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In vitro Methodologies for Environmental Risk Assessment: Comparison between methods for the isolation of marine fish primary hepatocytes.

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ABSTRACT

Interest of *in vitro* methods has grown exponentially throughout the years especially due to the ethical problems surrounding the use of animals in *in vivo* studies. Cell-based assays for the isolation of hepatocytes using enzymatic methods have been credited in fish toxicology and play a substantial role in the propagation of *in vitro* methodologies, having been implemented with various degrees of success to a variety of fish species, especially in freshwater species. Isolated hepatocytes provide an optimal system to study the role of hepatic metabolism in the activation of environmental pollutants, such as polycyclic aromatic hydrocarbons (PAHs). For these reasons, the present work focused on the optimization of the isolation of two marine fish hepatocytes (*P. maxima* and *S. aurata*) using two enzymatic methods of isolation, pancreatin digestion and 2-step collagenase perfusion, for posterior validation in cytotoxicity testing. The two main parameters studied were cell viability and yield after isolation. Cell viability was assessed via the Trypan blue exclusion test and cell yield was counted with the use of a hemocytometer. The pancreatin method accounted for better overall cell yield and also produced the highest viability out of all experiments. Pancreatin digestion isolations also produced purer cultures, largely with 100% viability, which was made possible by way of modifications made during isolations, such as the use of histopaque for blood cell separation and addition of another smaller mesh filter (60 µm) to achieve better purity and fewer cell debris, respectively. A cytotoxicity assay was also performed using the MTT colorimetric assay to access cell viability after contamination with Phenanthrene and Benzo[b]fluoranthene, two polycyclic aromatic hydrocarbons (PAHs) of interest for cytotoxicity studies. Viabilities after contamination with both compounds only exhibited small variability demonstrating no dose-response curve, likely due to factors such as intra-species individual variability and the precipitation of contaminants in the highest concentrations, which might have affected absorbance readings and could cause misleading cell viabilities. Therefore, we call for further studies to access the cytotoxic effect on *P. maxima* and *S. aurata* by Phe and B[b]f. In conclusion, the research showed that the best method for isolation was the pancreatin digestion and the marine fish primary hepatocyte cultures obtained are suitable for the application in cytotoxicity assays.

Key words: Hepatocytes, *in vitro*, 2-step collagenase perfusion, pancreatin digestion, cell viability, cell yield, cytotoxicity, PAHs.

RESUMO

O interesse dos métodos *in vitro* cresceu exponencialmente ao longo dos anos, principalmente devido aos problemas éticos relacionados com o uso de animais em estudos *in vivo*. Neste contexto, os métodos de isolamento de hepatócitos primários usando métodos enzimáticos foram creditados na toxicologia aquática e têm desempenhado um papel substancial na disseminação de metodologias *in vitro*, tendo sido implementados com sucesso em diversas espécies de peixes, especialmente em espécies de água doce. Os hepatócitos primários constituem um sistema ideal para o estudo do papel do metabolismo hepático na ativação de poluentes ambientais, tais como os hidrocarbonetos aromáticos policíclicos (PAHs, do inglês *polycyclic aromatic hydrocarbons*). Por estes motivos, o foco do presente trabalho recaiu na otimização do método de isolamento de hepatócitos a partir de dois peixes marinhos (*P. maxima e S. aurata*) usando dois métodos de dissociação enzimática - digestão com pancreatina e perfusão com colagenase de duas etapas - para validação posterior em testes de citotoxicidade. Após o isolamento, os dois principais parâmetros estudados foram a viabilidade celular e o número de celulas produzido. A viabilidade celular foi avaliada através do teste de exclusão do azul de Trypan e o rendimento celular foi determinado através da contagem de células num hemocitómetro. O maior rendimento e a melhor viabilidade celular foram obtidos através do método da digestão com a pancreatina. Além do rendimento, este método também gerou culturas mais puras, em grande parte com 100% de viabilidade, o que foi possível através de modificações realizadas durante os isolamentos, como o uso de histopaque para separação de células sanguíneas e a adição de um segundo filtro de malha menor (60 µm) durante a filtração para obter melhor pureza e menos detritos celulares. Um ensaio de citotoxicidade foi também realizado, utilizando o teste colorimétrico do MTT para avaliação da viabilidade celular após contaminação com Fenantreno e Benzo[b]fluoranteno, dois PAHs de interesse para estudos de citotoxicidade. A viabilidade após a contaminação com ambos os compostos exibiram apenas uma pequena variabilidade, sem uma evidente curva de dose-resposta, provavelmente devido a fatores tais como a variabilidade individual intraespecífica e precipitação dos compostos nas concentrações mais altas, que podem afetar as leituras de absorvência e introduzir erros nos cálculos da percentagem de viabilidade. Recomendamos a realização de mais estudos para avaliar os efeitos citotóxicos de Phe e B[b]f sobre *P. maxima e S. aurata*. Em conclusão, neste estudo o melhor método de isolamento foi a digestão com pancreatina e as culturas de hepatócitos primários isolados a partir de peixes marinhos são adequadas para a aplicação em ensaios de citotoxicidade.

Palavras-chave: Hepatócitos, *in vitro*, perfusão com colagenase em duas etapas, digestão com pancreatina, viabilidade celular, rendimento celular, citotoxicidade, PAHs.

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1. INTRODUCTION

The earliest known references for the use of animals for medical research date back to ancient Greek writings (Aristotle; 384–322 BCE and Erasistratus; 304–258 BCE) (Cohen and Loew, 1984). Since then, an overabundance of animals have been used for medical research, generally intending to protect the integrity of human life, tackling issues like mechanism of disease, genomic susceptibility, and new developments in diagnostics and therapy (Dey, Smith, and Leyl 2010). Laboratory animals are utilized every year in highly painful and distressing scientific procedures. An underlined responsibility for the welfare of these animals should exist, both in and out of the context of active experimentation to tackle *in vivo* experimentation (Ghasemi and Dehpour 2009).

1.1. CELL-BASED MODELS FOR ECOTOXICOLOGY

1.1.1. *In vivo* vs *In vitro*

In vitro testing is the answer to a lot of the ethical problems that plague *in vivo* testing. It is assumed that *in vitro* experiments can extrapolate toxicity of chemicals to the whole organisms due to the fact that most chemicals exert toxicity through the interference of natural processes common to most types of cells within an organism (Ekwall 1983). *In vitro* testing is also considered cheaper than *in vivo* but it is assumed not to take animal complexity in account, with results often varying depending on the test and chemical used, so they are considered to only being able to partially mimic *in vivo* methods (Benfenati et al. 2010). Although *in vivo* testing is still highly used due to being widely reliable while also being able to measure effects of contaminant mixtures, its ethical flaws are seen to overpower most benefits (Benfenati et al. 2010). Since the late 60s/70s the value of vertebrate cells as *in vitro* models for toxicological research was beginning to be established (Schirmer 2006), especially using fish, who are the dominant and most diverse group of vertebrates for the evaluation of ecotoxicity and for that reason are afforded the same legal protection than mammals (Isomaa and Lilius 1995,Walker, 1998). Understanding the effects of toxicants in fish is an important tool in assessing the health conditions of the aquatic environment (Zelikoff 1998), because fish comprise 48% of the known vertebrate species (Altman and Dittmer, 1972), something crucial in the development of vertebrate cell and tissue models for use in biomedical sciences (Lakra et al. 2011).

1.1.2. Primary cells vs Cell lines

Both the use of primary cells and immortal cell lines are important components of *in vitro* assays. Primary cells can be obtained directly from tissues and organs of interest and maintained in laboratory conditions for a slight number of days. The beginning of a primary culture is the attachment of cells to a physical support (petri dish, flasks or multi-well plates), yet if the cells are used upon isolation or immediately after, they are simply referred to as cell suspensions (Bols et al. 2005). Subculturing (also known as passaging) cells will result in the creation of immortal cell lines, which can be kept alive for extended periods, often indefinitely (referred to as permanent cultures) (Schirmer 2006). Cell lines are better suited for biological

studies on basic mechanisms such as the cell cycle (Pan et al. 2008). The main disadvantages of using cell lines would be that they originate from tumors and have to adapt to growth in culture leading to a lack of tissue architecture and heterogeneous population of cell types which disregards cell-cell interaction, secretion, and other functions based on tissue context. Moreover, genotypic and phenotypic drifting may also lead to cell line cultures being prone to lose tissue-specific functions and acquire a molecular phenotype that can differ from cells *in vivo* (Pan et al. 2008). On the other hand, they are cost effective, easy to handle and can provide an infinite number of a pure population of cells, which leads to consistent samples and reproducible results (Kaur and Dufour 2012).

Table 1 – Advantages and disadvantages of using primary cultures or cellular lines.

	Advantages	Disadvantages
Primary cells culture	• Keeps the original characteristics of the biopsied tissue and other in vivo properties, facilitating extrapolation of the results *in vivo* (Schirmer 2006; Zacchino et al. 2013) • More sensitive and with higher metabolic capability than cell lines (Chen et al. 2003).	• Samples typically only last a short number of days and are usually more heterogenic than cell lines (Bols et al. 2005). • Cell preparation can be impossible, e.g. if the species or organ is too small, hard to collect, only available seasonally or too expensive to keep in laboratory (Bols et al. 2005). • Preparations in different dates may result in conflicting results due to hard replicability of the dissociation processes or due to changes in the physiologic status of the fish (Bols et al. 2005). • For prolonged studies it requires regular sampling of cells (Roux 2015). • Requires specialized medium (often expensive) and has a low proliferation capacity (Eckerle et al. 2014).
Cell lines	• They are standardizable easily manipulated with low variability, ethical to use, provide unlimited supply of material and produce pure populations of cells (Kaur and Dufour 2012; Yanhong et al. 2008). • Can be maintained indefinitely through passaging, avoiding cell senescence (Schirmer 2006). • For fish it only requires a medium and an incubator at optimal temperature and ideal CO_2 concentration (Roux 2015). • Homogeneous when stabilized and can be cryopreserved indefinitely (Bols et al. 2005).	• Extremely simplified and standardized, often yielding questionable results (Roux 2015). • Grown in artificial monolayers (loss of original 3D disposition) (Roux 2015). • Can be attached to factors such as misidentified and contaminated cell lines (Barallon et al. 2010). • Can become contaminated with other cell lines and mycoplasma (Kaur and Dufour 2012). • Loss of original genetic and biochemical characteristics (Schirmer 2006; Segner and Braunbeck 1998)

Despite their increased complexity and possible variability in obtaining results, primary cells are preferred in some situations, due to their genetic integrity and biological reverence, in terms of retaining original characteristics of the biopsied tissue and closely mimicking the physiology of cells *in vivo* (Pan et al. 2008; Schirmer 2006). Some studies comparing primary cells with immortalized cells have been performed (Bols et al. 2005; Kaur and Dufour 2012; Pan et al. 2009; Roux 2015). A comparison between these cell types is made in table 1.

1.2. HEPATOCYTE ISOLATION

Hepatocytes are liver parenchymal cells that occupy 80% of the original liver volume, 60% of the number of total liver cells and perform numerous functions (Tanaka and Miyajima 2016). Isolation of these cells has proved to be optimal for evaluating conditions of hepatic metabolism, such as cellular processes involved in toxic chemical and environmental pollutant activation (Yanhong et al. 2008). According to the majority of existing literature the most widely used method for the isolation of hepatocytes are perfusion methods, namely the 2-step collagenase perfusion (Burdon and Knipperberg 1991; Ferreira et al. 2014; Scholz et al. 1998; Seglen 1976; Segner and Braunbeck 1998). Although, new interest has been found in the enzymatic method of pancreatin digestion. The basis of enzymatic methods is the introduction of a digestion mixture to minced tissue and later incubation at a specific temperature required by the mixture (Reichard and Asosingh 2018). The enzymatic-based method addressed in this study was previously implemented by (Yanhong et al. 2008) to establish primary cultures of *Cyprius Caprio* hepatocytes.

1.3. AQUATIC SYSTEMS POLLUTION – SPECIAL CASE OF PAHs

The marine environment is subject to a wide range of contaminants, such as organic compounds (Polycyclic aromatic hydrocarbons (PAHs), dichlorodiphenyltrichloroethane (DDT), polychlorinated biphenyl (PCB), heavy metals (such as mercury) pharmaceuticals, solid waste (e.g. plastics), nutrient excess (like nitrates and phosphates that can cause eutrophication) and radioactivity (Cuevas et al. 2018; Martins et al. 2012; Martins et al. 2008; Wilhelmsson et al. 2013; Figueiredo et al. 2014). The main sources for the pollution of aquatic systems are anthropogenic activities, such as agricultural runoffs, industry, deforestation, aquaculture, oil spills and sewage discharge (Noone et al. 2013). These pollutants upon reaching the water body, can become dissolved in the aqueous phase (DAP), suspended in particulate matter (SPM) or adsorbed to organic-rich sediments (Cuevas et al. 2018; Vidmar et al. 2017). Diversely, most PAHs are insoluble in water, meaning that their mobility in this medium is decreased but on the other hand they have the potential to be adsorbed by sediments and subsequently transported (Cano-Lerida et al. 2009; De Luca et al. 2004; Walker et al. 2013). In fact, sediments are viewed as the most significant reservoir of organic pollutants for PAHs in aquatic coastal environments (Martins et al. 2013). The effects these contaminants have on marine resources depend on their form, their intensity (acute or chronic), their location and the sensitivity of the surrounding environment or of the species itself (Noone et al. 2013). They can be released by point sources or diffuse sources, although the period that exists from emission to contact with the receptor

should also be taken into consideration since it's when transportation processes occur (Hoffman, 2003).

Their effects can also reach various ecological levels like cellular and/or molecular, individual or of a species, population or community. Cells can be used as biomarkers of exposure or response to contaminants, meaning that they are tools to detect or predict effects at an individual level and consequently at the population and community level (Weis et al. 2006). Regarding fish and other marine species, the intake of contaminants can sometimes be facilitated (for instance through the skin or gills) and potentially corrupt an entire food chain. This process is called bioaccumulation and is defined as an increase in the concentration of a substance in or on an organism or specified tissues thereof relative to the concentration of the substance in the surrounding medium (Bart et al. 2013). This means that the affected organisms will experience an increase in the accumulation of a certain contaminant that was taken up from food, water or suspended sediment (Wang and Fisher 1999). PAHs are amongst the compounds that fish and other marine organisms can uptake and bioaccumulate. When these organisms become prey to other organisms in higher trophic levels in the food chain, there is a tendency of pollutants to increasingly concentrate, in a process called biomagnification (Bleeker and Verbruggen 2009). Another phenomenon related to this is the bioconcentration, which is a specific bioaccumulation process by which the concentration of a chemical in an organism becomes higher than its concentration in its surrounding environment. The bioconcentration factor (BCF) is a measurement of the ratio between the uptake rate of a compound from water into the organism and its elimination rate to water and was used by Bleeker and Verbruggen (2009) to determine a varity of PAHs bioaccumulation in fish and marine invertebrates. Their findings determined that fish are capable of transforming PAHs into soluble compounds that can be easily excreted but on the other hand invertebrates are less capable of PAH transformation, resulting in higher BCF values.) Due to this processes of bioacumulation and biomagnification human, as the top predator, can be directly or indirectly affected by toxicants via food uptake or direct exposure, to land or of the water body itself (Adams and Greeley 1996).

PAHs are compounds composed of two or more benzene rings, often containing alkyl groups. They are listed on Annex II of Directive 2008/105/EC of the European Parliament and the Council on environmental quality standards in the field of water policy as priority hazardous substances, an amendment to Annex X of the European Water Framework Directive. They are also listed as priority substances by the World Health Organization (WHO) and by the United States' Environmental Protection Agency (EPA).

PAHs are usually subcategorized as low molecular weight PAHs (LPAHs), with 2 or 3 aromatic rings or as high molecular weight PAHs (HPAHs), with four or more aromatic rings. Distinguishing these PAHs groups is important because they exhibit very different physicochemical properties, bioavailability, bioaccumulation, and toxic potential (Cano-Lerida et al. 2009). HPAHs are deemed more dangerous since they degrade slower and have higher mutagenic and carcinogenic potentials, which have been reported to increase with increasing molecular weight (CWQO 1999; Martins et al. 2015). They are ubiquitously present in the environment and are very persistent at contaminated sites due to their low water solubility and high lipid solubility, being detected in the atmosphere, soil, water, sediment and food (Lu et al. 2008; Yang et al. 2010). They become dissipated in the environment by anthropogenic and

natural sources (WHO, 2003). The main sources are anthropogenic and include operations involving petroleum (such as drilling, refining, combustion, spills, etc.), combustion of most organic materials, incomplete burning of fuels, municipal and industrial wastewater (Cano-Lerida et al. 2009, Zhang and Tao, 2009). Notable natural sources are stormwater run-off, volcanic eruptions and forest fires (Cano-Lerida et al. 2009, Zhang and Tao, 2009).

PAHs are responsible for a profusion of adverse effects, being known to cause teratogenic (Hecht et al. 2011; Sogbanmu et al. 2016), immunotoxic (Reynaud and Deschaux 2006), genotoxic (Martins et al. 2006, 2018) histopathological changes (Al-Hashem 2017; Martins et al. 2016) and tumorogenic (LaVoie et al. 1993) and carcinogenic effects (Cavalieri et al. 1991; Wislocki et al. 1978).

2. OBJECTIVES

The present thesis intends to optimize the isolation and culturing of primary hepatocytes of marine fish to be used in cytotoxicity studies. Our focus in this study was mainly to optimize hepatocyte isolation methods but also to credit two teleost fish as viable marine options for primary hepatic cell cultures since the majority of existing studies tackle the optimization of the isolation methods on freshwater fish. It is expected that these methodologies will contribute to good practices in the use of animals in laboratory and, at the same time, maintain ecological and environmental relevance. All of the above-mentioned processes are directly corelated to the 3R's (Replacement, Reduction and Refinement) policies for more ethical use of animals, first described by W. M. S. Russell and R. L. Burch in 1959.

The primary hepatocytes will be obtained from two fish species with ecological and economic relevance, *Sparus aurata* (sea bream) and *Psetta maxima* (turbot) using these isolation methods. The comparison between these methods will be performed through the evaluation of cell yield and viability after isolation. The most promising method of isolation will be chosen for the establishment of the cultures and to validate cytotoxicity assays with two PAHs, usually present in aquatic environments, the 3-ring PAH phenanthrene and the 5-ring PAH benzo[b]fluoranthene. They were selected for their high availability in aquatic sediments and adverse effects on living beings, among them fish and humans.

Phenanthrene is a low molecular weight PAH formed out of three-benzenoid rings that consists of a colorless crystalline solid (Budaveri et al., 1989) and has a relatively high water solubility of 1.1 mg L^{-1} (Amellal et al. 2006). The International Programme on Chemical Safety (IPCS) determines that the highest concentrations of Phe are identified on aquatic samples. It was found to be one of the most available compounds in rivers and estuaries in different parts of China (Chen et al. 2004; Mai et al. 2002; Maskaoui et al. 2002; Zhang et al. 2004). It is abundant in fossil fuels and present in products of incomplete combustion (Torreiro-Melo et al. 2015). It is classified by the International Agency for Research on Cancer (IARC) as a group 3 compound, meaning not classifiable as to its carcinogenicity to humans. Phenanthrene is easily taken up by marine organisms (Anderson and Neff, 1981). It is regarded to contribute significantly to the adverse effects that PAH mixtures have on early stages of fish development, like craniofacial

deformities, swimming impairments, growth reductions and even mortality, as examined in pacific herring and pink salmon (Barron et al. 2004). Phe was also shown to be genotoxic to different species, like scallops *(Pecten maximus)* (Hannam et al. 2010), golden grey mullet *(Liza aurata)* (Oliveira et al. 2007) and freshwater fish like the estuarine guppy *(Poecilia vivipara)* (Machado et al. 2014) and the goldfish (*Carassius auratus*) (Sun et al. 2006).

Benzo[b]fluoranthene is a high-molecular-weight PAH compound that contains five rings and a water solubility of 0.0015 mg L^{-1} (Neff 2003), substantially lower than fluoranthene, due to its higher molecular weight, since each additional aromatic ring increases hydrophobicity (Kim et al. 2013). B[b]f is less toxic and carcinogenic than other PAHs like benzo[a]anthracene, dibenzo[ah]anthracene and benzo[a]pyrene but still has a substantial carcinogenic potential, being classified by the IARC as a group 2B compound, as possible carcinogenic to humans. The United States' Department of Health and Human Services (HHS) classifies B[b]f as a known animal carcinogen. This toxicant is largely present in the environment, being identified has the compound with the highest concentration out of 11 PAHs tested in Ologe Lagoon in Nigeria (Obanya et al. 2019). Also regarding B[b]f and Phe and their toxic effects, Martins et al. (2013) found that sediments spiked with low-moderate Phe and B[b]f triggered genotoxicity to *Ruditapes decussatus.* Martins et al. (2015) also established that the mixture of these PAHs, as well as individually, yielded synergistic toxicological effects to sea bass (*Dicentrarchus labrax*).

With high commercial value in most Mediterranean fisheries and aquaculture, the *S. aurata* species is a marine teleost fish of the *Sparidae* family and was chosen for this study for its availability and credibility in cytotoxicity results. They are found in temperate and tropical waters, mostly in the northeast Atlantic and Mediterranean and about 100 million tons are produced per year (Barros et al. 2011). Banni et al. demonstrated in 2009 that the *S. aurata* was a species particularly sensitive to acute Benzo[a]pyrene (B[a]P) exposure. This sensitiveness allows for a detailed study of the effects of different concentrations of B[a]P in the health of these sea breams. B[a]P was already demonstrated to be genotoxic in every concentration, triggering apoptosis and uncontrolled proliferation at even the smallest dose (Pastore et al. 2014). B[a]P is seen as marker of exposure for the effects of PAH mixtures and is the most widely studied PAH (WHO 2006). Phenanthrene was also shown to cause oxidative stress in this particular species (Correia et al. 2007).

The *Psetta maxima* (turbot) is a benthic fish of the *Scophthalmidae* family. It's the most important marine flatfish for Northern Europe aquaculture and East Asia due to its rapid growth rate and high market value, with a global production of 70000 tons per year (Bonaldo et al. 2015; Moksness, 2004). No studies were found for the isolation of hepatocytes of turbot either by two-step collagenase perfusion or by pancreatin digestion. Similarly, no studies were found correlating Benzo[b]fluoranthene with toxicity in turbots. Although, Phenanthrene was shown to not cause toxicity in turbot larvae either under artificial visible light or absence of light (Mhadhbi et al. 2010).

3. MATERIALS AND METHODS

3.1. Fish acclimatization and maintenance

The turbots (*Psetta maxima*, Linnaeus, 1758), with weights ranging from 46.80g to 75.51g and sea breams (*Sparus aurata*, Linnaeus, 1758), with weights ranging from 186.67g to 271.25g, were acquired from Aquanostra®, an aquaculture farm in Setúbal, carefully transported alive to the MARLab at Department of Environmental Sciences and Engineering (DCEA), NOVA School of Science and Technology (FCT NOVA) and maintained in 400l tanks at 16°C, in regular photoperiod, aeration close to 100%, periodically checked ammonia levels (0-2mg/l) and salinity kept around (34 ± 1.0). The fish were fed commercial fish food pellets at about 0.2 mg per turbot and 1mg per sea bream.

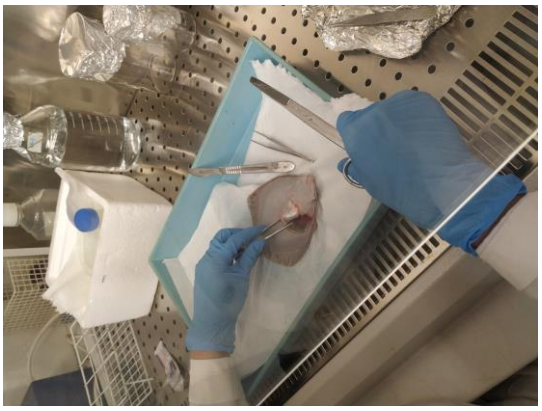

Fig. 1 – Dissection of a *P. maxima* sampling.

3.2. Reagents

The 2-phenoxyethanol (VWR Chemichals BDR™) was used as anesthesia for both fish, 70% alcohol for disinfection, Histopaque (Sigma-Aldrich™) to separate the blood cells from hepatocytes primary culture. HBBS Medium without Ca^{2+}, Mg^{2+} and Phenol red (Lonza™), HBSS Medium without Ca^{2+} and Mg^{2+}, with Phenol red (supplemented with 5% fetal bovine serum (FBS) and 1% of antibiotic-antimycotic 100X, containing amphotericin B 0.025 g/l, penicillin G 6.028 g/l and streptomycin 10.00 g/l) (Sigma-Aldrich™); PBS Medium (Lonza™), L-15 Medium (supplemented with 5% FBS and 1% of antibiotic-antimycotic 100X, containing amphotericin B 0.025 g/l, penicillin G 6.028 g/l and streptomycin 10.00 g/l) (Sigma-Aldrich™) and L-15 Medium without FBS (Sigma-Aldrich™) were used in the isolation process and culture of primary hepatocytes. MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromide) (Sigma-Aldrich™) was used in the cytotoxicity assessment. Dimethyl sulfoxide (DMSO) (VWR Chemichals BDR™) is commonly used as a solvent for water-insoluble substances (Onyegeme-Okerenta et al. 2018). In this case, DMSO was used as a solvent in the preparation of the PAHs solutions. Triton X-100 (Riedel-de Haën™) was used as a positive control for the cytotoxicity assay. The composition of solutions used in the isolation of primary hepatocytes is presented in table 2.

Table 2 – Composition of the solutions used in both methods for the isolation of primary hepatocytes.

Component	**Two-step collagenase perfusion** (Ferreira et al., 2014)			**0.1% Pancreatin digestive juice** **(**Yanhong et al., 2008)
	Sol. A	**Sol. B**	**Sol. C**	
NaCl mM	176	176	176	126
KCl mM	4.8	4.8	4.8	4.82
KH_2PO_4 mM	0.44	0.44	0.44	1.5
$NaHCO_3$ mM	3.6	3.6	3.6	-
Na_2HPO_4 mM	0.35	0.35	0.35	3.2
HEPES mM	10	10	10	-
Na_2EDTA, pH 7.6 mM	5	-	-	0.54
$CaCl_2$ mM	**-**	2.5	2.5	**-**
Collagenase IV mg/ml	-	0.02	-	-
1% BSA	-	-	1%	-
Pancreatin g/l	-	-	-	1
pH				

Buffer solution used in the Pancreatin Digestion method: Dissection Balanced Salt Solution (DBSS) – 126 NaCl mM, 4.82 KCl mM, 1.5 KH_2PO_4 mM, 6.1 Na_2HPO_4 mM, 21.9 HEPES mM, pH 7.2 – 7.6

3.3. Primary hepatocytes isolation

The two fish species used in the present work were irreversibly anesthetized with an aqueous solution of 2-phenoxyethanol (3 ml). After disinfection with 70% alcohol, a longitudinal incision from the anus to the gills was performed and the liver was removed and placed in a petri dish. The primary hepatocytes were then isolated using the two-step collagenase perfusion and the pancreatin digestion techniques according with Ferreira et al. (2014) and Yanhong et al. (2008), respectively, with some modifications (Fig. 2).

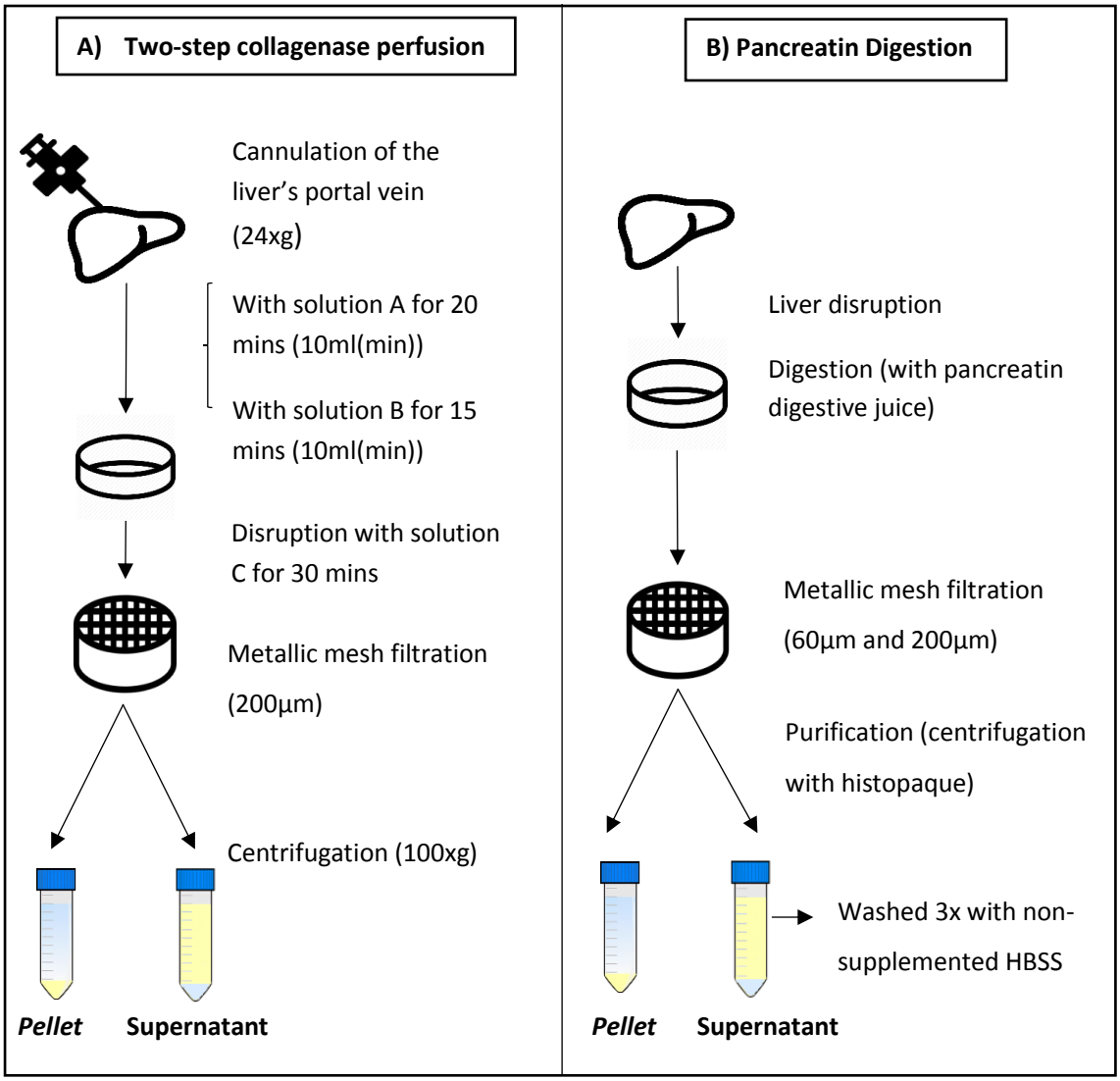

Fig. 2 - Scheme of two fish primary hepatocyte cell isolation methods used: A) two-step collagenase perfusion and B) pancreatin digestion.

3.4. Two-step collagenase perfusion

The liver was weighed and then cannulated (24G) in a petri dish with solution A (composition given in table 2) through the portal vein with a flow rate of 10 ml/min for 20 minutes (Fig. 2A). This technique is used to remove the blood cells, demonstrated through a gradual loss of the liver's color. After the 20 minutes, another 15 minutes (10 ml/min) of solution B (Table 2), previously warmed to 37 °C, was cannulated with the purpose of disrupting the intercellular connections. The liver surface was washed with phosphate buffered saline (PBS). After the washing process, the liver was added to solution C (Table 2), cut into pieces gradually using a scalpel while being let to settle for 30 minutes in a cool petri dish. The fragments were filtered through stainless-steel meshes (60 µm and 200 µm metallic meshes). The cell suspension was collected by centrifugation at 100 x g, 5 min and 4 °C and the supernatant was subsequently removed. The *pellet* was resuspended in cool non-supplemented Hank's Salt Based Solution (HBSS), without Ca^{2+}, Mg and Phenol Red (Lonza™) and washed 3 times in the same medium,

being the cells collected by centrifugation at 100xg, 5 mins and 4°C. After this, the *pellet* was resuspended in the same medium supplemented with 5% fetal bovine serum (FBS) and 1% of antibiotic-antimycotic 100X (containing amphotericin B 0.025 g/l, penicillin G 6.028 g/l and streptomycin 10.00 g/l).
The cell yield was assessed according with the methodology described in section 3.6.

3.5. Pancreatin digestion

The liver was removed from the fish and placed in a cool dish containing sterile DBSS (see the composition in the Table 2). The liver was then cut into 1.0 – 2.0 mm^3 pieces and washed 3 times with cool sterile DBSS and then clipped into fragments. The preheated (37°C) pancreatin digestive juice (0.1%) (Table 2) was added to the dish to digest the fragments for 30 min. During the digestion, the fragments were blown gently by use of a Pasteur pipette. The digested fragments were then filtered through stainless-steel meshes (60 µm and 200 µm) and collected by low-speed centrifugation (100 x g, 5 min and 4°C). The *pellet* was resuspended with 10ml of histopaque (Sigma-Aldrich™) and centrifuged two times (120 x g, 5 min 4°C followed by 140 x g, 5 min 4°C) to separate the hepatocytes from the remaining blood cells. The hepatocytes were collected from the supernatant and washed 3 times with non-supplemented HBSS, being the cells collected by low-speed centrifugation (100 x g, 5 min 4°C). As in the perfusion method, cell yield and viability were assessed using the methodology described in section 3.6.

3.6. Assessment of the number of cells extracted and their viability

The hepatocyte cells yield was monitored after the cell isolation procedures by counting the total hepatocyte cells with a hemocytometer. The viable cells were assessed by the Trypan blue exclusion test, mixing 100 µl of trypan blue (0.4%) to 900 µl of the cell suspension. If the cells take up trypan (dark blue), they are considered non-viable. The total and the viable hepatocyte cells were calculated according with the equations:

A		C
B		D

E		F

$\bar{x} = \frac{(\Sigma xi)}{n}$ = A+B+C+D+E+F / 6

Note: the mean value should be calculated for total cells and for dead cells.

$\bar{x}_{\text{viable cell count}} = \bar{x}_{\text{total cell count}} - \bar{x}_{\text{dead cell count}}$

% of viable cells = $\frac{Viable\ cell\ count}{Total\ cell\ count}$ x 100

Dilution factor = $\frac{Final\ Volume}{Cell\ Volume}$ ⇨ Total number (cell yield) = viable cells x dilution factor x 10^4

3.7. Optimization of hepatocyte isolation

During the process of the isolation of primary hepatocytes using the two aforementioned methodologies, some adjustments were made to achieve a better cell viability and better culture purity.

In the case of the two-step collagenase perfusion methodology, perfusion time was shortened. The perfusion is usually carried out at room temperature, which may compromise the cell viability. Thus, besides the solution B, which was kept at 37 °C, all other solutions were kept at 4 °C.

In the case of pancreatin digestion methodology, a major change was performed in the filtration process, the usage of two meshes (60 and 200 µm). A substantial adjustment was also performed in the centrifugation process, the adding of histopaque. This was considered since along the experiments it was noticed that some cultures might be displaying blood cells besides hepatocytes. For this reason, a blood cell screening test was performed with a turbot. A blood sample was isolated and added to a centrifugation tube with 5 ml histopaque. Two centrifugations followed at 120 x g and 140 x g for 5 min at 4 °C and the subsequently formed *pellet* was resuspended in non-supplemented HBSS and washed 3 times (120 x g for 5 min at 4 °C) with the same medium. The Trypan blue assay exclusion was used to observe the morphology of the isolated cells. This observation was then used as a control for blood cell contamination in the primary hepatocytes culture.

3.8. Liver weight and cell yield correlation analyses

The correlation analysis is a statistical method used to evaluate the strength of relationship between two quantitative variables (Gagné 2014). This analysis was performed to determine a correlation of the liver weights (g) of the *P. maxima* and *S. aurata* specimen used in the experiments to the quantity of viable cells produced by said livers.

3.9. Cell plating and monitoring

Cell confluence was observed by plating cells in a T-25 culture flask and taking daily photos for 5 days using inverted light microscopy with 10x and 20x lenses. The confluence was assessed on naked eye observation.

3.10. MTT assay

The cytotoxic effect of the Phe and B[b]f toxicants was assessed via the MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromide) reduction assay described by (Mosmann 1983) and modified by (Carvalho et al. 2008). The MTT Assay is a colorimetric assay which consists of a yellow water-soluble tetrazolium salt that is used to test cell viability. It relies on the absorption ability and metabolism of the MTT salts of the examined cells (Mosmann 1983). When the cells take the MTT salts through endocytosis, these are reduced in the mitochondria or in endosome/lysosome compartments and converted to purple water-insoluble formazan by the mitochondrial and cytosolic enzymes. The quantity of formazan can then be used to represent the reductive potential of cells and their viability (Lü et al. 2012). The primary hepatocytes were isolated from a turbot using the pancreatin digestion technique (and the

resulting cells (2.5 x 10^4 per well) in L-15 medium (with 5% FBS and 1% of antibiotic-antimycotic 100X, containing amphotericin B 0.025 g/l, penicillin G 6.028 g/l and streptomycin 10.00 g/l) were plated in 96 well plates. A total of 200 µl were distributed per well from a solution of 28,6 ml of L-15 a 9,8 ml of the cell suspension. After overnight incubation, the existing medium was then changed for L-15 without FBS, from which 198 µl were added per well. After this step, 2 µl of each contaminant was also added per well with different concentrations along with the wells containing negative and positive control substances (Annex I). The contaminants used were Phenanthrene (5, 25, 50, 100, 250 and 500 µM) and Benzo[b]fluoranthene (1, 5, 25, 50, 100 and 250 µM). MTT solution (2 mg/mL in PBS (1x) sterile; 50 µL per well) was previously heated to 37ºC, the optimal temperature for enzymatic activity, then administered to the wells for hour 0 and again at the 24th and 48th and kept in an incubator to allow the viable cells to metabolize the MTT. After 4 hours past each aforementioned time, the MTT solution was removed from every well and the plaques were frozen at -20 °C to induce cytolysis and the consequent release of the formazan crystals. The formazan crystals that formed were solubilized using 150 µl of a solution of 4:1 dimethyl sulfoxide (DMSO)/glycine buffer per well, and shaken for approximately 20 min at 200 rpm. Absorbance was measured with the use of a Synergy HTX multi-mode reader at 550 nm wavelength. Two independent assays were performed in the course of two weeks, for three experimental times (0h, 24h, 48h). The cell viability of treated cells is presented as percentage over cell viability of cells exposed to vehicle control (% control DMSO) ± STDEV (see Annex II).

4. RESULTS

4.1. Cell yield and viability

The cell yield and viability were assessed after the isolation with both the pancreatin digestion and two-step collagenase perfusion methods (Table 3).

Table 3 – The cell yield and viability of hepatocyte primary cultures obtained sea breams and turbots livers by 2-step collagenase perfusion and pancreatin digestion methods.

Method	Species	Weight	Liver Weight	Exper.[1]	Cell Yield	Viability	Morphology
2-step Collagenase Perfusion	*S. aurata*	244.70g	-	I	7.40 x 10^6	71.1%	Pure[2]
	S. aurata	186.67g	0.25g	II	3.60 x 10^6	72.8%	Mixed[3]
	S. aurata	271.25g	5.96g	III	3.16 x 10^6	70.7%	Pure
Pancreatin Digestion	*S. aurata*	254g	-	IV	2.15 x 10^6	95.1%	Pure[2]
	S. aurata	205.11g	2.29g	V	1.38 x 10^6	100%	Pure
	S. aurata	235.14g	4.10g	VI	6.58 x 10^5	100%	Mixed[3]
	P. maxima	65.95g	0.63g	VII	1.22 x 10^7	100%	Pure
	P. maxima	46.80g	0.35g	VIII	1.52 x 10^7	100%	Pure
	P. maxima	67.98g	0.37g	IX	1.76 x 10^7	100%	Pure
	P. maxima	75.51g	0.48g	X	2.12 x 10^7	100%	Pure

[1]Experiment number.

[2]Blood cell culture.

[3]Mixed culture of blood cells and hepatocytes.

The cells yield ranged from 6.58 x 10^5 to 2.12x10^7 and the viability from 71.1% to 100% (Table 3). Experiment VI yielded a lower viability (6.58 x 10^5) than any other isolation experiment performed. Moreover, the highest cell yield (2.12x10^7) was achieved in the last experiment performed, with the use of the pancreatin digestion method modified (Table 3). The lowest viability was observed with the 2-step collagenase perfusion, with all experiments demonstrating a lower than 75% viability. The highest viability was 100%, which was achieved in all but one experiment using the pancreatin method (Experiment IV), which still amounted to an excellent 95.1%. On average, the turbot was the fish that yielded the most cells (1.66 x 10^7), comparing to the sea breams (3.58 x 10^6).

4.2. Purity and morphology

Photos were taken microscopically to show the morphology of the cells and purity of the cultures during the experiments. These photos also served the purpose of analyzing the amount of cell clumps and debris in the cultures.

Blood cells are identifiable in Fig.3 (a) which makes this culture mixed. On the other hand, very few blood cells were detected in Fig.3 (b) which makes it a pure culture, both exhibit cell clumps and debris.

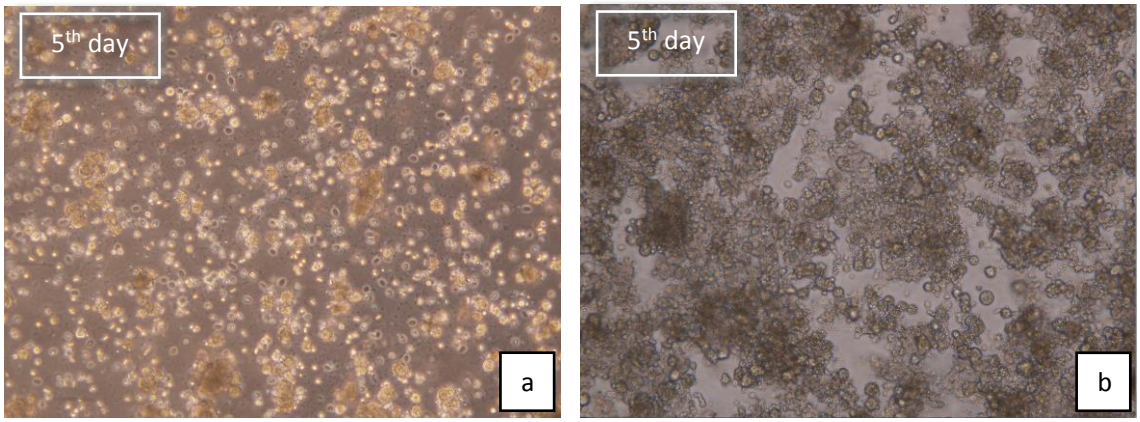

Fig. 3 - Microscopic photos taken from the hepatocyte primary culture isolated from *S. aurata* by 2-step collagenase perfusion, after 5 days of incubation. (a) mixed culture with blood cells and (b) pure culture of hepatocytes.

To observe blood cell morphology, a blood screening assay was performed with a *P. maxima* (Fig. 4). The blood cells observed in this assay were helpful in confirming their presence in some isolated hepatocytes primary cultures (Fig. 3 (a) and Fig 5).

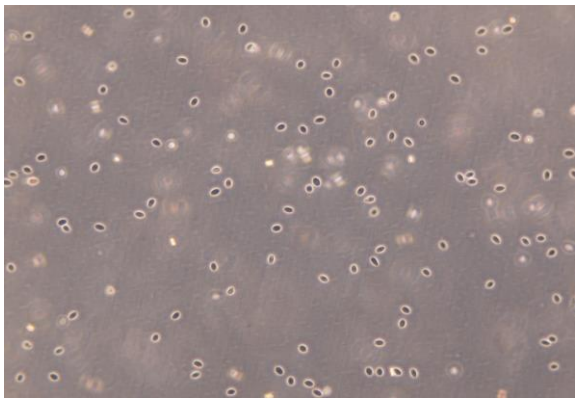

Fig. 4 - Microscopic observation of the blood screening assay

In Fig.5 it is noticeable that the hepatocytes are still in their primordial round shape (a) and start to spread and expand their pseudopods toward extending their shapes (b), which happens when hepatocytes complete an attachment process on a culture surface (Takagi et al. 2012).

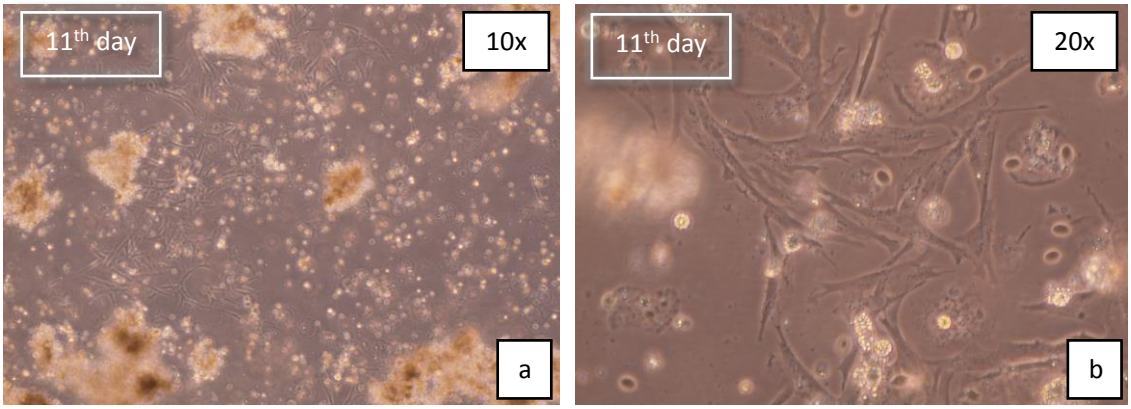

Fig. 5 - Microscopic observation of a near-pure culture of *S. aurata* achieved through the 2-step collagenase perfusion for 10x (a) and 20x (b).

A latter experiment is presented in Fig. 6 (a) and (b) which shows an entirely pure culture, exhibiting no blood cells mainly due to the fact that these photos were taken after the optimizations described in section 3.7 of the present thesis, which included the use of histopaque in the centrifugations.

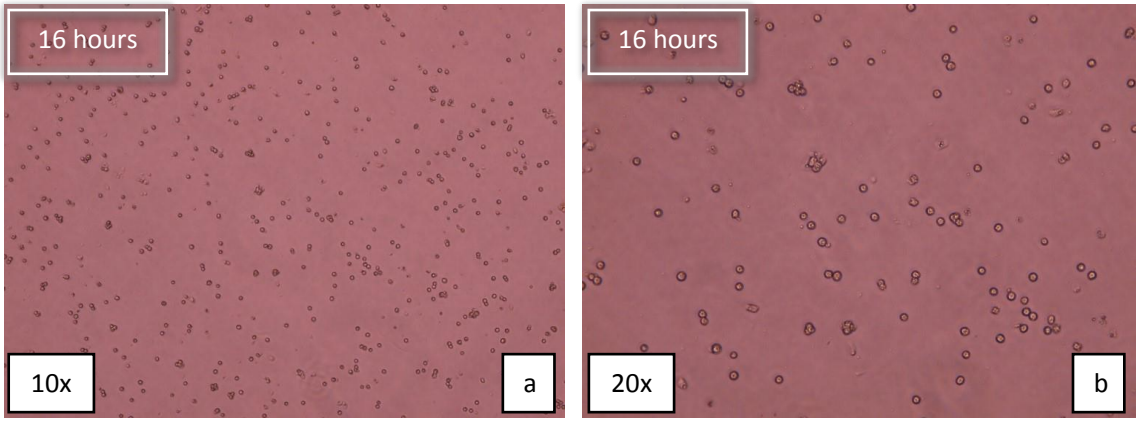

Fig. 6 - Microscopic photos taken after 16 hours of a pure culture of *P. maxima* hepatocytes achieved through the pancreatin digestion method with modifications.

Microscopic observations of all cultures also demonstrate that the latter experiments showed little to no cell debris or clumps (as can be observed in Fig.6) caused by the adding of the 60 µm mesh filter to the filtration process.

4.3. Liver weight and cell yield correlation analyses

As observed in picture 7 there is no relation between liver weight and cell yield.

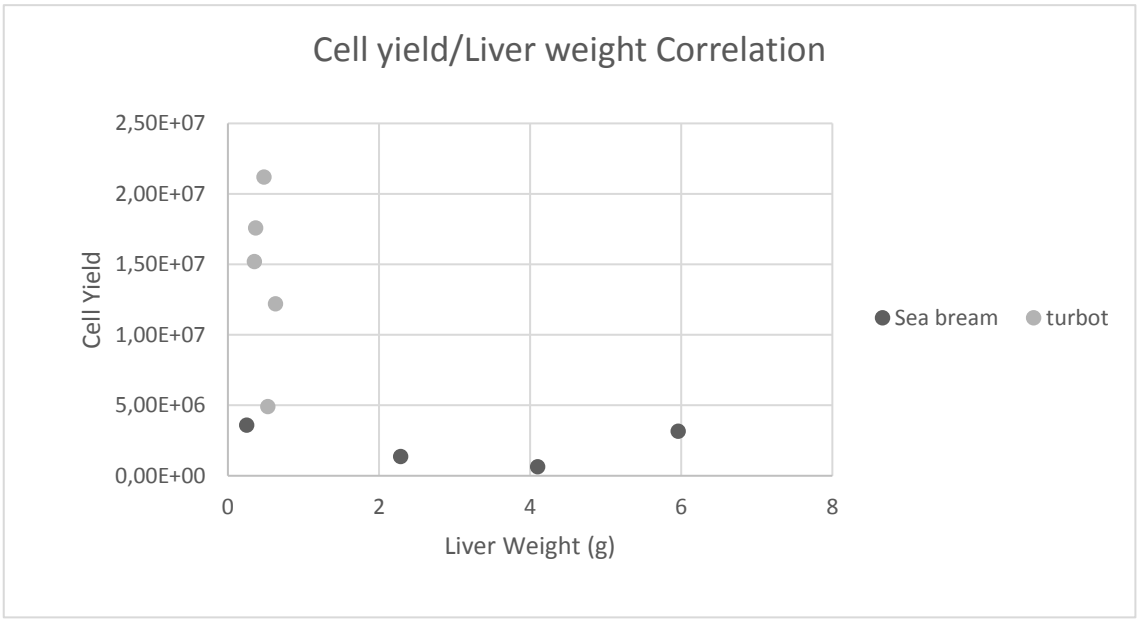

Fig. 7 - Correlation assessment between cell yield and liver weight of sea bream and turbot.

4.4. Cell confluence

The culture of primary hepatocytes in T-25 culture flask was monitored every day up to 5 days (and on the 8th day) to evaluate cell confluence (Fig.8).

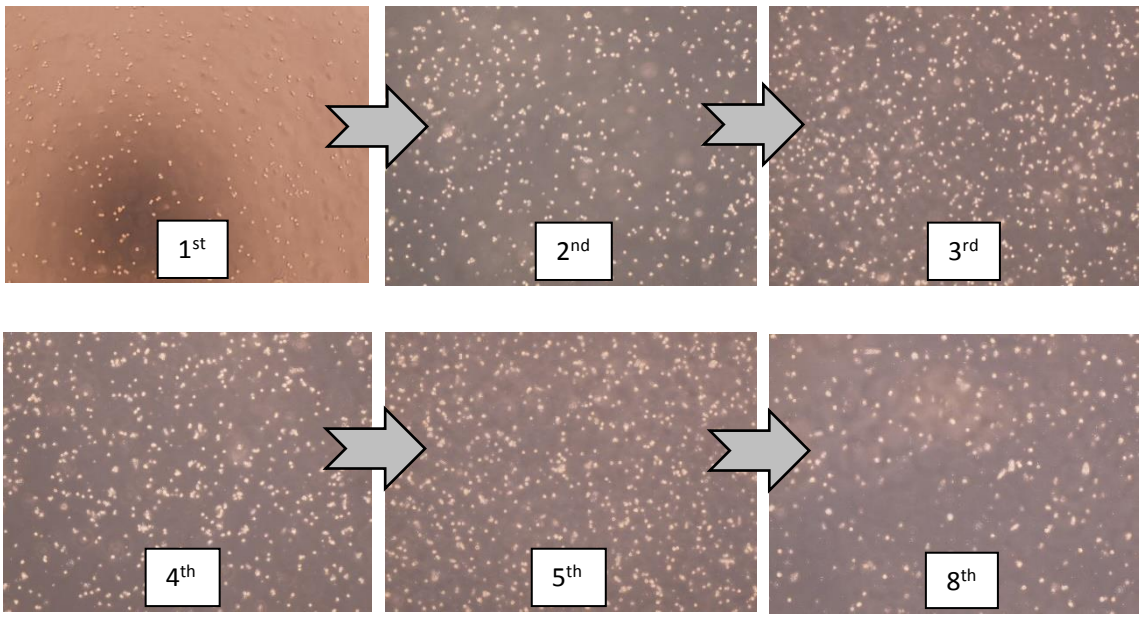

Fig. 8 - Chronological evolution of cell confluence within a culture flask.

The photos demonstrate that the cells were adhering properly to the wells and starting from the second day, the majority of the cells were attached to the flask with only a very small number of suspended cells, a condition that remained until the fifth day of the experiment. Noticeable decrease in confluence could only be seen in the last microscopic observation, done 8 days after the start of the experiment (Fig. 8).

4.5. Cytotoxic effect of Phe and B(b)f assessed by MTT

Fig. 9 and 10 show the cytotoxic effect of Phe and B[b]f on fish primary hepatocyte cells exposed for 24 and 48 h and evaluated by MTT assay.

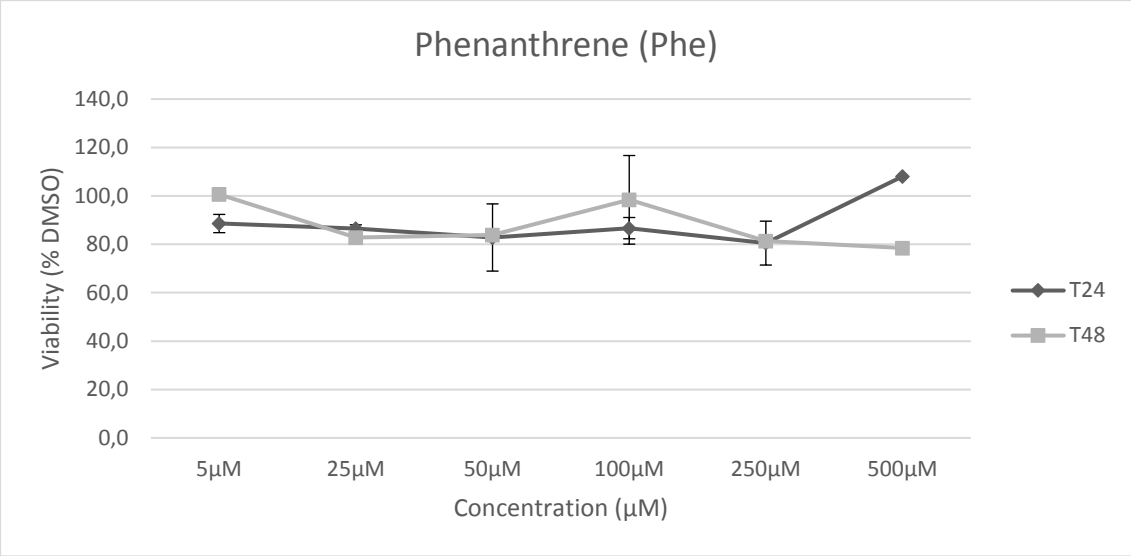

Fig. 9 - Viability of fish primary hepatocyte cells exposed to phenanthrene for 24 and 48 h. Cell viability was assessed by the MTT assay and the results are expressed as the mean percentage of 2 independent replicates relative to vehicle control (% DMSO) ± stand.

The results show that the viability of cells exposed to phenanthrene ranged from 80.5% to 108% after 24h of exposure and from 78.5% to 100.6% after 48h of exposure. In general, the viability decreased to about 80% with the exception of two concentrations, 100 µM and 500 µM, which prompted sudden increases. The differences regarding dose and time of exposure are minimal, with both times showing a seemingly similar trendline with minor deviations.

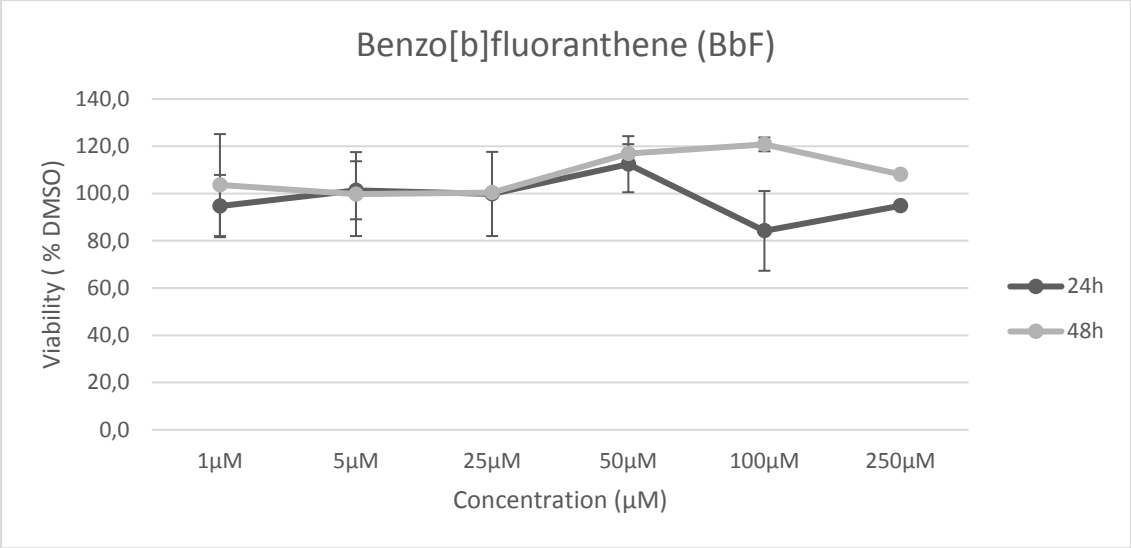

Fig. 10 – Viability of fish primary hepatocyte cells exposed to Benzo[b]fluoranthene for 24 and 48 h. Cell viability was assessed by the MTT assay and the results are expressed as the mean percentage of 2 independent replicates relative to vehicle control (% DMSO) ± stand.

Regarding the Benzo[b]fluoranthene exposures of the primary hepatocyte cells, viability ranged between 84.2% and 112.4% and between 99.8% and 116.9%, for 24 and 48 h of exposure, respectively. The variation between viabilities in the different times are minimal and congregate around 100%, with the sole exception on the 100 µM concentration after 24h of exposure, which saw an inhibition in the order of 16% of viability.

5. DISCUSSION

5.1. Comparison of pancreatin digestion and 2-step collagenase to obtain better yield of viable fish primary hepatocytes cells

The aim of the present work was to optimize the isolation and culturing of primary hepatocytes of marine fish to be used in cytotoxicity studies, complying with the 3R's principle (Replacement, Reduction, Refinement) in toxicity-based tests as a tool for Environmental Risk Assessment. In this study, primary hepatocyte cultures were isolated using two different primary cell isolation procedures, the pancreatin digestion and 2-step collagenase perfusion.

The results show that the pancreatin digestion prompted 100% viability in all but one of the extractions. All isolations using this technique were also pure except one, that yielded a mixed culture of hepatocytes and blood cells. On the other hand, 2 step-collagenase perfusion technique evoked less satisfactory results for the extraction of *S. aurata* primary hepatocytes due to the lack of a good percentage of viable cells. Comparative to perfusion, pancreatin digestion seems to be the better method regarding cell viability percentages after extraction. This can be attributed to the fact that pancreatin digestion is a simpler method that requires less handling and shortens the liver's exposure time to conditions different than those of *in vivo* (e.g.

temperature) and thus producing better viability (Yanhong et al. 2008). The average cell yield (1.14×10^7) also proved to be 3.4-fold higher than that as of the 2-step collagenase perfusion (3.38×10^6). However, in fish, cell yields also vary with species, strain, sex and age of the liver's donor (Segner and Braunbeck 1998). It was also found that there was no correlation between the liver weights and the cell yield and since isolations were made using the pancreatin digestion in small turbot livers and did not result in low cell yields, the notion is added that this method is ideal for small fish because it does not compromise cell yield numbers.

Coincidentally, Yanhong et al. (2008) also found the pancreatin digestion to be the best method for hepatocyte isolation from *Cyprinus carpio* freshwater fish with a viability of 98.4%. On the other hand (Ferreira et al. 2008) isolated *Dicentrarchus labrax* hepatocytes using the two-step collagenase perfusion with viabilities between 90% and 98% and Nabb et al. (2006) isolated *Oncorhynchus mykiss hepatocytes* with viabilities over 95%. Therefore, despite the results of the present thesis, 2-step collagenase perfusion should not be considered as an underperforming method of isolation of fish hepatocytes. Although, there is a notion that using perfusion on small species like *P. maxima* is unadvisable due to the less robust livers with more feeble vascular channels, which lead to worries of bursting the thin connective tissue surrounding the liver (Glisson's capsule) which may lead to a deficient flow of liquid within the capillary bed of the liver (Cabral et al. 2018). The other difficulty with small livers is inserting the cannula in the portal vein which can lead to increased handling time and/or result in deficient perfusion. This method requires trained personnel to perform and is also deemed more expensive since it requires a peristaltic pump and wastes a lot of collagenase (Yanhong et al. 2008).

Another alternative for tissue dissociation is the mechanical disruption method. Mechanical methods of dissociation include the use of filters, chopping techniques, microfluidic devices, and various trituration strategies (Jager et al. 2017). Although, it was not considered for this thesis due to reported damage to cell membranes and liver parenchymal and also loss of function of the isolated hepatocytes (Lee et al. 2013; Li et al. 2017; Yanhong et al. 2008). Furthermore, when compared with pancreatin digestion and 2-step collagenase perfusion, mechanical isolation was found to be the underperforming method, yielding 1.3×10^5 cells and 87.3% cell viability of *Cyprinus carpio* hepatocytes (Yanhong et al. 2008). Comparing to the results achieved in this study, it can be concluded that cell yields were high in both methods, but a remarkable difference was observed in terms of cell viability, which was higher when using the pancreatin digestion method.

To note that this thesis was a process of optimization of the method for the isolation of marine fish hepatocytes and that, as stated previously (section 3.5), various optimization steps were taken to secure better viabilities upon extraction. The biggest overall changes were the use of histopaque and a second smaller mesh filter upon the filtration processes. The first and forth experiment yielded culture contaminated with blood cells and some of the other cultures appeared to have a few blood cells among the hepatocytes (as verified by the blood screening assay performed). Histopaque helped separate blood cells which lead to purer cultures of hepatocytes. The adding of another mesh filter was performed due to too much tissue passing through the mesh which was thought to negatively affect the disruption of intercellular connections. The shortening of the enzymes concentration (in this case pancreatin) has also been considered to possibly lessen injury to hepatocytes (Yanhong et al. 2008). Additionally, the

duration of the perfusion was also shortened to decrease vascular pressure and avoid the bursting of the Glisson's capsule, but mainly to decrease the time of exposure to room temperature. These optimizations could be the sole reason that the 2-step collagenase perfusion and the use of the *S. aurata* yielded less satisfactory results, since they were the first performed.

A test was also performed plating cells without these contaminants in a T-52 culture flask to assess cell death. Cells maintained at a good confluence level for 5 days, demonstrating their potential for *in vitro* cytotoxicity studies for this period.

5.2. Primary hepatocyte application for toxicity evaluation of Phe and B[b]f:

The other part of this thesis focused on the study of the effects of the 2 model PAHs, Benzo[a]fluoranthene and Phenanthrene on the hepatocytes extracted by the selected pancreatin digestion method. A cytotoxic assessment was performed using the MTT assay and the results show that there is no significant decrease in the viability of the cells when exposed to a wide range of concentrations of Phenanthrene and Benzo[b]fluoranthene.

Sogbanmu et al. (2016) found that phenanthrene induced the highest toxicity out of 4 single compound PAHs and a PAH mixture, causing a dose-dependent increase in mortality and abnormalities to *Danio rerio embryos* (zebrafish) at 72 hours post-fertilization. Sublethal effects of phenanthrene have also been reported at concentrations as low as 2.4 µM (Butler et al. 2013). Based on these results, the concentration range used in this thesis should be enough to trigger cell death, at best at the higher concentration (200 µM). Although, it was not possible to assess phenanthrene toxicity since viabilities showed little variability. Moreover, the small variations observed can be caused by factors such as an interference in the absorbance readings due to precipitation of the contaminants, which occurred, mainly for the higher concentrations. A future consideration for this problem might be adding a nonvolatile cosolvent to the DMSO, such as glycerol, to aid in resolubilization (Waybright et al. 2009). The intra-species variability aspect of a test subject should also be considered as a possible interference in toxicity results since fish differ in morphology, liver size and other characteristics.

Benzo[b]fluoranthene yielded similar results and similar trendline. The only notable variation of viability was a small decrease of 16% in viability that can also be attributed to the reasons aforementioned for phenanthrene.

In these experiments, the MTT assay worked but was not expressive which might be attributed to the tested compounds possible interaction with MTT. Despite interference of MTT with Phe or B[b]f not being documented, other compounds have been reported to interfere with mitochondrial dehydrogenase activity, which may lead to overestimation (activation of the MTT reducing dehydrogenases) or underestimation (inhibition of mitochondrial dehydrogenases) of the MTT assay results (Jaszczyszyn and Gasiorowski 2008). Despite being widely used, the MTT reagent may exhibit cytotoxic effects that can result in cell death during an experiment, being found to induce toxic effects in eukaryotic cells (Sittampalam et al. 2019). A recent report also indicated that formazan crystals might be able to harm cells by puncturing membranes during exocytosis (Lü et al. 2012). Even though none of these processes was detected with certainty in this thesis, it is suggested that further works implement another assay for cell viability to complement MTT results.

Overall, the cytotoxicity assay with B[b]f and Phe produced inconclusive results. This leads to the underline imperative that further work is needed to assess viability after contamination with these compounds on fish hepatocytes.

6. CONCLUSION

The present study attempts to make a contribution to the optimization of the dissociation methods discussed by way of modifications that improved purity of cultures and helped reduce cost and duration of experiments. The optimized pancreatin digestion method appeared to be optimal for marine fish hepatocyte isolation mainly due to exhibiting perfect viability and good cell yield. This result is expected to contribute to the proliferation of *in vitro* enzymatic methods for hepatocyte extraction which leads to the reduction of *in vivo* assays being performed, in line with the 3R's principles of Replacement, Reduction, Refinement that provide a framework for more animal research practices.

The good viability and state of the cells isolated by the methods discussed in this thesis, especially with the optimized pancreatin digestion method, showed substantial potential for their future use in cytotoxicology studies to screen chemical contaminants, especially PAHs.

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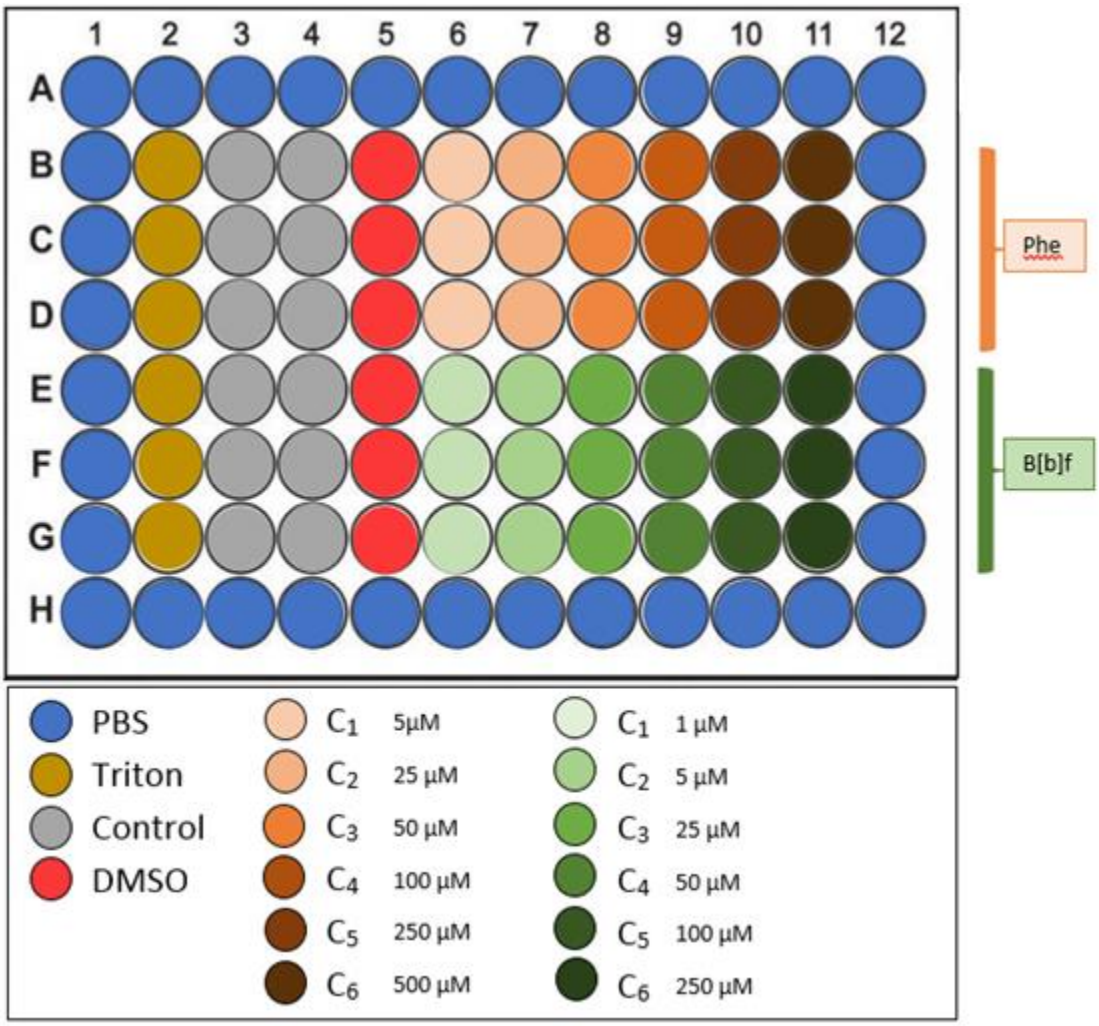
ANNEXES

Annex I - Plate configuration of the MTT assay with different concentrations of Phe and B[b]f.



Annex II – Average viabilities after 24h and 48h of contamination with different concentrations of Phe and B[b]f for 2 replicate experiments.

Phe Concentrations	**T24**			
	R1	R2	average	st.dev.
1µM				
5µM	85,9	91,2	88,6	3,8
25µM	87,6	85,4	86,5	1,5
50µM	106,2	73,0	89,6	23,5
100µM	83,6	89,8	86,7	4,4
250µM	86,9	74,1	80,5	9,1
500µM		108,0	108,0	

Phe Concentrations	**T48**			
	R1	R2	average	st.dev.
1µM				
5µM	101,7	99,6	100,6	1,5
25µM	82,7	83,0	82,9	0,2
50µM	120,2	83,9	102,0	25,7
100µM	121,3	126,0	123,7	3,3
250µM	80,5	107,2	93,8	18,8
500µM		78,5	78,5	

BbF Concentrations	**T24**			
	R1	R2	average	st.dev.
1µM	104,0	85,4	94,7	13,2
5µM	110,1	92,7	101,4	12,3
25µM	112,4	87,2	99,8	17,8
50µM	120,8	104,0	112,4	11,9
100µM	96,1	72,3	84,2	16,9
250µM		94,9	94,9	
500µM				

BbF Concentrations	**T48**			
	R1	R2	average	st.dev.
1µM	88,3	118,8	103,6	21,6
5µM	87,2	122,0	104,6	24,6
25µM	140,3	100,4	120,4	28,2
50µM	130,2	119,7	125,0	7,4
100µM	118,7	122,9	120,8	2,9
250µM		108,1	108,1	
500µM				