs and the importance of considering environmental factors, such as temperature, when evaluating the effects of cigarette butts' residues on organisms. It is particularly relevant to evaluate potential interactions between environmental pollutants and environmental conditions such as temperature (as a consequence of global warming) that may alter the sensitivity and susceptibility of organisms to chemical exposures.

4.5 Energy metabolism

LDH activity is an indicator of metabolic rate, and is a biomarker that can be used to evaluate energetic metabolism disturbances since is a crucial enzyme of the anaerobic pathway for producing energy (Diogo *et al.*, 2023). This biomarker can be modulated by several chemical stressors (Rodrigues *et al.*, 2018) and is temperature-dependent (Zakhartsev *et al.*, 2004).

In this study, at 25 °C there was a decrease in LDH activity at some concentrations (0.03125, 0.0625 and 0.5 SCB/L and 0.5 nSCBs/L), indicating that exposure to these concentrations of CBs at lower temperatures (25 °C) may have an inhibitory effect on LDH activity, affecting cell energy metabolism or causing cellular damages, inhibiting cellular growth and triggering cell death (Le *et al.*, 2010; Zhang *et al.*, 2022). And the opposite was found at 30 °C, i.e., an increase in LDH activity was observed, but only occasionally in a concentration of SCBs and nSCBs (0.5 SCBs/L and 0.25 nSCBs/L). Michalaki *et al.*

(2022) exposed individuals of *Daphnia magna* to toxic chemicals separately, including nicotine (9.99 mg/mL), for 24 hours, to evaluate acute toxicity and effective concentration, in a range of dilutions from low (10% EC₅), medium (20% EC₅) and high (30% EC₅). Although there is a general lack of data on the impact of nicotine in freshwater and marine organisms that focuses also on energy metabolism, Michalaki *et al.* (2022) showed that nicotine can inhibit LDH activity, which could indicate disruptions of energetic metabolism in daphnids (freshwater organisms). An increase in LDH activity could mean the demand for anaerobic energy production (to maintain cellular functions), caused in this case in the *A. franciscana* individuals by CBs components.

The present results showed that organisms had a different response to CBs at each temperature, indicating that the temperature can change the toxic effects, as previously mentioned. These results indicate that the impacts are not also resulting from the CBs concentration, but mainly from the sensitivity of the species that alter at different temperatures.

Analyzing the global results of biomarkers, CBs components can have a significant effect on *A. franciscana*, causing disturbances in various metabolic pathways and cellular functions, affecting the activity of AChE and the antioxidant enzymes such as CAT and GSTs, inducing oxidative damage and influencing energy metabolism. In addition, the toxicity and volatility of CBs compounds, and their concentration, worsened by climate change can trigger several and different biological responses. Sub-individual parameters as biochemical biomarkers are crucial because they can detect impacts before lethal or organismal effects occur (individual parameters such as growth), allowing for early identification of environmental problems. The variation of biomarkers under different temperature conditions and concentrations of CBs provided insights into how the individuals tested respond and adapt to these environmental stressors.

Although some results indicated that SCBs had a more significant impact on *Artemia franciscana* on parameters such as mortality and oxidative stress, compared to nSCBs, it is important to highlight that the difference in toxicity was not statistically significant in all cases so, under the conditions tested here, it is not possible to say that one leachate is more toxic than the other. It is also highlighted that different organisms may have different sensitivities to the same chemicals; therefore, the results of this study may not be directly comparable with previous studies. The organisms' responses in several parameters, such as neurotoxicity, oxidative stress and energy metabolism, were not linear in relation to the concentrations of SCBs and nSCBs, which also does not help in a clear definition of which will be the most toxic. However, the most influential factor seemed to be temperature: it affected the toxicity of the chemicals and influenced the

results significantly, making it an important variable to consider when evaluating the toxicity of CBs in a climate change scenario. Global warming is causing changes in water temperatures in aquatic ecosystems, which may affect the response of organisms to CB pollution.

5. Conclusions

Both smoked and non-smoked cigarette butts have been shown to be toxic to *Artemia franciscana* individuals. The results of the present study showed that the increase in temperature combined with the exposure to CBs represents a stress for *A. franciscana* causing effects on growth but also in sub-individual parameters, such as disturbances in various metabolic and cellular functions, inducing oxidative damage and influencing energy metabolism. Temperature can alter the toxicity of CBs components, varying the intensity of the effects under different temperature conditions. Moreover, temperature appears to have modulated the activity of enzymes involved in different metabolic pathways. In fact, our results indicate that the impacts are not resulting only from the CBs concentration, but mainly from the sensitivity of the species to CBs that alters with temperature. This has important implications in light of climate change, as it suggests that rising temperatures could worsen the negative effects of CBs on marine organisms, highlighting the complexity of biological responses to multiple environmental factors.

Based on the results presented, it is not possible to definitively conclude that SCBs are more toxic than nSCBs, since the toxic effects appear to vary depending on several factors, including temperature and concentration. However, both SCBs and nSCBs represent environmental risks and that the toxic response can vary under different environmental conditions. Although this study provides valuable information, it would be useful to carry out more ecotoxicological tests (exposing the organisms to longer periods; investigate how the organisms process each CB component; study the combined effect of each component with temperature; use different organisms and compare the consistency of results), in order to understand the impacts of CBs under realistic contamination conditions. Although a 7-day study provides important short-term information, longer-term studies may be needed to evaluate the cumulative and chronic impacts of cigarette residue on marine organisms. It is clear that to fully understand the impacts of CB waste, studies that investigate its environmental impact in natural ecosystems (*in situ*), rather than just in laboratory conditions, may be necessary.

In conclusion, this study highlights the need to consider the interaction between chemical pollutants and environmental conditions, such as temperature, when assessing

environmental risks and impacts, namely global warming. The results also emphasize the importance of addressing cigarette butts' pollution as a serious environmental issue that can negatively affect marine biodiversity and, potentially, global ecosystems. Mitigation measures and public awareness are essential to address this growing problem and protect aquatic environments.

6. References

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