

Faculty of bioscience engineering

The impacts of pressure and trimethylamine N-oxide on pollutant metabolism in European eels

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ABBREVIATIONS

3-MC	3-methylcholanthrene
AChE	Acetyl cholinesterase
AhR	Aryl hydrocarbon receptor
ARNt	AhR Nuclear translocator
ATPase	Adenosine triphosphatase
CAR	Constitutive Androstene
CAT	Catalase
ChE	Cholinesterase
COX	Cytochrome oxidase
CYP	Cytochrome P450
CYP1A	Cytochrome P450 1A
DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetraacetic acid
EROD	Ethoxyresorufin-O-deethylase
FMO	Flavin containing monooxygenase
FW	Fresh water
GLC	Gas liquid chromatography
GPX	Glutathione peroxidase
GR	Glutathione reductase
GST	Glutathione-S-transferase
HHP	High hydrostatic pressure
HP	Hydrostatic pressure
HSP	Heat shock protein
MPa	Megapascals
NADPH	Nicotinamide adenine dinucleotide phosphate
OCs	Organochlorines
PAHs	Polycyclic Aromatic Hydrocarbons
PBDEs	Polybrominated diphenyl ethers
PCBs	Polychlorinated biphenyls
PCLS	Precision cut liver slices
POPs	Persistent Organic Pollutants
POR	Cytochrome P450 oxidoreductase
PXR	Pregnance X Receptor
PY	Protein yield
ROS	Reactive oxygen species
SOD	Superoxide Dismutase
SULT	Sulfotransferase
SW	Seawater
TMA	Trimethylamine
TMAO	Trimethylamine N-oxide
UGT	Uridine 5'-diphospho-glucuronosyltransferase
XAP2	Hepatitis B Virus X-associated protein 2

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ABSTRACT

The aim of this master thesis was to assess the role of trimethylamine N-oxide (TMAO) in the inhibitory effect of pressure on pollutant metabolism in European eels.

By measuring TMAO levels in liver slices exposed to pressures going up to 30MPa, we first wanted to prove correlation between increasing pressure and TMAO levels in eel liver cells. Further, we wanted to compare transcript levels of associated genes implicated in pollutant metabolism under high pressure (30MPa) with those remaining at atmospheric pressure (0.1MPa). This would have allowed us to evaluate whether or not pressure inhibits the metabolism of pollutants in the liver of the European eel. As safety measures related to the COVID-19 crisis prevented us from carrying out the planned experiments, this thesis explains our intention and the importance of this work.

First, the complete life cycle as well as the different life stages of the eel have made it possible to understand the importance of its spawning migration towards the Sargasso Sea, announcing the end of its life but simultaneously the beginning of that of its offspring. Over years, wild eel stocks have been gradually declining to a point where they are now classified as “Critically endangered species”. Among the different lethal obstacles involved in this decline, POPs are largely responsible. Indeed, scientific reports have shown that pollutants in high concentrations in the bloodstream are able to cause disturbances in the reproductive, immune, endocrine and nervous systems. They are also able to induce lipolysis thus depleting energy reserves needed for migration and so, endangering European eel’s life. After questioning which factor was preventing eels from getting rid of those persistent pollutants, high hydrostatic pressure appeared to be overwhelmingly implicated.

The second part of this work has therefore focused on the effects of pressure on global fish, and more precisely, pollutant metabolism. As pressure experiences had not yet been done on eels, we relied on results from other fish species. These data demonstrated the inhibitory effect of pressure on the expression of genes involved in metabolic reactions of pollutants. Thus, CYP1A, HSP70 and HSP90 expression levels decreased drastically after being exposed to high pressure (15MPa representative 1500 m depth) and pollutant (3-MC 25 μ M) for 6 and 15 hours in both pressure and non-pressure adapted species.

Consequently, third part of the work focused on the potential implication of the osmolyte TMAO in the inhibitory effect of pressure. It was demonstrated that accumulated TMAO levels were significantly higher in deep-sea fish, making it possible to establish a linear relationship between TMAO concentrations and depth (and thus pressure). These experiments conducted on other species than eels have led to the postulate that HHP induces the production of FMO₃ in fish liver cells and that the increased production of TMAO may interfere with phase 1 detoxification process thus affecting the migration of European eels.

In conclusion, only by carrying out the planned eel experiments will we be able to validate the hypothesis of the involvement of TMAO in the pressure inhibition of detoxification process in European eels. These experiments would thus help to explain the current decline in European eels.

INTRODUCTION

Known for its unusual snake-like appearance and its legends, the European eel seems familiar to everyone, even though its lifestyle and life cycle remain unknown for most people. However, this animal has the supernatural capacity to cross the Atlantic ocean for over 5 000 km to reach freshwater aquatic systems, thanks to its adaptability to different saline environments.

Over years, the European eel has unfortunately been in decline: wild eel stocks have been dropping since the 1980s all over Europe. Multiple causes are at the origin of this decline: diseases, predators, overfishing, pollution, ...

The aim of this research is to evaluate the effects of pollutants, commonly called POPs (Persistent Organic Pollutants), on the European eel and more precisely on its reproductive migration. The reproductive migration is an essential element in the eel's life cycle because it represents its journey to its spawning ground: the Sargasso Sea. This is where eels will lay their eggs to give birth to their offspring before dying.

POPs can be ingested by eels during their growth period and can accumulate in eel lipid reserves thanks to their lipophilic properties. During migration, lipids are metabolized and POPs are released into the bloodstream. At high concentrations, they can reach toxic levels and disrupt reproductive, nervous and endocrine systems of adult eels.

We will focus on the different conditions encountered during eel's migration and thus the factors or compounds that could possibly be involved in the inhibition of the detoxification process. The effects of pressure on this metabolism will be discussed as well as the effects of trimethylamine N-oxide (TMAO), a common osmolyte in fish.

This master thesis is a bibliographical review of what has already been done on eels or other fish species. Each chapter will attempt to provide answers to the main questions based on experiences and knowledge acquired to date in the scientific and marine world.

CHAPTER 1: INTRODUCTION OF THE EUROPEAN EEL

1. Portrait of the European eel

1.1 Characteristics

The European eel (*Anguilla anguilla* Linnaeus 1758) is a teleost and catadromous fish, typical of Western European waters, from the order of the Anguilliforms. The European eel has a wide geographic range of distribution, from the coasts of the Mediterranean Sea to the Northern Atlantic Ocean and from Mauritania to Iceland (Ringuet, Muto, & Raymakers, 2002). They live in marine, freshwater and brackish aquatic environments at depths ranging 0-800 m on average (Tsukamoto et al., 2011).



Figure 1: European eel (*Anguilla anguilla* L.) (Steffen Zienert, 2011)

The European eel is characterized by an elongated, cylindrical body composed of 150 vertebrae (Figure 1). Unlike most fish, the European eel doesn't have separate pelvic, ventral, caudal or dorsal fins. It only possesses a single continuous fin known as the "odd fin" starting from the front of the back to the end of the body and a pair of pectoral fins. Furthermore, its skin is also extremely thick and resistant (Kuroki & Tsukamoto, 2013).

Female eels are generally larger than males and the largest recorded mass of an eel was 6,599 kg. The average length is about 80 cm and the maximum length ever recorded is about 148,7 cm (Tutman, Glamuzina, Bartulović, & Dulčić, 2007).

1.2 Life cycle

The life cycle of eels is very particular and research is still needed to fully describe it. Over their complete lifespan, they pass through many different stages. They start their life as “leptocephalus larvae” then become “glass eels”, then “yellow eels” and finally they complete their cycle as “silver eels” (Figure 2). Although the European eel spends most of its growth (recruitment area) in inland waters, the lifecycle is still primarily dependent on oceanic conditions as most stages of the cycle (sexual maturation, migration, spawn and transport of the larvae) occur in the marine realm. The capacity of the European eel to evolve in environments with different salinity levels is characteristics of amphihaline migratory species (Kuroki & Tsukamoto, 2013).

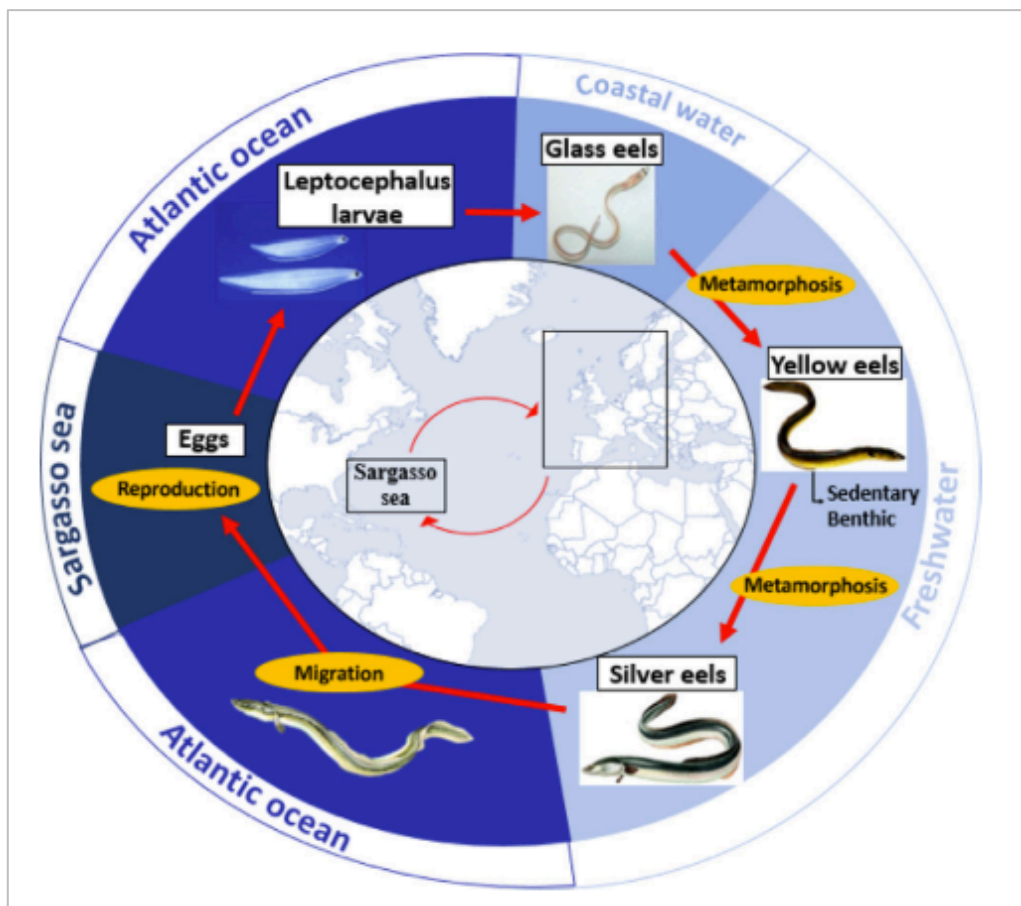


Figure 2: Life cycle of the European eel (Lortholarie et al., 2020)

a) Spawning

The term “spawn” designates the action of depositing eggs. Spawning will allow the eggs to hatch therefore starting the life cycle of young European eels. Unlike most fish species, eels are only able to spawn once in their lifespan. The exact way and location where the spawning of the European eel occurs has not been clearly defined although to fully understand the life history of the European eel, it is crucial to know the exact location of their spawning grounds

as well as to understand how the larvae are then carried to the appropriate area for maturation. About one century ago, Johannes Schmidt, a Danish scientist, first studied the spawning location of this eel species. He organized a research expedition which intended to undertake a survey of the breeding grounds of the eel. His research team was able to track down larvae at their youngest age and so chart the area where eels first entered their existence (Schmidt, 1923). Those historical collections showed that small leptocephali larvae (<10mm) were collected in a region of the North Atlantic Ocean leading to the theory of Schmidt which states that the European eels spawn their eggs in the Sargasso Sea (Miller et al., 2019; V. J. T. van Ginneken & Maes, 2005). This theory remained controversial as the spawning process of the eggs by mature adult eels had not been properly observed.

In 2014, the hypothesis was however confirmed by two research groups which further studied the eel in specific areas of the Sargasso Sea (Ayala & Munk, 2018). During this study, <12 mm leptocephali larvae were found over a 2000 km distance in the Sargasso Sea. These results prove that spawning can occur across an extensive region within one spawning season.

By catching the larvae it was also observed that spawning occurs south of where warm southern surface water meets cold northern water and fronts are created (Miller et al., 2019). By observing the reproductive behavior of the European eel in laboratory, it was also shown that spawning is possibly stimulated by pheromones and is simultaneous and collective (V. J. T. van Ginneken & Maes, 2005).

b) Leptocephalus Larvae

After the eggs have hatched, the young eels start their life cycle as transparent and leaf-shaped larvae. Their bodies, compared to their heads, are large and gelatinous which helps them drift on ocean currents since they are still unable to swim. The improved hydrodynamic performance conferred by this physiognomy is essential as leptocephali undertake their first migration of more than 4000 km to the coasts of Europe (Kuroki & Tsukamoto, 2013). So the leptocephali start a 8 to 9 months journey where they are transported along the Gulf Stream and North Atlantic Drift to finally drift to the eastern Atlantic coast; their recruitment areas (Lecomte-Finiger, 1992; Tesch & Rohlf, 2003).

c) Glass eels

The larvae reach Europe in winter, however the accurate duration of this first migration is unknown. Approaching the coasts, they undergo their first metamorphosis into glass eels acquiring the ability to swim. Once these eels get to the coastal margins, they gather in huge shoals to wait for the water to warm up. Once entered their continental habitat, the glass eels metamorphose into elvers, miniature versions of adult yellow eels. Finally, in February and

March, they start to colonize the brackish waters and the first eels are found in the estuaries. Some of them will remain in that environment while others will leave the estuaries to rapidly progress upstream and transform into pigmented yellow eels (Kuroki & Tsukamoto, 2013).

d) Yellow eels

Once the eels reach a length of 30cm, they can be described as yellow eels. Turned into yellow eels, they enter the growing phase during which they gain weight until reaching partial maturity. The duration of this phase varies a lot due to gender differences. Indeed, for female eels, this phase lasts between 4 and 20 years, while for males, this phase can last from 2 to 15 years (Lecomte-Finiger, 1992).

Yellow eels feed on benthic fauna. After their period of growth, they will undergo the last metamorphosis called “silvering process”, in preparation for their return to the spawning areas for the reproduction. As indicated by its name, the 3rd transformation leads to the eel’s skin turning silver. This pigmentation takes place to allow the eel to cope with the lack of solar light at depth and also helps for the differentiation between silver eels and yellow eels which are not yet migrating. On top of that, the silvering process leads to a large increase in body fat content; going from 8 to 28% (Lecomte-Finiger, 1992). In the book “Eels and Humans”, Boëtius and Boëtius state that the energy supply of the eels during the silver phase mainly consists of triglycerides i.e. 80% of the total energetic supply of the body (Boëtius & Boëtius, 1980; Kuroki & Tsukamoto, 2013).

During this last metamorphosis, eels undergo a lot of important physiological changes in order to prepare themselves to temperature, salinity, hydrostatic pressure and light variations (Tesch & Rohlf, 2003). The pectoral fin becomes longer to increase the stabilization in the water column and salinity-tolerance is improved thanks to better osmoregulatory capacities and by gill chloride cell multiplication (Durif, Dufour, & Elie, 2005). An increase in the developed muscular power output allows better swimming abilities and enlarged eye size promotes visibility for the dark environment (Carlisle & Denton, 1959; Spierts, Ellerby, & Altringham, 2001).

Finally, during the silvering multiplication process the eel reaches sexual maturation preparing for spawning. These events explain the important fat storage as the shrinking of the stomach and gut so that the fish can only absorb water (Kuroki & Tsukamoto, 2013).

e) Reproductive migration

Reaching the silver stadium, the European eel is not able to live in freshwater anymore and therefore starts migrating south. At this stage, the lifestyle of the eel changes completely: it stops feeding and acquires salt tolerance. In order to reach the Sargasso Sea to spawn, eels

migrate across the Atlantic Ocean traveling more than 5000 km (Figure 3 : blue arrows represent the migration route of the European eel) (Bruijs, Aquatic, & Durif, 2009).

Each year, adult European eels start their migration from European rivers in autumn (October-December). The duration of this migration is not known but larvae analysis and other field

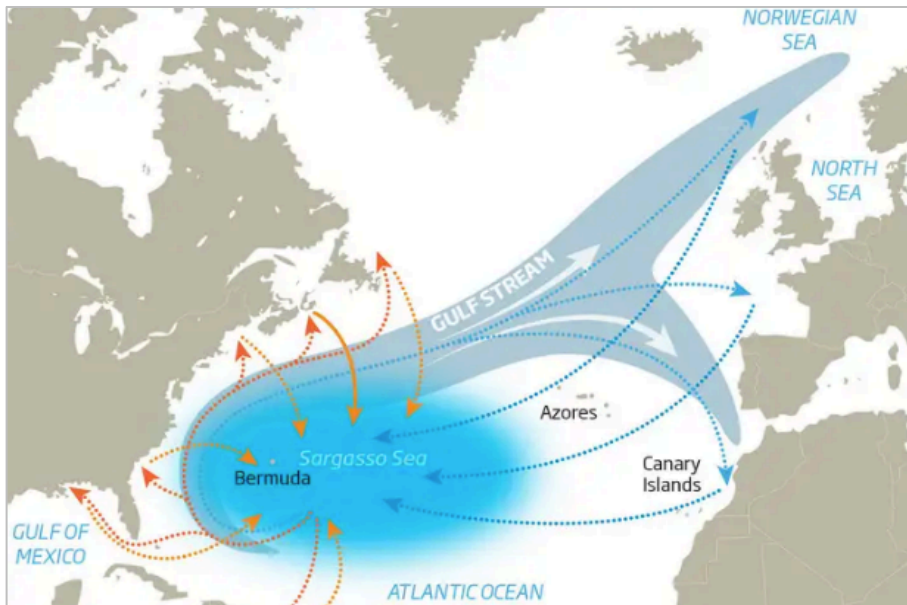


Figure 3: Migration of the silver eel to the Sargasso Sea (Pearce, 2016)

studies suggest that the smallest larvae (< 5 mm) are found between February and May. Therefore, it can be assumed that the eel migration lasts between 80 and 170 days. As eels are semelparous (spawning once in their lifespan), they will quickly die after laying eggs (Pearce, 2016).

Migration route

A study was made to reconstruct the reproductive migration route of the European eel. Data collected over 5 years tracking the journey of eels released at 5 different locations in Europe were used.

The results showed a vertical migration behavior in eels moving between depth of 200m to 1000m in day/night cycles. This type of migration is the result of a daily change in temperature with cooler temperatures during the day than at night. Eels are found in warm, shallow water at night and cool, deep water during the day. Thereby, the daily temperature range experienced by eel varies between 0° and 11°C. This vertical migration regulates their body temperature and is not intended to protect them from predators (Lennox et al., 2018). Furthermore, it is highly possible that this vertical migration allows eels to access different salinities and photic conditions (Lennox et al., 2018).

Thus far, the scientists succeeded to reconstruct the full migratory path of the eels and confirmed that eels follow routes all converging on the Azores Region. Even eels released from the Swedish west coast and the Baltic outlet followed a route towards northeastern parts of the Atlantic Ocean (Righton et al., 2016). It is likely that European eels use selective tidal streams transport to cross the continental shelf as well as to cross the Atlantic Ocean (V. J. T. van Ginneken & Maes, 2005).

An important characteristic to take into account is the acclimation of eels to seawater. As J. Cliff Rankin describes it in the “Spawning migration of the European eel” collection; teleost fish are used to adjust the osmotic regulation of their body fluids at about 30-40% of the level of the oceanic sea water (named osmoregulation). This principle leads to two problems: osmotic entry of water and diffusional loss of salts. This is where it is essential to distinguish two types of teleosts: the majority, called stenohaline, only possess the mechanisms to osmoregulate in either sea water or fresh water. The other minority is able to move between fresh and salt water either at certain stages of their life cycle; they are called euryhaline. Eels are part of this minority thanks to basic features of osmoregulation and their endocrine control mechanisms (Rankin, 2009).

What about the energy costs of this reproductive migration?

One of the principal questions about the spawning migration remains how the European eel is able to swim for about 180 days at the same low energetic cost? Ginneken and Maes conducted a study to determine the energy cost of a 5500 km migration. They thereby proved that eels can swim four to six times more efficiently than non-anguilliform fish.

A 5500 km laboratory swim trial, officially demonstrated for the first time that European eels are physiologically able to reach the Sargasso Sea without feeding (V. J. T. van Ginneken & Maes, 2005).

Boëtius and Boëtius made estimations about the total body lipid stock and found out it should exceed 20% of the body weight to cover the migratory needs of the silver female European eel (Boëtius & Boëtius, 1980). van den Thillart et al. found that fat consumption for a 5500km run would be 126,5 mg/g wet weight which corresponds to 60% of the total fat reserve of most silver eels. Moreover, based on the fat percentage in oocytes, measurements show that 40% of the total fat reserve is required for incorporation in oocytes. Animals with less than 13% of the body weight would therefore not be able to swim 5500km (additional 7% of the body weight is needed for oocytes) (Geeraerts & Belpaire, 2010). Finally, other studies showed that energy supply are, in principle, sufficient and that fat, proteins and hydrocarbons are consumed in the same proportions (V. van Ginneken et al., 2005).

What are the different obstacles encountered during migration?

Migratory species are exposed to additional risk because they are crossing multiple marine habitats. This long-distance journey through estuaries, rivers and fjords also exposes them to considerable interference from human infrastructure (Lennox et al., 2018). Thus, their migration route to the Sargasso Sea is fraught with obstacles.

Flood control dams, flood gates, hydropower stations or pumping stations which are very abundant in European river systems are all examples of the obstacles encountered by eels

during their migration. Those structures have an important impact on the river regulation but also on the spawning migration of the eels. Fisheries and pumping stations seem to be the most lethal for eels as modern pumps are 100% lethal for any incoming fish (Bruijs et al., 2009).

The consequences of those barriers are very tragic for downstream passing fish populations. In addition to the danger of massive hydropower stations, silver eels also can be affected by wide drops and by the entrainment at pumping stations and industrial cooling waters. Even though the downstream migration is known for many years, only very few publications concern standard technology for fish protection.



Figure 4: Eels collected from trash rack at Wahnhausen power station (Bruijs et al., 2009)

In addition to these obstacles, predators also play a role in slowing down the downstream of European eels. A lot of data suggested that eels released from Ireland and the Mediterranean region are exposed to the highest risk of predation (Bruijs et al., 2009).

Although the abundance of predators in the Sargasso Sea is approximately low, it could bring disadvantages if the shoal of fish slows their migration pace because it could strongly affect the reproductive process (Maes, Pujolar, & Volckaert, 2009; Righton et al., 2016).

2. Fisheries

The European eel is widely distributed across European and North African coastal areas and represents an important craze in fisheries. Eels are mostly exploited as a food source and at all stages of their life cycle they represent an important interest for the fishing industry (ICES, 2017). They are traded both locally and internationally.

As they arrive in Europe during winter, the exploitation of the glass eels mainly takes place from the middle of winter until early spring. The eels caught are of interest for aquaculture, local consumption or storage (ICES, 2016). In Europe, a total of 25 000 tons of eels are consumed per year (Usui, 1991).

With regard to yellow and silver eels, most fishing activities take place during the emigration period in the late summer and autumn, when most of the eels are migrating (ICES, 2016).

2.1 Economic issues

Eel fisheries in Europe are covering an area of approximately 90 000 km². This represents a significant economic outcome generating income for roughly 25 000 people (Dekker, 2004). The eel trade started in the early 1900s with a small-scale subsistence fishery supplying a localized market. This was quickly followed by a commercial explosion leading to the exploitation of new areas and new fishing methods (Dekker, 2019). Over time, wild eel stocks have declined exerting increasing pressure on fisheries and aquaculture especially as it is not possible to artificially reproduce European eels (Palstra, Cohen, Niemantsverdriet, Van Ginneken, & Van Den Thillart, 2005).

2.2 Evolution of wild eel stocks

The total landings of yellow and silver eels decreased from 18 000 – 20 000 tons in 1950 to 2000 - 3000 tons since 2009 (ICES, 2017).

The landings of European eels reflect the stocks of wild eels in the different countries of Europe. In the ICES advice of 2016 it is demonstrated that the landings have diminished significantly over the last 60 years in most European countries (Figure 5) (ICES, 2016).

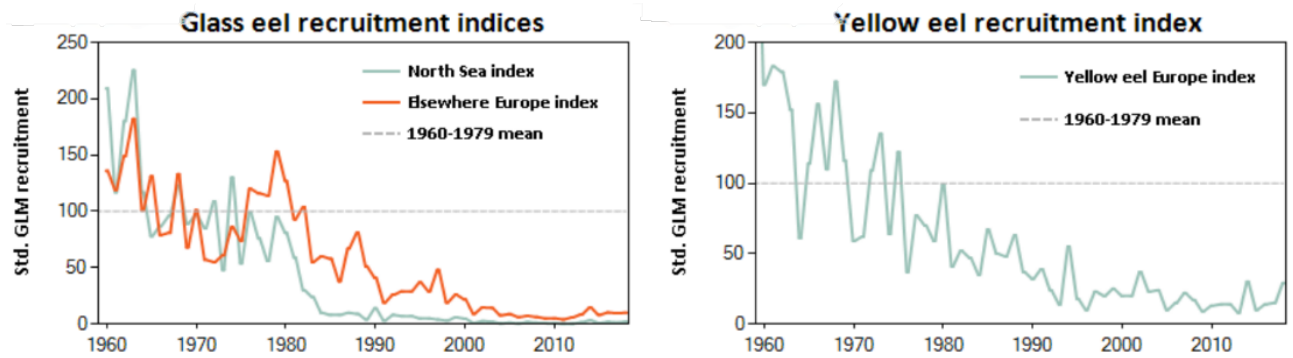


Figure 5: Geometric mean of estimated glass & silver eel recruitment for both continental “North Sea” and “Elsewhere Europe” series (ICES, 2018)

Figure 5 describes the indices of both glass and yellow eel recruitment. Obviously, they have strongly declined from 1980 to 2010 and have remained at a low level since. The annual recruitment of yellow eels in 2018 was 29% of the 1960-1979s level. Concerning the glass eel recruitment, the level in 2018 was 2.1% of the 1960-1979s level in the “North Sea” series (including Norway, Sweden, Germany, Denmark, the Netherlands and Belgium). In the “Elsewhere Europe” series (including UK, Ireland, France, Spain, Portugal and Italy) the level in 2018 was 10.1% of the 1960-1979s level.

The ICES (International Council for the Exploration of the Sea) actions taken for the protection of the European eel are mainly aimed at all anthropogenic impacts contributing to the reduction of silver eels populations. Those impacts should be reduced as close as possible to zero for 2019-2020 (ICES, 2018).

CHAPTER 2: POLLUTANTS EXPOSURE AND EFFECTS ON THE ENDANGERED EUROPEAN EEL

Since 2008, the drastic decrease of wild eel stocks in Europe has led to its classification as a “Critically endangered species” following the IUCN red list criteria (OSPAR Commission, 2010). This means it is not yet threatened with extinction but may become unless trade is controlled. Thus, since 2010, import and export of eel from the EU has been prohibited.

1. Possible causes of decline

According to the report of the Joint EIFAAC/ICES/GFCM Working Group on Eels (WGEEL), eel’s mortality is mostly attributed to fisheries, hydropower turbines and pumps. In fact, there are many other causes that are also mainly implicated in this decline (ICES, 2019).

Therefore, it is essential to identify these causes, in order to be able to act for their survival.

Habitat damage

According to the project INDICANG1, the wetlands and backwaters are being massively destroyed since the second half of the past century. This is problematic since those habitats are the most convenient for eels. Destruction of those areas is responsible for increased mortality but also for the modification of sex ratio in the remaining population. Indeed, sex determination is density-dependent in eels. This means that when their habitats are destroyed, the eels are forced to concentrate in smaller remaining areas. As density increases, the male proportion in the population increases thus reducing the reproductive ability of the population (OSPAR Commission, 2010). Moreover, obstruction of downstream migration reduces the capacity of the silver eel to reach its spawning location (Ringuet et al., 2002).

Diseases and parasites

In the last years, eels have been affected by many parasites and viruses. In the 1980s, a parasite called *Anguillicoloides crassus* (swimbladder nematode) infected wild eel populations after being accidentally introduced from East Asia (Ringuet et al., 2002). The repercussion of *Anguillicoloides* infections is responsible for damaging swim bladders and impairing eels swimming competence. Those contaminations actually result from poor health and can, in extreme cases, cause death (OSPAR Commission, 2010). Besides, viruses such as the Eel Virus European X (EVEX), the *Herpesvirus anguillae* (HVA) and the Eel Virus European (EVE) can also be detected in wild and farmed eels. If fish are under stressful conditions, they can cause disease or even mortality. Such viruses were isolated from eels and showed skin and gill

erythema and necrosis of the skin and the liver possibly leading to fatal consequences (V. van Ginneken et al., 2004).

Climate change in the ocean

In 1996, Knights et al. (Knights, 1996) suggested that a northward deviation in the north barrier of the Gulf Stream pushes some leptocephali larvae to follow a more northerly route. This could then lead to the exposition of the larvae to less convenient temperatures and a less food-rich environment. Consequences could then be fatal if the larvae are not carried safely to their recruitment areas.

Pollution by contaminants

The greatest threat to the eel population is the water pollution by contaminants. Even though multiple eel species are threatened by anthropogenic activities, pollution and climate change have an even greater impact on European eels as every stage of their life cycle is affected. Due to the wide range of habitats in which they evolve, eels are continuously in contact with many contaminants such as PCBs, pesticides, heavy metals as well as endocrine disruptors. The latter are specifically impairing the eel's reproductive capacity. Given the important impact that contaminants can have on eel functioning, it is essential to further investigate what are the main pollutants encountered by the eel and how these chemicals affect its life cycle and metabolism.

2. Persistent organic pollutants (POPs)

2.1 What are POPs ?

Persistent organic pollutants (POPs) are a large group of synthetic organic compounds that resist photolytic, chemical and biological degradation. Even if some of them are unintentionally produced by human, most of the POPs are formed as a result of anthropogenic activities, principally industrial processes (synthesis of industrial chemicals, pesticides or solvents). They are very persistent in the environment and can last over decennia or centuries before being degraded. Furthermore, they are lipophilic which gives them the capability to remain and accumulate in fat-rich tissues. POPs become toxic when living organisms are exposed to them for long periods of time even at low concentrations. It has been suggested through research that even low levels of contaminants have the capacity to affect the development, behavior and reproduction of fish and wildlife, and potentially humans as well (Nowell, Capel, & Dileanis, 1999).

PBDEs & PCBs

POPs include several categories of compounds that vary according to their origin and chemical conformation. Chemicals such as polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs) belong to the class of flame retardants. Flame retardants are chemicals used in paints, cable insulation, electric equipment and interior textiles to inhibit flammability.

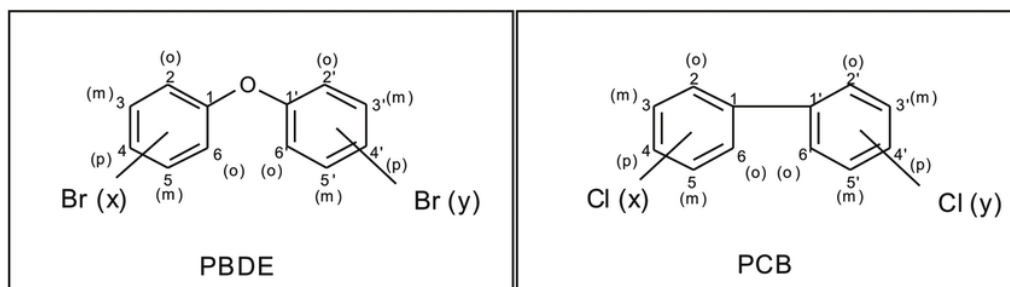


Figure 6: Chemical structure of PBDE and PCB congeners (Fernandez Cantón et al., 2006)

The significant threat of PCBs to human and environmental health has led to the gradual prohibition of their production, in the 1970s (Fernandez Cantón et al., 2006). Between 1970 and 1990, they were only found in closed systems and no longer in open systems like paints. Despite the ban of the industrial use of these chemicals, they are still omnipresent in the environment, partly because of their high resistance to biotransformation conferred by the position and number of chlorine and bromine atoms in the molecules. They also have high affinity for binding to particles such as water sediments and dust particles. Stockholm convention focused on the elimination of PCBs and PBDEs and recommended phasing out the use of those pollutants in equipment by 2025 and ensuring their elimination by 2028. Despite the efforts that have already been put in place, the 2025 and 2028 goals appear to be difficult to reach given that only 17% of the total amount of PCBs have been eliminated so far whereas 83% (which corresponds to 14 million tons) have yet to be eliminated from our environment (Fernandez Cantón et al., 2006; Hernik et al., 2011).

OCs & DDTs

The organochlorine pesticides such as dichlorodiphenyltrichloroethane (DDTs) are old pesticides intensively used after the Second World War to protect cultures and people from insect-borne diseases. Like PCBs, they are hardly degradable mainly due to their lipophilic character (Waliszewski, Aguirre, Infanzón, Rivera, & Infanzón, 1998).

Dioxins & furans

Dioxins and furans are, for their part, involuntarily synthesized by human activity. They have no specific use but are byproducts resulting from the production of other chemicals. Current sources are waste incineration, fuel burning and steel industry (Charnley & Doull, 2005).

PAHs

Finally, polycyclic aromatic hydrocarbons are the major POPs and the most toxic ones (Davies & Vethaak, 2012). PAHs belong to the group of aromatic compounds composed of 2 to 13 aromatic rings. They are carcinogenic compounds that derive from oil and drilling activities, cars and truck emissions but also forest fires, volcanic eruptions and incinerations. As also reported for the other chemicals, the carcinogenicity and toxicity of PAH varies with their specific chemical and molecular characteristics (Manzetti, 2013). The functioning of this category of pollutants will be detailed later.

2.2 Water contamination by POPs

Despite increasing restrictions on the production of PCBs, large amounts of these compounds and other POPs are still present in the environment (WHO, 2008). POPs are released to the atmosphere from hot areas of high industrial and urban activities. As they persist in atmosphere, land and aquatic environments, they can be transported over long distances to colder regions of the earth (Jiménez, Dachs, & Eisenreich, 2015). In surface water, POPs can penetrate with precipitation or wastewater. They oxidize, volatilize and bind to suspended particles or sediments, or accumulate in aquatic organisms. In the atmosphere, heavier chemical compounds are absorbed on dust particles. POPs can then be carried by wind and remain in the atmosphere until they are removed by precipitation (Wenning & Martello, 2013). Deposition at the air-water interface is the main source of pollution of the marine ecosystems. Oceans can also be directly contaminated by the release of chemicals due to the exploitation of petrochemical resources or even via boating activities or accidental discharges (Wania, Axelman, & Broman, 1998). Pollutant particles absorbed on sediments will settle on river bottoms for a long time with half-lives of 0.2-5 years in sediment and soils. About two-thirds of persistent organic pollutants in aquatic systems are associated to particles whereas one-third is suspended in the water column (Wenning & Martello, 2013).

Marine environments are affected in the same way as freshwater environments. Recent studies assessing POPs amounts absorbed or present in the ocean have been performed: The levels of PCB in the ocean are estimated at 370 kilotons (Sobek & Gustafsson, 2014), 354 kg of dioxins are absorbed every year by the ocean (Dopico & Gómez, 2015) and the input of atmospheric PAHs to the oceans is estimated at 90 000 tons/month (Gonzalez-Gaya et al., 2016). Although the oceans are large and vast, POPs accumulate in pelagic zone, at the sea-bed level. Unfortunately, even the most inaccessible habitats of the Earth are affected by these pollutants (Jamieson, Malkocs, Piertney, Fujii, & Zhang, 2017). Researchers were indeed able to identify considerable amounts of POPs in the deepest ocean trenches (approximately 10 000m deep). In 2018, PCBs were found in sediment core samples (0-2cm) at water depth of 7000-11000m in the Mariana Trench at levels much higher than those recorded in surface waters at the same location (Dasgupta et al., 2018).

2.3 Chemical pollution in eels

Belpaire et al. suggested the following mechanism to explain the decline in the eel population. Since the 1950s, a lot of new chemical agents have been produced and released into the environment. Those contaminants are lipophilic and accumulate very easily in fat tissues and adipocytes (Claude Belpaire & Geeraerts, 2010).

During their growth phase as yellow eels in freshwater, eels accumulate large amounts of lipid reserves and reside for long periods in contaminated brackish waters (Geeraerts & Belpaire, 2010). Environmental pollutants can either be ingested and enter the digestive system or can be absorbed by the skin or the gills (Johnson et al., 2013). The lipophilic character of POPs allows them to pass through cell membranes by passive diffusion. They will reach the liver where they will be metabolized and then excreted by the kidneys. In polluted freshwaters, POPs absorption is far more important than their elimination because metabolic rate in the liver is not high enough to cope with such high levels. The high amounts of pollutants ingested cause an important accumulation of contaminants in lipid-rich tissues of the fish. Among fat storage, it has been shown that lipid droplets are the largest targets for pollutants (Elskus, Collier, & Monosson, 2005).

As the European eels are semelparous, they are not disposed to eliminate contaminants by laying eggs or by releasing gametes which are mechanisms observed in other animal species (Claude Belpaire, Hodson, Pierron, & Freese, 2019). So, there is no loss of contaminants and since they can remain for prolonged period in freshwater (5.9 years for males and 8.7 years for females), they will continuously bioaccumulate xenobiotics so that their concentration levels increase with age (Geeraerts & Belpaire, 2010).

When eels begin their long-distance migration to the spawning area, catabolic reactions will lead to the release of stored contaminants to the bloodstream. The contaminants can then cause disturbances in the reproductive, immune, endocrine and nervous systems.

As highest concentrations of pollutants are usually found in liver and muscles, a research decided to focus on brain and neurotoxic properties of those xenobiotics on the European eel (Bonnineau et al., 2016). The concentrations found in the eel's brain were proportionally similar to those measured in the muscles and liver. This reveals that the brain of eels is highly impacted by organic and inorganic contaminants. The secondary effect of those pollutants has yet to be discovered but these contaminants may generate injuries and behavioral deficits while also impairing the eels' cognitive performance thus affecting the migration of silver eels (Bonnineau et al., 2016).

2.1 Effects of pollution on eel migration and reproduction

It has been established that a sufficient quantity of energy stored as lipids is essential for silvering process, reproductive migration as well as gonad maturation for the European eel. Thus, a significant drop in lipid contents could have a major negative impact on the eel's life cycle. As discussed earlier, the total stored lipids must exceed 20% of the total body weight to cover the migratory needs of the female European silver eel (13% needed for the 6000km swim and 7% for oocytes). Eels with insufficient fat content (<13% of the body weight) would hypothetically not be able to continue their migration activity (Geeraerts & Belpaire, 2010).

In yellow eels from Belgium and the Netherlands, a decrease in muscle lipid content has been monitored in a 2009 experiment led by Belpaire et al. (C. G. J. Belpaire et al., 2009). The total body lipid content of those eels decreased from 20% of total body weight to 12.3% since 1994 in Belgium over a period of 13 years. In the Netherlands, the decline ranged from 20.6 to 13.1% of the total body weight over the same 13-year period questioning the ability of these eels to start silvering and to achieve their spawning migration.

Several scientists described negative effects of chemical contaminants on the lipid metabolism of the eel. So, Céron et al. (Céron, Ferrando, Sancho, Gutierrez-Panizo, & Andreu-Moliner, 1996) have shown that, after pesticide exposure, lipid accumulation in eel was disturbed due to the inhibition of acetyl cholinesterase (AChE) activity. It was then shown that ChE-activity affects lipogenesis by inducing lipid mobilization by involuntarily muscular activity and simultaneously reduce migration efficiency.

In Belgium, studies exposed clear relationships between lipid content and environment variables by analyzing various dataset of contaminants. They demonstrated that PCBs and DDTs could act as endocrine disruptors in many freshwater and marine fish species. Those disruptors could also affect fat storage due to specific chemicals imitating the steroid hormone estrogen (Turner & Sharpe, 1997). Precisely, pollutants are able to induce lipolysis and thus disturb the lipogenesis process. This causes a decrease in the silver eels' fat content therefore decreasing the amount of energy accumulated during the yellow stage. This lack of energy resource could prevent the eel from completing its migration (C. Belpaire & Goemans, 2007).

According to the importance of lipids for the gonad maturation process, a fat deficiency could also have negative consequences on the reproductive success of eels (Henderson & Tocher, 1987). In addition to a lack of lipid reserves, contaminants can also directly affect reproductive process. While lipid reserves are depleted during migration, contaminants are released into the blood. Larsson et al. then confirmed that pollutants, once released in blood, could damage reproductive organs and affect embryogenesis (Larsson, Hamrin, & Okla, 1990).

Such effects are evidence of the impact of pollutants on marine wildlife and are one of the main indirect causes of the decline of European eels.

3. POPs metabolism

Knowing the metabolic the effects of POPs on the environment and aquatic wildlife, it is imperative to develop the way metabolism of affected fish acts to eliminate the absorbed toxic particles. The aim here is to understand how marine species are able to metabolize pollutants found in the aquatic environment.

Once absorbed by the organism, POPs undergo different reactions to enzymatically be converted to more hydrophilic substances that will increase the rate of clearance. This conversion is a two-phase process: Phase I (functionalization) and Phase II (conjugation).

3.1 Phase I

Biotransformation of xenobiotics mainly takes place in the liver. The absorbed chemicals, drugs or other endogenous compounds undergo a biotransformation in the liver before the elimination through the kidneys. They will first be taken up by the hepatocytes via passive diffusion or transporters and will then be converted through various processes into more hydrophilic compounds which will be excreted in urine (Figure 7) (Cui & Li, 2018).

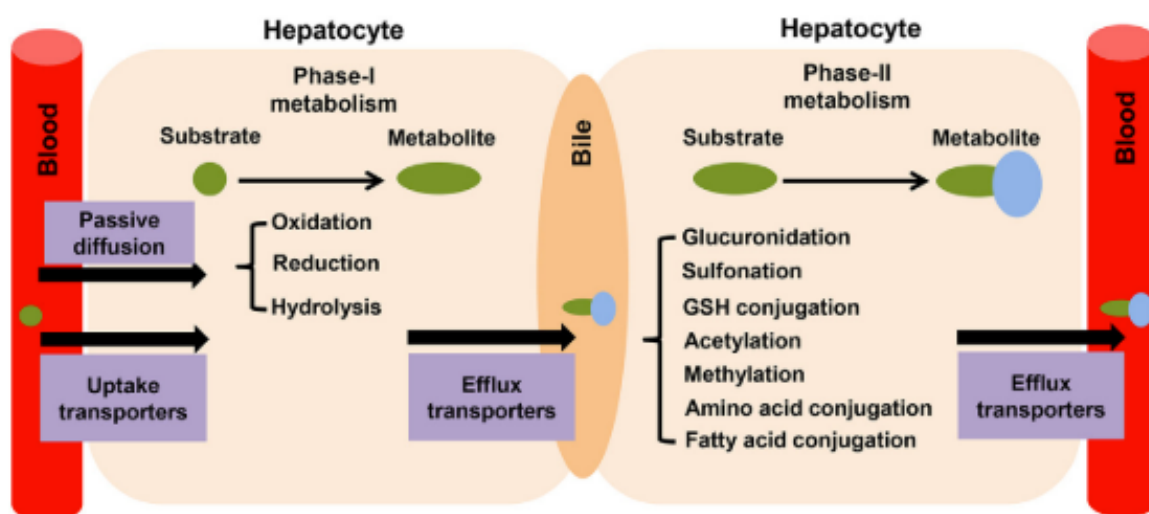


Figure 7: Biotransformation of xenobiotics in liver; diagram of hepatic drug metabolism and transport (Cui & Li, 2018)

Phase I reactions result from the action of several enzymes. They catalyze oxidation, reduction and hydrolysis reactions which will increase the water solubility of the endogenous compounds. This is done through the introduction of functional groups such as OH, SH, NH₂, COOH to the existing group. The most important enzyme involved in this phase is the monooxygenase cytochrome P450 which is found at the highest concentrations in the membrane of the smooth endoplasmic reticulum of liver (Ibor, Adeogun, Regoli, & Arukwe, 2019).

a) CYP450 monooxygenase

Monooxygenase cytochrome P450 is dependent on 2 enzymes: cytochrome P450 and NADPH-cytochrome P450 reductase (POR). The general reaction catalyzed by cytochrome P450 is the following:



Figure 8 shows the membrane-bound monooxygenase complex of liver. Bound to the membrane of cytoplasmic reticulum, a two-electron donor called NAD(P)H-cytochrome P450-

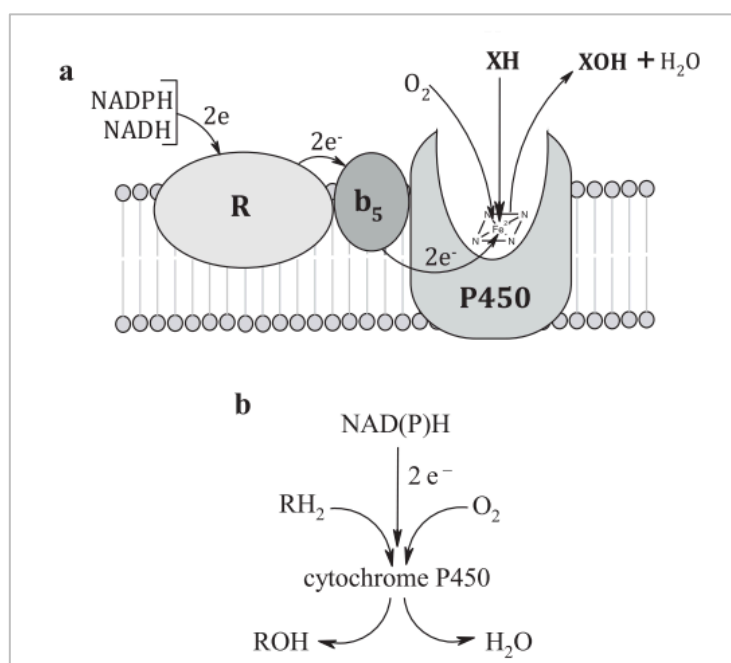


Figure 8: Membrane-bound monooxygenase complex of liver
(Gordeziani et al., 2016)

reductase is going to transfer 2 electrons to an intermediate electron carrier (b5 - cytochrome). The final component of the system is the electron acceptor, cytochrome P450. Part b of the figure describes the basic mechanism of cytochrome P450 resulting from the formation of a hydroxylated molecule by the reduction of one oxygen atom. The other oxygen atom is reduced in water (Gordeziani, Varazi, & Pruidze, 2016).

Cytochrome P450 is a unique family of heme proteins. Its substrates include both endogenous and exogenous compounds. "P" comes from "pigment" and "450" is the wavelength in nanometer at which the highest absorbance is observed. CYP designates the Cytochrome P450 family which consists of a very large number of genes and approximately 400 different genes have been identified. In total, we can distinguish 18 families of CYP (CYP1, 2, 3, 4, 5, 7, 8, 11, 17, 19, 20, 21, 24, 26, 27, 39, 46 and 51). Within a family, we can find several isoforms that differ by a letter. In fish, the CYP1 family can still be divided in 4 sub-families: CYP1A, CYP1B, CYP1C, CYP1D.

CYP1A subfamily is a useful biomarker for the aquatic pollution as it plays a crucial role in the metabolism of xenobiotics. Many cDNAs have been described in various fish species such as the European sea bass, the Atlantic salmon or the Rainbow trout among others. An example of a biomarker is ethoxyresorufin-O-deethylase (EROD) activity (Heinrich, Diehl, Förster, &

Braunbeck, 2014). This biomarker is a widely applied method to assess the dioxin-like and PAH exposure in the environment by the measuring the CYP1A1 activity. (Uno, Ishizuka, & Itakura, 2012). CYP1A1 can transform EROD into a fluorescent compound. The measurement of fluorescence level will then indicate the expression level of CYP1A1 in liver (Catteau et al., 1995).

Mainly, Phase I of xenobiotic metabolism consists in the oxidation of contaminants as to increase their hydrophilicity and further facilitate their clearance. Even though cytochrome P450 plays an essential role in this process, the role of other enzymes should not be neglected. (We have seen in depth the cytochrome P450 enzymes and mechanism but it is important not to lose sight that other enzymes are involved). Indeed, these enzymes are also involved in the catalysis of the reactions taking place in phase I, notably dehydrogenases, aldehyde dehydrogenases, molybdenum hydrolases, amine oxidases, flavin monooxygenases and peroxidases (Cui & Li, 2018).

Although CYP450 enzymes are essential for biotransformation of pollutants, they can potentially be a source of Reactive Oxygen Species (ROS) by their use of a molecular oxygen for the oxidation of NADPH (Bondy & Naderi, 1994).

b) Reactive oxygen species (ROS)

Reactive oxygen species are reactive chemical species containing oxygen. They are formed in biological systems as by-products of the normal metabolism of oxygen and include peroxides, superoxide, hydroxyl radical, singlet oxygen and alpha-oxygen. The reduction of molecular oxygen (O_2) produces superoxide ($^{\bullet}O_2^-$) which is the precursor of most other reactive oxygen species (Hrycay & Bandiera, 2015). Reactive oxygen species can be produced by endogenous or exogenous sources. Among the endogenous sources, there is the mitochondrial electron transport chain and the cyp-dependent microsomal electron transport system (Hikita et al., 2015; Hrycay & Bandiera, 2015). Intracellular reactive oxygen species are found in all cells in amounts that balance with intracellular antioxidant activity to prevent cellular damage. Nevertheless, if too much ROS are produced, the balance could break and cause damage to the cells. Too high concentrations of POPs can cause an increase in ROS and thus perturb the cellular balance. Cell damage can affect proteins, DNA and lipids.

Protective agents can be induced by the cell to combat some reactive oxygen species because a quick detoxification of those species is critical for the survival of all aerobic life-forms.

A system of antioxidants is put in place by the cell to keep these species under control. Antioxidants constitute the primary defense of the cells with the main enzymes superoxide dismutases, catalase, glutathione peroxidases, glutathione reductase, glutathione-S-transferases and peroxiredoxins. Their major function is avoiding radical-induced tissue damage by preventing formation of reactive oxygen radical species (Sies, 1997).

Reactive oxygen species can target molecular and mitochondrial DNA. The major damages are deoxyribose oxidation, strand breakage, base modifications and DNA-protein crosslinks. Mitochondrial DNA changes can lead to the formation of unexpected components of the mitochondrial chain and thus lead to the generation of more ROS and therefore more cellular damage (Bandyopadhyay, Das, & Banerjee, 1999).

ROS are known to be cytotoxic and have been implicated in various human diseases including cancer. They are potential carcinogenic as they facilitate mutagenesis, cancer promotion and progression (Manda, Nechifor, & Neagu, 2009; Waris & Ahsan, 2006). Still, the association between ROS and cancer is complex and not yet fully understood. At moderate concentrations, ROS influence the tumor microenvironment and are known to activate cancer cell survival signaling cascade. Paradoxically, at high concentrations, ROS can cause cancer cell apoptosis. Hence, it depends on the ROS levels to stimulate tumorigenesis or apoptosis and additional research is required to understand the potential of ROS as therapeutic target for treating malignancies (Aggarwal et al., 2019).

As mentioned earlier, ROS can generate double-stranded DNA breaks, purine, pyrimidine or deoxyribose modifications whether they are induced by CYP enzymes or by mitochondrial electron transport chain (Halliwell, 2006). Changes brought in the DNA can be repaired by DNA repair enzymes but some DNA damages can persist and thus lead to replication errors, activation of oncogenes or inactivation of tumor repressor genes. These genomic instabilities are associated with initiation of various cancers (Petros et al., 2005).

3.2 Phase II

Phase II consists of the conjugation of the hydrolyzed xenobiotic with a polar molecule. The addition of a polar molecule on the primary metabolite allows a better excretion of the compound. A lot of enzymes are involved in this transformation, but the three major ones are the sulfotransferase (SULT), the glutathione-S-transferase (GST) and the uridine 5'-diphosphoglucuronosyltransferase (UGT). Sulfotransferases conjugate metabolites through addition of sulphate. These reactions result in the production of water-soluble sulphate esters which can easily be eliminated. The glutathione-S-transferases conjugate the primary metabolite with glutathione while the glucuronyl transferases conjugate the substrate with a glucuronic acid (Croom, 2012).

3.3 Polycyclic Aromatic Hydrocarbons (PAHs)

Among the different categories of POPs are the PAHs and we will focus on this type of POPs in later experiments. PAH will be the POP model to understand the issue of pollutants on deep-

sea fish. This category of POPs is part of the most absorbed pollutants in the oceans as about 90 000 tons of PAHs are incorporated every month in the oceans. Moreover, PAHs are known to be among the pollutants that can become even more toxic once metabolized which is not the case for the majority of POPs. This is why the main studies exposed in this paper focus on fish species exposure to this type of pollutant.

Once polycyclic aromatic hydrocarbons enter the cell, they can be processed through different pathways but are still considered as procarcinogen because their metabolism promotes cancer formations. During phase I and II metabolism, PAHs are transformed into phenols, quinones and catechols. At the same time, byproducts are generated such as diol epoxide, o-quinone and radical cations which may induce the formation of DNA and proteins adducts and thus increase ROS production. By increasing ROS concentration, the PAHs metabolism is involved in the alteration of DNA, proteins and lipids (Moorthy, Chu, & Carlin, 2015).

a) AhR-dependent pathway

The induction of cytochrome P450 can be realized in different ways. Non-coplanar PCBs, DDTs and PBDEs will preferentially induce CYP2B and CYP3A enzymes via interactions with the Constitutive Androstene Receptor (CAR) and Pregnancy X Receptor (PXR). In contrast, dioxins and dioxin-like PCBs with co-planar conformation can induce the synthesis of CYP1A via the interaction with the Aryl hydrocarbon Receptor (AhR). PAHs have a high affinity to the Aryl hydrocarbon Receptor. Once entered in the hepatocyte, PAH binds to the AhR complex (Figure 9).

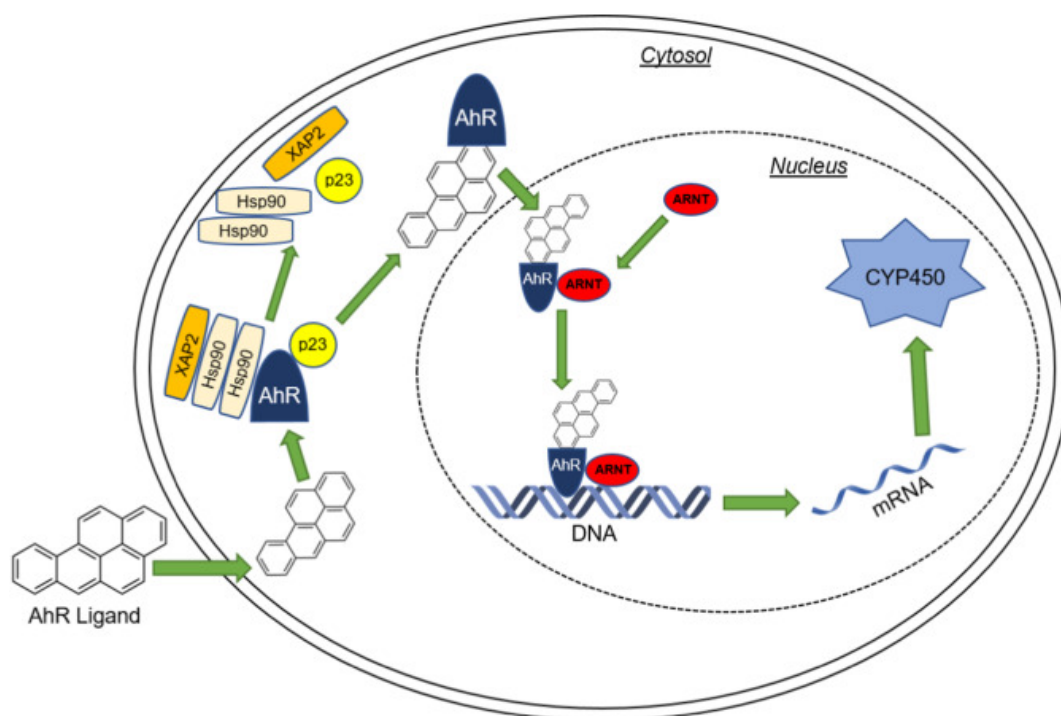


Figure 9: AhR-dependent pathway (Franco & Lavado, 2019)

The binding releases the hepatitis B Virus X-associated protein 2 (XAP2) from the complex, which allows the receptor to enter the nucleus. The released HSP 90 also allows the dimerization of AhR with the AhR Nuclear translocator (ARNT). This dimerization is essential for the creation of the AhR/ARNT heterodimer which is the final active form of the AhR transcription factor. This factor can regulate the transcription of AhR-regulated genes such as CYP1A1 and other phase II enzymes (Moorthy et al., 2015).

3.4 Heat shock proteins

During their life, fish are exposed to biological and abiotic stressors coming from the environment. Stress can also occur at the cellular level and is called “cellular stress response” (Iwama, Vijayan, Forsyth, & Ackerman, 1999). Among the generalized cellular stress responses, there is the production of heat shock proteins (HSPs) against stressors that threaten the life of the cells. Heat shock proteins are part of a family of chaperones proteins helping in the unfolding of damaged proteins in all organisms even under non-stress conditions (Lindquist, 1986). Chaperones are proteins which are expressed to prevent the aggregation of histones proteins. Their production appears to increase in response to chemically and physically stress such as oxidative stress, heat shocks, heavy metals or amino acids analogues. The HSP family is constituted of many different proteins classified according to their molecular weight: HSP10, HSP40, HSP60, HSP70, HSP90 (Iwama, Thomas, Forsyth, & Vijayan, 1998).

HSP90 and HSP70 are essential proteins of eukaryotic cells present in the nucleus and the cytoplasm. HSP90 is essentially known to prevent damage during the transcription phase of the cellular cycle. Additionally, it acts in well characterized signaling pathways for a large amount of proteins, for example HSP90 regulates the AhR activation and hence activates CYP1A transcription. On the other hand, HSP70 is mainly known for its ability to help proteins to adopt and maintain native conformation (Becker & Craig, 1994). HSPs are also regulated by transcription factors and can be affected by post-transcriptional modifications and regulations. For example, HSP90 co-chaperone XAP2s aim is to stabilize the non-active AhR protein complex in the cell (Basu et al., 2002).

4. Objectives

At this stage of the research, the report states that the European eel is officially an endangered species. The significant declines in stocks since 1980 are a clear indication of its gradual disappearance from our European waters. Although overfishing and other anthropogenic obstacles encountered during their migration are inevitably fatal to them, another element disturbs their migration as well as their reproduction.

Large quantities of pollutants are recovered in eel's freshwater life stage environment. Even though those lipophilic compounds are metabolized by their liver, they accumulate in impressive ways in their lipid reserves. Too high concentrations of pollutants induce lipolysis thus depleting migration energy needs. Moreover, pollutants in blood can also affect reproductive organs and embryogenesis thus disrupting the eel's entire life cycle.

The question at this stage is to determine what could be the factor preventing the eel from getting rid of persistent pollutants affecting its reproductive migration and, at the same time, endangering its life ?

CHAPTER 3: HIGH HYDROSTATIC PRESSURE EFFECTS ON FISH

During its migration, the European eel is going to face high depths due to its vertical migration. These depths will bring the eel to encounter significant high pressures and therefore it is important to evaluate the effects of pressure on the overall metabolism of fish, more precisely on their detoxification process.

1. Environmental characteristics of the deep-sea

The ocean can be divided in 5 layers:

- The **Epipelagic zone** goes from 0 to 200 meters deep. This zone is called the highlight zone because it is the most illuminated area.
- From 200 to 1000 meters is the **Mesopelagic zone** also called midwater zone because the light penetrating at this depth is extremely low.
- Bioluminescent creatures appear from 1000 meters down, in the **Bathypelagic zone** which extends to 4000 meters. This zone is where the dark zone starts and where the only visible light is produced by creatures themselves.
- From 4000 meters begin the abyss, characterized by no light and temperatures as low as 0°C. This is where the **Abyssopelagic zone** starts and extends to a depth of 6000 meters.
- Finally, the **Hadalpelagic zone** goes from 6000 meters to the bottom of the deepest parts of the ocean (the deepest point in the ocean is in the Mariana Trench at 10 911 meters) (Sea and Sky, 2016).

During their vertical migration, eels can go down to a depth of 2000 meters, thus reaching the Bathypelagic zone (Robins, Cohen, & Robins, 1979). This zone marks the beginning of the deep-sea zone starting at around 1000 meters below the sea level (Woodward, 2013). In this area, light does not reach water, pressure can rise to 40MPa, temperature drops to 5 or 6°C, food resources are limited, and the oxygen levels are relatively low (Mozhaev, Heremans, Frank, Masson, & Balny, 1996).

2. Action of high hydrostatic pressure on aquatic organisms

To understand how deep-sea fish cope with the extreme conditions of their environment, it is fundamental to assess the effects of high hydrostatic pressure (HHP) on these organisms.

Deep-sea is considered to be the largest biotope of our planet. About 40% of the surface of the earth is found under important water masses. Liparid snailfishes are, for example, the most common hadal vertebrate species and are present in the widest depth ranges (at 8100 meters deep) (K. Wang et al., 2019). An increased depth of 100 meters results in a pressure rise of 10 atm (1.01325 MPa) therefore, at 2000 meters depth, pressure reaches 200 atm.

The physico-chemical effects of high pressure in deep-sea mainly manifest themselves through a compression of the seawater as depth increases. The biological consequences of this compression on marine organisms are volume fluctuations of the protein system. Indeed, the effect of pressure on protein structure can be related to volume changes for the denaturation of those proteins (Heremans & Smeller, 1998; Royer, 2002). Water is only partially compressible because the hydrogen bonds are not much affected by HP. These effects are observed in such remote areas and can lead to major changes in cellular and reproductive systems.

As depth increases, fish population density decreases indicating the adverse effects exerted by extreme conditions on fish physiology. In fact, increasing water pressure slows down metabolic rate and sexual development of deep-sea fish. Thus, an inverse relation exists between pressure and the complexity of marine organisms. This implies that, as the level of organization increases, there is a decreasing pressure resistance (Schlieper, 1968).

A lot of adaptations are necessary to cope with such an environment because high pressure can lead to stress for most living organisms. Even though effects of HP on hydrogen bonds of water molecules are not significant, they have an important effect on noncovalent bonds in proteins and membranes. The main effects are observed on the cell structure (Paul H. Yancey, 2020).

2.1 HHP effect on proteins and enzymatic reactions

HHP has important impacts on protein structures, they can be compressed, dissociated and even denatured by water penetration into protein's lipophilic interior (Huang et al., 2016).

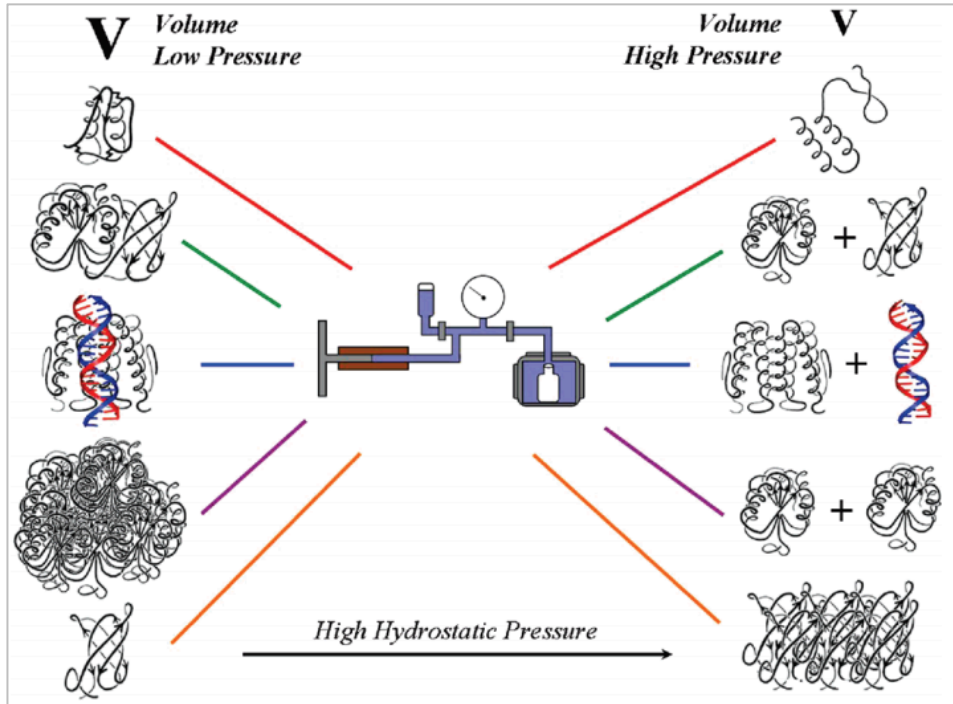


Figure 10: Hydrostatic pressure effects in different protein systems (Silva et al., 2010)

Pressure acts on proteins by the infiltration of water. This results in denaturation, subunit dissociation, protein-DNA unbinding, separation of protein aggregate or, in some cases, formation of these aggregates and unfolding (Figure 10). The final states are then more hydrated, have fewer non-hydrated cavities and, therefore, occupy smaller volumes. The equilibrium is thus shifted to smaller volumes (Silva et al., 2010).

However, depending on the structural characteristics of the protein, the pressure range for denaturation will differ: while primary and secondary structures are not affected by pressures as high as 10 kbar (1000MPa), oligomeric proteins can easily dissociate at pressures below 3kbar (300MPa) (Boonyaratankornkit, Park, & Clark, 2002).

Pressure can affect the enzymatic reactions by affecting the equilibrium constant (K_{eq}) which is governed by the volume change of the process, ΔV . The relation between ΔV and the equilibrium constant is the following:

$$\left(\frac{\Delta \ln K_{eq}}{\Delta P}\right) = -\left(\frac{\Delta V}{RT}\right)$$

K_{eq} represents the equilibrium constant where P is the pressure, ΔV is the volume change from the initial state, R is the gas constant and T is the absolute temperature. Volume changes can be positive, negative or null depending on the type of reactions. Thus, a pressure application will cause a shift of the equilibrium in favor of the state with the overall lowest volume. As follows, an increase in pressure favors the reduction of the volume of the system (Gross & Jaenicke, 1994; Mozhaev et al., 1996).

Regarding the enzymes, pressure is applied to the entire system which means that each equilibrium is affected and shifted in a way that the total ΔV is negative. In this way, the catalytic reactions will be affected: changes in protein conformation will lead to changes in the hydration state of amino acids residues which will alter the total volume of water-protein-ligand (Ohmae, Murakami, Gekko, & Kato, 2007).

a) Adaptations to HHP in protein structure in deep-sea fish

Intrinsic adaptation: In response to high pressures, deep-sea animals have managed to adapt. Because of the affectation of pressure on the protein functioning, an intrinsic adaptation of the structure evolved to reduce adverse effects. This adaptation is made at the level of the inherent organization of the protein. It results in changes in amino-acid sequences leading to a decrease in volume changes and thus pressure sensitivity.

Actin protein is a major structural component and participates in important cellular processes such as muscle contraction, cell signaling and cell division. Actin is the monomeric subunit of microfilaments (one of the components of the cytoskeleton) and thin filaments (part of the muscle cells structure).

According to Swezey and Somero (1982), the assembly of actin units into the filamentous form allows muscle contraction but structure is inhibited by high pressure. Their study revealed that the polymerization of actin requires ATP and Ca^{2+} binding, two components which are strongly pressure sensitive. In spite of this, they noticed that actin from deep-sea fish appeared much less sensitive to pressure than those from shallow fish (Swezey & Somero, 1982). As a fact, as reported by T. Morita's study on actins from abyssal fish (*Coryphaenoides* species), three amino acids substitutions are responsible for the reduced pressure sensitivity in comparison with carp actin (shallow fish). The first observed substitution is glutamine to lysine at position 137 and the second is alanine to serine. Those two substitutions are able to reduce the volume change involved with ATP and Ca^{2+} dissociation. The third substitution is valine to alanine and reduces the volume change associated with the interaction of subunits undergoing polymerization (Morita, 2003).

Extrinsic adaptation: Despite intrinsic structural changes and in vivo amino acids exchange assays, some deep-sea animals still showed a 20% inhibition of the actin polymerization (Morita, 2003). Thus, it might be that deep-sea species aren't yet totally able to adapt to pressure with amino-acids substitutions only. Some extrinsic factors, such as osmolyte accumulation, might help to protect proteins against other disruptors like urea or temperature. Osmolytes are uncharged molecules of low molecular weight. Their ability to stabilize proteins under stress conditions often leads to refer them as chemical chaperones (Singh & Dar, 2017).

The concentration of different osmolytes were measured in three different studies conducted by Yancey and revealed that trimethylamine N-oxide (TMAO) was the osmolyte with the highest levels and that its contents increased linearly with depth in muscles of 12 species teleost (Gillett, Suko, Santoso, & Yancey, 1997; Kelly & Yancey, 1999). Indeed, TMAO appears to be present in all muscle tissues of marine organisms and to have protein-stabilizing and other protective properties (Paul H Yancey, 2007). The accumulation of TMAO inside cells provide protection against protein shrinkage or swelling (Singh & Dar, 2017).

2.2 HHP effect on biological membranes

Cellular membranes are also affected by HP. Their fluidity, required for proper function, is reduced while their thickness increases due to compression and extension of phospholipids. HP may also induce the production of reactive oxygen species or other highly reactive species as well as promoting lipid peroxidation (Holmstrup et al., 2010). Both low temperature and high pressure can affect rigidity of the lipid bilayer of membranes and thus, affect their transport proteins. For example, a 100 MPa pressure equals a cooling in membrane temperature of 13 to 21°C below the ambient temperature which is 2°C (to give -11 to -19°C) (A. R. Cossins & Macdonald, 1989).

In deep-sea conditions, both temperature and pressure are affecting the living conditions of fish and can act in synergy on the membranes to reduce rigidity. While pressure decreases distance between the acyl chains, low temperatures rigidify membranes. Those modifications can have an important impact on the normal functioning of the Na⁺/K⁺-ATPase in gills of marine teleosts. Indeed, it has been demonstrated that HHP decreases Na⁺/K⁺-ATPase activity proportionally to the reduction of membrane fluidity. This alters the membrane permeability to substrates just as it can affect a large part of the signal transduction system (Gibbs & Somero, 1989).

Membrane-associated proteins and enzymes are very sensitive to HP (Siebenaller & Garrett, 2002). Figure 11 is a summary of the pressure effects on a membrane-bound protein dimer.

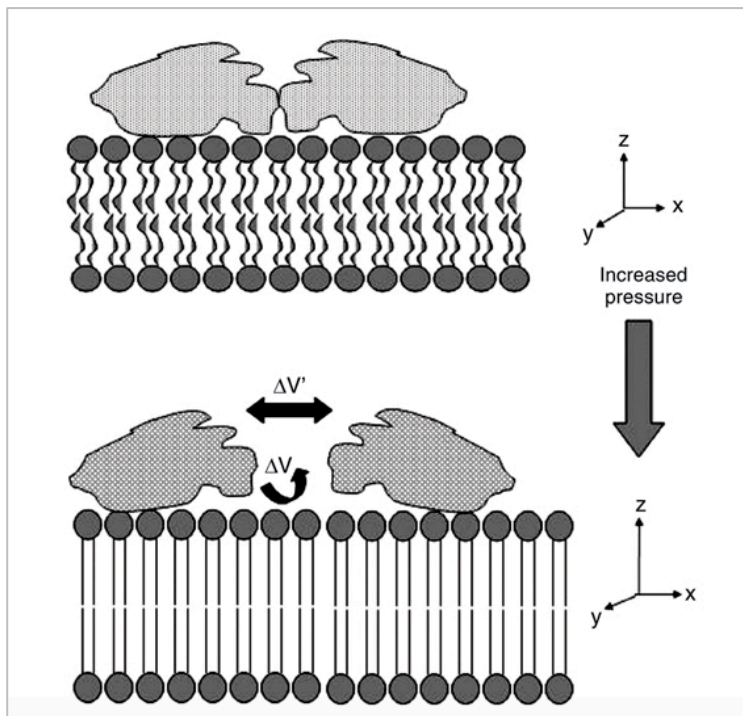


Figure 11: High pressure effects on membranes (Scarlata, 2005)

Pressure application on the membrane results in the dimer dissociation due to the permeation of solvent into the dimer interface, where the dissociation is given by $\Delta V'$. Pressure compresses the lipids, resulting in the elongation of the bilayer of the membrane. HP is also expected to promote dissociation of membrane-bound proteins from membrane. This dissociation depends on the size and the extent of packing voids between protein-membrane interface (Scarlata, 2005).

a) Adaptations to HHP in membrane structure in deep-sea fish

A major adaptation of HHP on cell membrane is its fatty acid profile. Cell membranes are mainly constituted of phospholipids which have an important role in the maintenance of the membrane order and its fluidity. Some biochemical analyses have shown that, either at low temperature or high hydrostatic pressure, the membranes of deep-sea-adapted species have a higher unsaturated to saturated fatty acid ratio in comparison with shallow-sea species (A. Cossins & MacDonald, 1984).

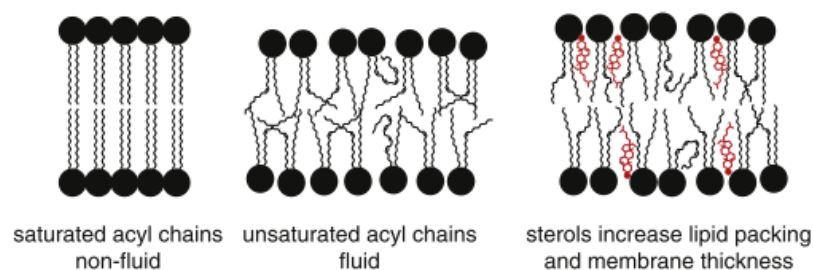


Figure 12: Hydrophobic matrix and membrane fluidity (Ernst et al., 2016)

This adapted membrane composition (Figure 12), rich in mono-and/or polyunsaturated fatty acids and sterols, allows the fish to remedy the negative effects of pressure. Indeed, it is

proven that docosahexaenoic acid (DHA) are able to alter the properties of membranes, including fluidity, compressibility, permeability and protein activity: double bonds of unsaturated fatty acids induce the curvature of acyl chains allowing the phospholipids to be more spread and thus to increase the membrane fluidity usually lost with pressure (Ernst, Ejning, & Antonny, 2016; K. Wang et al., 2019).

2.3 HHP effect on eels

Just like deep-sea fish, eels are able to acclimatize to high pressure. Since they have to migrate over 6000km to their spawning area, the scale of depth encountered during this journey would be around 1800 – 2000 meters given that some eels have been observed up to 2000 meters deep (Robins et al., 1979). This demonstrates they are able to adapt their organism to high pressure environments (Tesch & Thorpe, 2003). Pressure is assumed to modify activities of the respiratory chain but also the oxidative phosphorylation complexes located in the mitochondria inner membrane. The physical activity required for the reproductive migration could thus be affected by the exerted pressure on aerobic metabolism.

a) Comparison between pressure resistance of yellow and silver eels

In 2006, an observation was made by Vettier et al. about non-migrating yellow eels exposed to high pressure (10MPa). First, the exposure revealed a decrease in respiratory chain and oxidative phosphorylation activities as well as in oxygen consumption, associated to a decreased membrane fluidity (Philippe Sébert, Simon, & Barthélémy, 1993; Vettier et al., 2006). After a longer period of exposure to the same pressure (1month at 10MPa), the aerobic metabolism reverted to normal level. The improvement in the respiratory chain and oxidative phosphorylation activities were a consequence of the restoration of the membrane fluidity and a greater incorporation of polyunsaturated fatty acids, such as in most of deep-sea species membranes. Thus, this reported that non-migrating yellow eels are able to adapt their membranes and metabolism to high pressure even if they do not encounter hydrostatic pressure, ordinarily (Sebert, 2002; P. Sébert, Scaion, & Belhomme, 2009; Philippe Sébert et al., 1993).

After this experiment, the same research group wanted to know if the silvering process had an influence on the pressure resistance and the energy production process of eels in order to prepare them to “real pressure exposure”. So, silver eels were also exposed to 10MPa and muscle cytochrome oxidase (COX) activities measurements were performed. The results showed that yellow eels had a lower pressure threshold and thus a higher sensitivity to pressure fluctuations than silver eels. Moreover, silver eels presented a significant higher energy charge under pressure as well as a higher red muscle oxygen consumption than in

yellow eels. Finally, higher COX activities were recorded in silver eels (Vettier et al., 2006). As the COX activity increased, the cytochrome c oxidase seems to play an important role in pressure acclimations, enabling an optimization of the respiratory chain (Theron, Guerrero, & Sebert, 2000).

In summary, the silvering process results in a lot of physical transformations (swimming abilities, eye size, pigments) and metabolic adaptations to high pressure.

After Vettier et al. compared eels of two different life stages (yellow and silver) during and after pressure acclimatization they hypothesized that the silvering process allows the eel to be pre-adapted to hydrostatic pressure through changes in the membrane fluidity. In addition, the higher mitochondrial membrane fluidity was correlated with a lower proportion of saturated fatty acids in silver eels. This makes sense since it has been demonstrated that this is how deep-sea species have adapted their membranes to HHP (Vettier et al., 2006).

3. What is the influence of HHP on the response to xenobiotics in fish ?

Since POPs represent a major threat of the 21st century for fish, it is important to find the relationship between elevated pressure and detoxification reactions in fish, more precisely in eels. Because eels are facing more and more POPs during their growth, it would be interesting to assess the role of pressure on their ability to metabolize xenobiotics.

According to Le Chatelier's principle, for all systems under high pressure, a change in temperature, concentration, volume or pressure can shift the system out of its equilibrium state to a state that counteracts the imposed change. Thus, by applying pressure, the equilibrium will be shifted so that the system occupies a smaller volume, causing the acceleration of processes (Macdonald, 1997; Somero, 1992).

Holmstrup et al. suggested that the interactions between toxicants and natural stressors could magnify the effects normally observed if there were no stressors (Holmstrup et al., 2010). As pressure affects chemical reactions as well as the physiology of marine organisms, it is pretty sure it would also affect the toxicity of contaminants released into the sea by human activities (Mestre, Calado, & Soares, 2014).

3.1 Xenobiotic metabolism in fish with different trophic strategies

In 2009, Solé et al. analyzed the expression of potential markers of 9 different fish species to pollutant exposure at shelf (53m) and slope (660m) depths. Choosing species that belong to six teleost families and two elasmobranch species of thus made it possible to provide results for species from different depths habitats. This was one of the first studies designed to relate biochemical activities to ecological markers and thus provide a perspective on pollution monitoring studies in the Mediterranean region.

The hepatic biomarkers were targeted on a set of enzymes involved in metabolizing xenobiotics such as antioxidant defense catalase (CAT), glutathione reductase (GR), CYP4501A-related EROD and glutathione S-transferase (GST). EROD activity is the most accepted biomarker for “dioxin-like” compounds such as PAHs. Protein yield (PY) was also used as general marker of hepatic protein synthesis.

The antioxidant enzyme activity reported showed an interesting link between enzymatic activity and depth: antioxidants CAT, GR and GST were significantly higher in fish from slope depth (660m) than shelf depths (53m). No clear relationship between these physiological conditions and antioxidant activity could be established but a significant positive relationship between swimming capacity and CAT was observed in this study: there could be an existing association between high oxygen consumption and enhanced ROS production. However, swimming capacity was negatively correlated with the other hepatic markers leading to the conclusion that the meaning of this remains to be established.

Unlike antioxidant enzymes, EROD activity and PY were higher in the five species sampled at 53m depth than in the slope-dwelling species sampled at 660m. These results were unexpected considering that slope species might accumulate more pollutants due to biomagnification and thus induce stronger hepatic activity.

To conclude, hepatic EROD activity was negatively correlated with depth while the other antioxidant activities were positively correlated with depth. These results highlighted the questioning of the relationship between EROD and antioxidant activity under pressure. Indeed, Lemaire et al. demonstrated in 2010 that EROD activity increased with PCB levels as well as SOD and CAT enzyme activities regardless of pressure. Thus, indicating that PCBs strongly affect the redox balance of *C. rupestris* through increased CYP1A activity (Lemaire et al., 2010).

This study was innovative by being one of the first to note the impact of pressure on EROD activity in fish exposed to contaminants. It can be considered as a good predecessor for the experiments that were done later (Solé, Rodríguez, Papiol, Maynou, & Cartes, 2009).

3.2 Deep-sea fish PCLS exposed to POP model 3-MC and HHP

In 2012, Lemaire et al. proposed an innovative experience based on precision cut liver slices (PCLS) from the roundnose grenadier *Coryphaenoides rupestris* exposed to a persistent organic pollutant. This was the first toxicological investigation carried out on living-hepatic cells of a deep-sea fish (because deep-sea fish usually do not survive the important drop in pressure when brought to the surface). The essence of this experiment was to expose liver slices to pressures of different intensities in order to evaluate the expression of enzymes specific to contaminant detoxification processes (Lemaire et al., 2012).

The liver slices were immediately generated after the fish were sampled and exposed at atmospheric pressure. The POP model, which was used, 3-methylcolanthrene, is a polycyclic aromatic hydrocarbon agonist for the AhR inducing CYP1A transcription and acting as a principal biomarker of the exposure to POPs (Kawajiri & Fujii-Kuriyama, 2007). Moreover, the transcription levels of some stress responsive genes, such as CAT, GPX, HSP70 and HSP90 were recorded.

Slices were first incubated at atmospheric pressure for 6 hours. A significant induction of mRNA CYP1A was observed in PCLS exposed to 3-MC pollutant (Figure 13). Among the heat shock proteins, both mRNA contents increased compared to T₀ controls, regardless of the presence of 3-MC in the media.

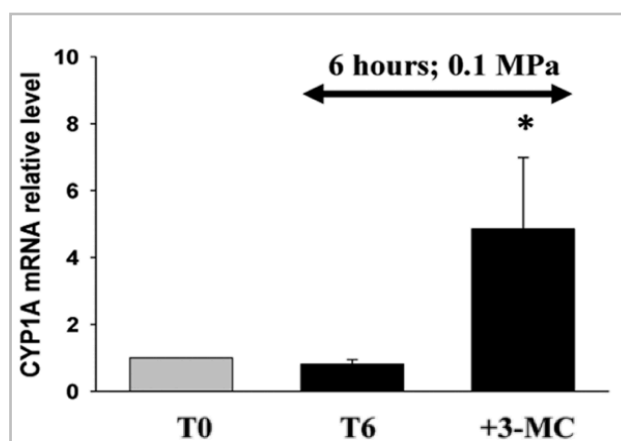


Figure 13: CYP1A transcript levels after 6h incubation at 0.1MPa (Lemaire et al., 2012)

A second part of the experiment consisted in the incubation of the slices in hyperbaric chambers at 0.1 (shallow), 5 and 15 MPa (representative of 500-1500 m depth) for 15 hours with the 3-MC (25µM) model (Figure 14).

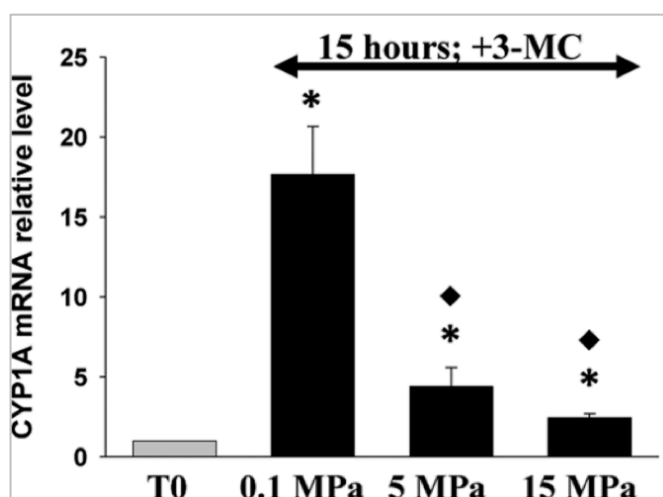


Figure 14: Effects of atmospheric and deep-sea pressure on CYP1A transcription after 15h incubation (Lemaire et al., 2012)

Figure 14 clearly exposes that CYP1A transcript levels were significantly increased above T₀ controls. The induction at atmospheric pressure (0.1 MPa) was considerably higher than that at high pressure levels (5 and 15MPa).

Further, same effects of decrease were observed for both HSP70 and HSP90 transcript levels. Transcription was highly induced at atmospheric pressure (0.1 MPa) compared to the control slices. Nevertheless, the increases were essentially marked at atmospheric pressure and decreased at elevated pressures.

HSP: The obtained results showed an increase in mRNA level of both HSP70 and HSP90 in the slices maintained at atmospheric pressure. Conversely, slices incubated at 5 and 15 MPa showed a less important induction of the chaperones. According to Lemaire et al., this observation could reflect the stabilizing properties of membrane fluidity and protein structure in deep-sea fish cells (Lemaire et al., 2012).

CYP1A: Concerning cytochromes, data indicated that CYP1A induction was meaningfully lower at high pressure. The same effects were observed for the induction of HSP 90 which seemed very low. Smaller amounts of transcription at 15MPa reflect the effects on the AhR transduction mechanism. The stable levels of transcript levels for the antioxidant enzymes CAT and GPX seemed to exclude the hypothesis of a global repression of transcription.

This is clear evidence that CYP1A is pressure-dependent and that the transcription inhibition could be due to a reduced efficiency of the AhR signaling pathway (Lemaire et al., 2012). This study reveals conclusive results to the advance of the research and raises important questions about the mechanisms of reduced responsiveness of AhR.

3.3 Shallow fish PCLS exposed to 3-MC and HHP

Evidence of the pressure-dependent expression of CYP1A in deep-sea fish *C. rupestris* were exposed by Lemaire et al. in 2012. The observations made about this experiment could suggest that HP might influence response of xenobiotics in deep-sea fish. Besides, they decided to conduct same scenario experiment on *Dichentrarchus labrax*, a species presumably not adapted to HP because living at 10-100m depth.

The same PCLS method was used, just as the pollutants exposure, gene's expression by qPCR and the hyperbaric exposure method.

Slices were first exposed to atmospheric pressure for 21hours (0,1MPa at 15°C) with increased levels of AhR agonist to cause the recruitment of cellular detoxification systems at this pressure. At this stage, slices exhibited a concentration-dependent increase of CYP1A transcription level. When exposed to 3-MC (25µM) for 6h and 15h, transcript level of CYP1A was increased 3 and 6 times that of T₀ controls at 0,1 MPa. Nevertheless, when slices were exposed to higher pressures, CYP1A expression was similar to that of T₀ controls (Figure 15) (Lemaire et al., 2016).

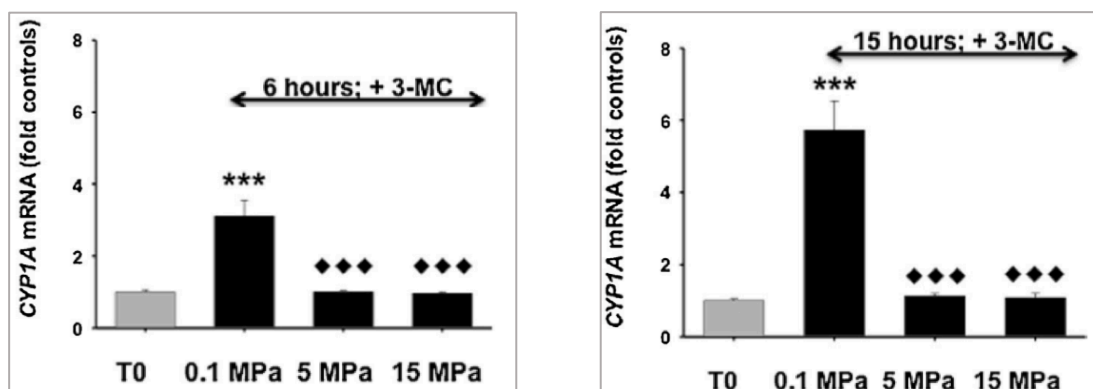


Figure 15: CYP1A transcript levels for the 6h and 15h exposure to 3-MC (25µM) at atmospheric (0.1MPa) and high pressure (5-15MPa) (Lemaire et al., 2016)

Just like the 2012 experiment of the same author, strong increases of HSP70 mRNA content were observed compared to T₀ controls at atmospheric pressure (0,1MPa): about 170 and 620 fold controls after 6 and 15 hours of incubation respectively. Once exposed at high pressure for 6 and 15 hours, HSP70 expression levels decreased to much lower levels. Same observations were made for HSP90 after 6 and 15 hours of high pressure exposure.

The general repression on CYP1A, HSP70 and HSP90 gene expressions suggest that a general mechanism could be at work. As the expression of other genes (Glutathione peroxidase, catalase) was, again, not affected in the *D. labrax*, it can be concluded that observed effects

on CYP1A did not reflect a global inhibition of transcription in hepatocytes (Lemaire et al., 2016).

Thus both experiments conducted by Lemaire et al. in 2012 and 2016 may clearly indicate that HHP inhibitory effects could not be specific to pressure-adapted species. A general effect appears to inhibit the expression levels of HSP70, HSP90 and CYP1A under pressure.

3.4 How does HHP affect xenobiotic metabolism in fish?

In order to assess the potential effect of HHP on the ligand activated AHR-CYP1A target, Lemaire et al. tried, in 2018, to determine the differences in gene sequences between *C. armatus* (deep-sea species) proteins (AHR, CYP1A and POR) and those of shallow species both at atmospheric pressure (0.1MPa). After sequences analysis of both shallow and deep-sea species, a significant proportion of substitutions turned out to be located in or near functional areas important for protein-protein interaction or ligand binding.

Regarding the impact of HP on AhR efficiency, it appeared that the sequence modifications are more complex than for CYP1A. The researchers suggested that, the transactivation region of abyssal receptor contained unique repetitive motifs of possible importance that were particularly enriched in serine proline and threonine (Lemaire et al., 2018). This indicates that specific adaptations to pressure of proteins involved in xenobiotic metabolism are present in deep-sea fish. There is a strong possibility that the pressure-dependent repression of CYP4501A could be associated to other mechanisms in both adapted and non-adapted fish species.

In order to find answer to this question, concentrations of different osmolytes in fish were analyzed depending on the surrounding pressure. It turned out that trimethylamine N-oxide (TMAO) synthesis increased with depth in a very large amount in a wide range of fish categories. This osmolyte could thus play a role in the repressive effect of HHP observed on fish proteins involved in detoxification processes.

CHAPTER 4: TRIMETHYLAMINE N-OXIDE ACCUMULATION IN FISH

1. Trimethylamine N-oxide (TMAO)

Trimethylamine N-oxide is a colorless organic compound in the class of the amine oxides of the formula $(\text{CH}_3)_3\text{NO}$. TMAO is a product of the oxidation of trimethylamine (TMA), a common metabolite found in animals and derived from choline (Baker & Chaykin, 1962). TMAO has an important role in the organism in counteracting the unfolding effects of urea. TMAO's protective properties protect organisms from denaturation through environmental stress (Zou, Bennion, Daggett, & Murphy, 2002).

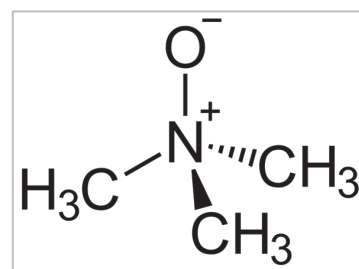


Figure 16: TMAO structure formula
(Trimethylaminoxid.svg, 2008)

Even though known in marine organisms for a long time, TMAO has been lately associated with cardiovascular disease in humans (Velasquez, Ramezani, Manal, & Raj, 2016). Substantial evidence supporting the hypothesis of TMAO as protective factor in humans were observed as well as evidence against these hypotheses; the concrete role of TMAO in cardiovascular risk thus still remains controversial. According to Ufnal & Nowiński's hypothesis, increased plasma TMAO in humans may be a marker of cardiovascular risk but also a compensatory response in order to protect cells from hydrostatic and osmotic stress (Ufnal & Nowiński, 2019).

2. TMAO: important osmolyte in fish

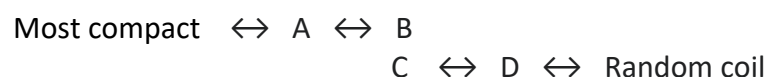
Deep-sea organisms are well adapted to extreme depths and may not experience the stress effect of pressure on protein structure. Indeed, HP only affects species colonizing new depths and those who undergo vertical movements (Dulvy et al., 2008). Pressure is therefore an important factor limiting species depth ranges.

In this way, species that are not accustomed to HP have to modify their metabolism to cope with protein compression and loss of function. To protect themselves against transformations, cells accumulate different organic molecules called osmolytes. Those osmolytes can fall into different categories: sugars and polyols, neutral amino acids (e.g. glycine and taurine), methylsulfonium compounds and methylamines (glycine and betaine) and trimethylamine N-oxide (TMAO) (P H Yancey, Clark, Hand, Bowlus, & Somero, 1982).

The principal observed adaptation is the accumulation of TMAO in marine animals' tissues in order to protect them against protein denaturation. Trimethylamine-N-oxide is the main produced osmolyte in marine elasmobranchs and is intended to avoid deleterious effects of urea. Indeed, it has been widely observed that TMAO can increase the melting temperature of proteins (Mashino & Fridovich, 1987; Paul H Yancey & Somero, 1979) and restore proteins to their native structure recovering their lost enzyme function due to the presence of concentrated urea (P. Yancey & Siebenaller, 2000). In addition to protecting against urea, TMAO overcomes the inhibitory effects of hydrostatic pressure on proteins in deep-sea creatures (Paul H. Yancey, Blake, & Conley, 2002).

2.1 Effects of urea on enzyme activity and stability

Urea synthesis is the major pathway for the metabolism of ammonia. Organisms are able to convert nitrogen, such as ammonia, into a less toxic substance such as urea. This happens due to the urea cycle that converts excess of ammonia into urea in the mitochondria of the liver. It has been confirmed that urea has a tendency to destabilize the structure of some proteins and to inhibit some enzymes. Organisms which were accumulating a high concentrations of urea were also shown to increase TMAO levels. Such compounds have been seen to oppose the effects of urea, note for example (1) the renaturation of lactate dehydrogenase, (2) the thermal inactivation of ribonuclease A, (3) the revival of several enzymes activity and (4) the reactivity of thiol groups of glutamic dehydrogenase (Mashino & Fridovich, 1987). Considering the different conformations of proteins along the continuum, from the compact structure to the fully expanded structure:



Urea is assumed to shift the series equilibria to the right leading to the denaturation of the protein. In contrast, TMAO shifts the equilibria toward the left preferring the stabilization of the more compact and stable form of the protein. This confirms the protective effect of TMAO towards the harmful impacts of urea on the body (Baskakov, Wang, & Bolen, 1998; Mashino & Fridovich, 1987).

The mechanism by which TMAO stabilizes the folded state of proteins is not entirely resolved yet (Velasquez et al., 2016). However, it is known that the protein unfolding is caused by urea's ability to accumulate around protein backbone and sidechains leading to a stronger dispersion between urea and protein backbone (Hua, Zhou, Thirumalai, & Berne, 2008). On the other hand, TMAO molecules appear to interact inversely with protein backbone and sidechain units, thus destabilizing the unfolded state (Canchi, Jayasimha, Rau, Makhatadze, & Garcia, 2012).

This would be due to the amphiphilic structural arrangement of TMAO thus forming H bonding network with water, resulting in protein stability (Ma, Pazos, & Gai, 2014; Velasquez et al., 2016).

3. Biosynthesis of TMAO in fish

Although the physiological role of TMAO has already been discussed, its origin in fish still must be known. TMAO in fish may originate from their natural diet, as it is produced by animals of the lower levels of marine food chains such as herbivorous copepods (Strøm, 1979). Still, TMAO may have an endogenous origin through a synthesis pathway from the oxidation of a precursor (Hebard, Flick, & Martin, 1982).

In that way, Bilinski E. (1964) decided to study the synthesis of TMAO in marine teleosts by testing diverse C^{14} -labelled compounds potentially involved in TMAO synthesis as precursors. A total of 11 C^{14} -labelled compounds were tested. The results of experiments are presented in table 1 (Bilinski, 1964).

Fish		C^{14} -labelled precursor	Amount administered per 100 g body weight ^a		Specific radio- activity of TMAO (counts per minute) ^b
Species	Total weight(g)		μ curies	μ moles	
Starry flounder	346	Trimethylamine- C^{14} hydrochloride	2.9	0.7	12000
Starry flounder	313	Methylamine- C^{14} hydrochloride	3.2	1.0	<5
Lemon sole	316	Betaine-methyl- C^{14} hydrochloride	4.8	2.8	50
Starry flounder	140	γ -Butyrobetaine-methyl- C^{14} hydrochloride	1.0	35.5	70
Starry flounder	198	DL-carnitine-methyl- C^{14} hydrochloride	0.9	15.1	<5
Lemon sole	227	L-methionine-methyl- C^{14}	6.6	0.7	20
Starry flounder	96	Glycine-2- C^{14}	10.7	2.8	<10

Table 1: Utilization of various C^{14} -labelled compounds for the formation of TMAO in fish liver (Bilinski, 1964)

Results indicate that the administration of labelled TMA resulted in the formation of very highly radioactive TMAO levels in the muscles whereas only small amounts of radioactivity were detected after administration of the other precursors. It should be noted that γ -butyrobetaine-methyl- C^{14} is the second TMAO precursor in this table reaching a radioactivity of 70. However, the obtained results distinctly demonstrate that in fish, TMA is oxidized in vivo to TMAO confirming the assumption made by Baker et al. in 1963. Thus, the oxidation of TMA might be considered as the ultimate step in the formation of TMAO in fish. However, the accurate pathway leading to the formation of TMA still must be specified (Bilinski, 1964).

3.1 Microbial conversion of choline to trimethylamine (TMA)

The biosynthetic pathway of TMAO from dietary precursors in fish has been questioned for a long time in order to identify the major compounds at the origin of TMAO. An experiment conducted by Niizeki et al. (2003) consisted of the introduction of quaternary ammonium compounds (choline, glycine betaine and carnitine) into isolated stomach and intestine of tilapia teleosts. Formation of trimethylamine was not detected in the stomach but in the intestine of fish that received choline.

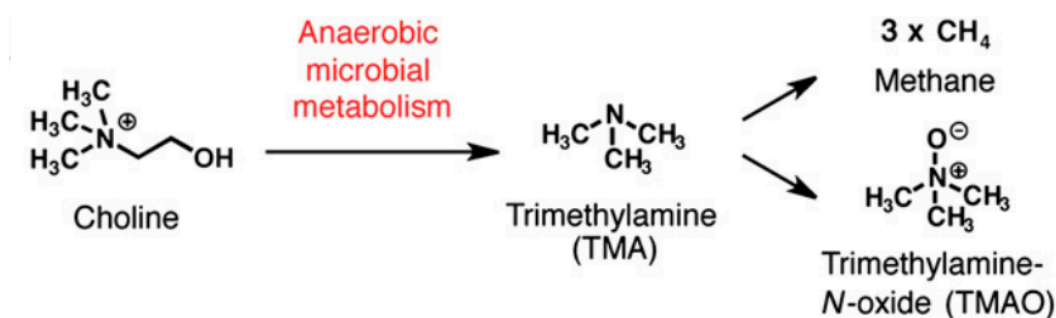


Figure 17: Generation of trimethylamine (TMA) from choline and its subsequent processing by microbial organisms (Craciun & Balskus, 2012)

So, it would appear that choline is the major quaternary ammonium compound involved in the synthesis of TMA (Niizeki et al., 2003). Furthermore, it has been asserted that the only biochemical reaction directly responsible for the formation of TMA is the metabolism of choline by anaerobic microorganisms (Figure 17). This exclusively microbial transformation plays a major role in the TMAO pathway as it is now recognized that symbiotic gut microbes in vertebrates generate TMA from choline (Bain, Fornasini, & Evans, 2005; de la Huerga & Popper, 1951; Zeisel, Wishnok, & Blusztajn, 1983).

In addition to the synthesis of TMAO through choline and its dietary origin, it turns out there was a greater content of TMAO in fish when their diet was supplemented with TMAO compared to the basal diet. Thus, TMAO could also be directly incorporated from diet into the tissues of the fish for storage.

On the other hand, diet supplemented with TMA also induced an accumulation of TMAO suggesting that much of the dietary TMA is oxygenated directly after its absorption in the intestine. It can therefore be said that the origin of TMAO in fish can result from direct incorporation in fish or can be biosynthesized from dietary choline pathway (Craciun & Balskus, 2012).

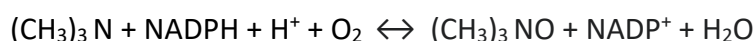
Besides evidence leading to the importance of choline in this synthesis, Koeth et al. (2014) have identified an additional pathway for the synthesis of TMA involving the catabolism of L-carnitine through microbial reactions. According to them, following L-carnitine ingestion, intermediate metabolite γ -butyrobetaine (γ BB) is produced and then converted into TMA by gut microbes. In fact, remember γ -butyrobetaine was the precursor producing the 2nd highest levels of C¹⁴-TMAO in Table 1. Thus, dietary choline and L-carnitine would be the major precursors for TMA through gut microbial transformation in fish (Koeth et al., 2014).

3.2 Oxidation of TMA into TMAO



Figure 18: TMA oxidation into TMAO (Trimethylamine structure.svg, 2008)

As TMA oxidation is known as the only pathway for the endogenous production of TMAO, Baker et al. described, in 1963, the presence of an enzyme, the TMA monooxygenase (also called TMA oxidase) catalyzing the following reaction (Baker, Struempler, & Chaykin, 1963) :



TMA monooxygenase would be the principal enzyme involved in this oxidative reaction. Likewise, Goldstein and Dewitt-Harley described in 1973 the presence of the enzyme in the liver of fish such as marine teleosts and elasmobranchs, also referring to TMA monooxygenase (L. Goldstein, Dewitt-Harley, & Foster, 1973). In 2003, Niizeki et al. focused on the monooxygenation of TMA to TMAO. They measured the monooxygenase activity and the distribution of the enzyme in fish (including the Japanese eel). Tested throughout the whole fish body, the greatest TMA monooxygenase activity was observed in the liver followed by the kidney considering TMA oxidation principally takes place in those organs (Niizeki et al., 2003).

In mammals, the responsible enzymes for TMA oxidation are the flavin-containing monooxygenases (FMOs), more precisely FMO₃ enzyme. Indeed, this enzyme is principally known as one of the most abundant enzymes involved in TMA oxidation (Fennema, Phillips, & Shephard, 2016). Monooxygenases are enzymes that transfer one atom of oxygen from molecular O₂ to substrates during oxidation of a substrate (Samanthi, 2011). Flavin-containing monooxygenases differ from other monooxygenases by utilizing host co-substrates such as iron-containing heme (e.g. CYP450s) and flavins, requiring the same cofactors as CYP450 monooxygenase (NADPH and oxygen) (Schlenk, 1998).

FMOs also have a toxicological importance since they have been primarily studied as xenobiotic metabolizing enzymes (mainly nitrogen and Sulphur substrates). As for CYP4501A, the upregulation of mRNA induction levels of FMO3 is principally performed by the Aryl hydrocarbon Receptor (Celius et al., 2008).

Since the TMAO production pathway has been studied in marine animals, TMA oxidase has always been identified as an enzyme activity such as in the liver of the guppy *Poecilia reticulata* and the Japanese eel *Anguilla Japonica* but without ever mentioning FMOs making it quite confusing (Toshiko, Michiyo, & Morihiko, 1988). Association between TMA oxidase and FMOs was not observed until Goldstein and Dewitt-Harley demonstrated a similarity between TMA oxidase and FMO enzymes in the nurse shark liver (Leon Goldstein & Dewitt-Harley, 1973).

Finally, the paper written by Raymond J. in 1998 clearly exposes his experiments conducted to characterize the TMA oxidase activity in Kotzebue herring. He sets out the hypothesis that most of the TMA oxidase activity would be assumed to be due to FMOs. According to him, TMA oxidase correlates with FMO-like protein expression and FMOs are at the origin of the oxidation reactions in the liver of fish. Thus, FMO₃ would be one of the most abundant substrates of the TMA enzyme, allowing the oxidation of TMA (Fennema et al., 2016).

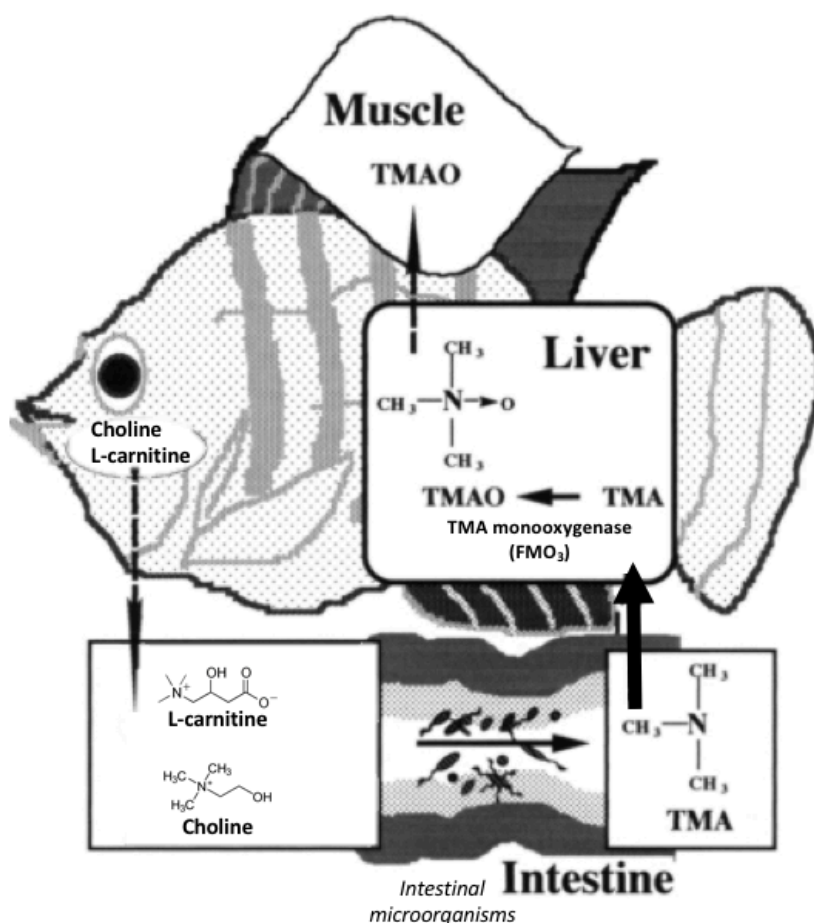


Figure 19: Biosynthesis of TMAO from dietary precursors in fish (Niizeki et al., 2003)

Since then, the scientific world considers that the oxidation of TMA is made possible by FMOs (more particularly FMO₃), facilitating the knowledge of this important metabolic pathway (K. Wang et al., 2019). Finally, figure 19 gives a global overview of TMAO pathway. Starting from dietary L-carnitine and choline passing through their metabolism by gut microbiota producing TMA and finally the oxidation of TMA into TMAO by FMO₃ (Niizeki et al., 2003).

3.3 Distribution of TMAO in tissues

Another study performed by Niizeki et al. focused on the distribution of TMAO in fish tissues of seawater teleosts, Nile tilapia. Once synthesized, TMAO can accumulate in several locations in the organism such as liver, intestine, kidney, muscle, brain and gills. According to the results, it turned out TMAO was localized in large quantities in the muscle (Table 2). Kidney and liver contained much smaller amounts and no TMAO was detected in brain, gills or intestine (Niizeki et al., 2003).

Tissue	Average amount $\times 10^2$ (mmol/100 g)	
Liver	(3)	1.6
Kidney	(3)	4.0
Eye	(3)	N
Muscle	(2)	4.5
Brain	(3)	ND
Gill	(3)	ND
Intestine	(3)	ND
ND; not detected. Numbers of individuals in parentheses.		

Table 2: Distribution of TMAO in tilapia (teleost) (Niizeki et al., 2003)

4. TMAO accumulation in deep-living fish species

As the purpose of this thesis is to assess the role of TMAO in the effect of pressure on the detoxification process in eels, it is crucial to estimate the effects of pressure on the accumulation of TMAO in fish. Given that TMAO accumulation measurements of deep-living eels have never been done before, we will use data from other deep-sea species to draw conclusions.

4.1 Elevated levels of TMAO in muscles of deep-sea Gadiform teleosts

As TMAO typically occurs at 20-70mM in shallow fish muscles, Yancey et al. (1997) conducted one of the first experiments to measure TMAO levels in deep-living fish species (Hebard et al., 1982) by collecting data from two families of benthic gadiform teleosts. Species were collected from the continental slope (1800 -2000 m) and abyssal plain (2850 m) of Oregon coast. One shallow-living species was also caught: *Merluccius productus*. For all species, samples from dorsal white muscles were immediately cut and stored at -70°C. TMAO contents were processed following the method of Wekell and Barnett (Wekell & Barnett, 1991).

The exposed results from table 3 revealed high osmolalities (mosm/kg) and high TMAO muscle contents going from 80 to 200 mmol/kg wet weight, exceeding the highest values recorded for close related cod family Gadidae (P.H. Yancey, Gillett, Suko, & Santoso, 1997). Only the shallow gadiform (*M. productus*) showed a much lower osmolyte content (72 mmol/kg wet weight) which is, on average, the standard rate for this variety of shallow gadids (Hebard et al., 1982). So, they were able to discern a positive correlation with capture depth of fish: the three abyssal macrourids showed significant higher TMAO levels and plasma osmolalities than *M. productus*, the shallow species.

Taurine was also measured in muscle and was found to be significant in some of them. It turns out taurine also acts like an osmolyte in some non-muscle teleost tissues but at very low levels (Brill, Horodysky, Place, Larkin, & Reimschuessel, 2019; King & Goldstein, 1983). This study was therefore the first to highlight that the sum of TMAO and taurine in muscle and TMAO and Na⁺ in plasma strongly correlates and accounts for the elevated osmolalities of body fluids. Moreover, it gave clear evidence of plasma and muscle TMAO values exceeding those mostly reported for teleosts (P.H. Yancey et al., 1997). In 2001, the same experiment was done on deep-sea teleosts providing similar observations confirming Yancey's assumption on increased TMAO accumulation in deep-living species (P H Yancey, Fyfe-Johnson, Kelly, Walker, & Aun, 2001).

Family	Species	Capture depth ²	Depth range ³	Plasma			Muscle	
				Osmotic pressure (mosm/kg)	Na ⁺ (mM)	TMAO (mM) ⁴	TMAO (mmol/kg)	Taurine (mmol/kg) ⁵
Moridae	<i>A. microlepis</i>	Both sites	530–2940 m	557 ± 20 (5)	219 ± 30 (4)	159 ± 46	211 ± 14 (3)	6.8 ± 0.5
Macrouridae	<i>C. leptolepis</i>	Abyssal	2000–3900 m	479 ± 15 (5)	270 ± 19 (4)	1.5 ± 0.5	158 ± 20 (6)	9.8 ± 1.8
	<i>C. fillifer</i>	Abyssal	2000–3000 m	488 ± 22 (5)	249 ± 12 (3)	15.0 ± 4.0	177 ± 8 (3)	nd
	<i>C. armatus</i>	Abyssal	2000–5200 m	479 ± 21 (5)	273 ± 34 (3)	4.4 ± 2.8	173 ± 5 (5)	nd
	<i>C. cinereus</i>	Bathyal	225–2830 m	444 ± 14 (5)	224 ± 19 (4)	15.1 ± 6.1	121 ± 11 (3)	1.8 ± 3.1
	<i>A. pectoralis</i>	Bathyal	200–2200 m	423 ± 16 (5)	225 ± 25 (3)	3.8 ± 1.7	83 ± 10 (5)	nd
Merlucciidae	<i>M. productus</i>	Shallow	0–900 m ⁶				71.9 (1)	nd
P value ⁷				<0.001	0.02	<0.001	<0.001	

Table 3: Osmotic pressures, osmolyte compositions, depth ranges and capture sites of gadiform species (M.B. Gillett & Yancey, 1997)

4.2 TMAO and other osmolytes in deep-sea fish

As high levels of TMAO were found in macrourid (grenadier) fish (about 160–180 mmol/kg wet weight) from depths in previous researches, this study focused on other macrourid fish from greater depths (4800 m deep). This way, they could pursue their hypotheses that TMAO and other organic osmolytes accumulate in deep-sea animals thus counteracting the inhibitory effects of HHP on proteins (P.H. Yancey et al., 1997).

A global analysis of osmolytes was therefore made on these fish. Abyssal grenadiers (*Coryphaenoides armatus*) were caught at 4.8 km deep and frozen at -80°C. The osmolytes quantifications and measurements were done on white muscle tissue. Other species as

Anemones were also collected at different depths (*Stomphia didemon* at 90m depth and *Actinauge abyssorum* at 1900 and 2900 m depth).

Results of the osmolyte composition of the anemones showed, once again, a distinct positive correlation between the TMAO content in the muscles and capture depth (P. H. Yancey, Rhea, Kemp, & Bailey, 2004). Concerning the muscle contents of cods from shallow (*Gadus macrocephalus*) and grenadiers (*Coryphaenoides* spp), the evolution of the TMAO values with depth clearly follows a linear regression.

Figure 20 gathers data from several experiments over time. It represents the TMAO contents in muscles of cods (*Gadus macrocephalus*) from shallow water and different grenadiers species (*Coryphaenoides* spp) from deeper waters. Open symbols are data from Gillett et al. (P.H. Yancey et al., 1997) and Kelly and Yancey (Kelly & Yancey, 1999) and the black filled circle is the data obtained from the study itself.

Following the progression of the points with pressure, it is possible to determine the following equation (P. H. Yancey et al., 2004):

$$\text{TMAO} = 0.0437 \times \text{depth} + 44.0 \quad (r^2 = 0.99)$$

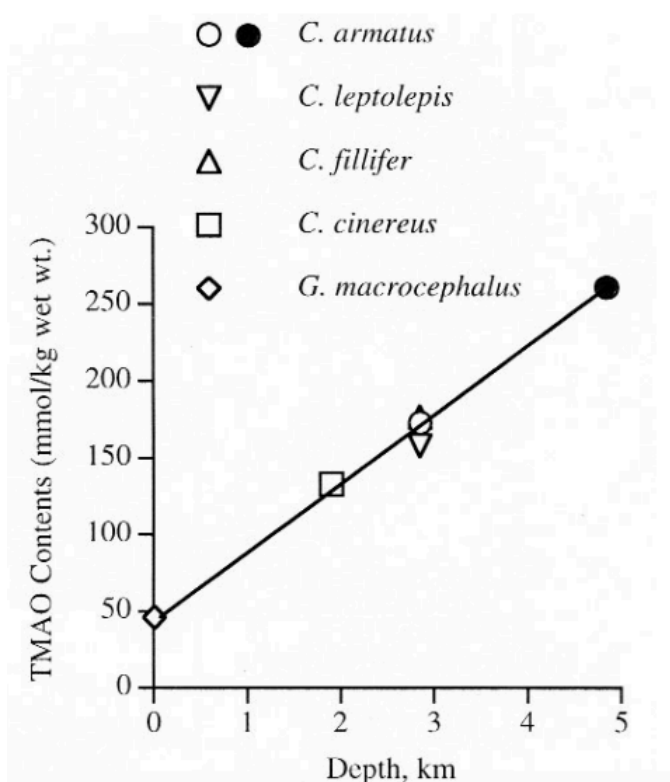


Figure 20: TMAO contents in muscles of cods and grenadiers (Yancey et al., 2004)

This equation is proof of the positive correlation between TMAO concentrations in fish muscle and depth. Moreover, the coefficient of determination of 0.99 supports the prediction quality of this equation.

The previously reported linear correlation between depth and TMAO in various animals (P.H. Yancey et al., 1997) has been extended to other species and fish from greater depths. It is worth pointing out that two of the points, one at 2.9 km and the new data at 4.8 km are from the same species (*C. armatus*).

More recent studies include a study done on snailfish from the Mariana Trench (1100 m depth) in 2019. This study indicated very elevated expression levels of genes coding for the FMO₃ enzyme catalyzing TMAO synthesis. Those results suggest that high hydrostatic pressure stimulates FMO₃ expression through AhR transcription factor thus increasing TMAO production (K. Wang et al., 2019).

Other studies have also put forward the possible crosstalk between TMAO, HSPs and pollutants response in fish cells. Primarily, FMO₃ and CYP450 genes both seem to be partly under control of the Aryl hydrocarbon receptor and are activated by 3-methylcholanthrene dioxin-like pollutant (Petriello et al., 2018). Further, a reported crosstalk between TMAO and HSPs expression has been reported in fish such as the dogfish where the incubation of red blood cells with TMAO repressed the expression of HSP70 by thermal stress preventing damages on cells (MacLellan et al., 2015). Finally, Graziano G. reported in 2012 conformational changes of HSP90 induced by TMAO confirming the relationship between TMAO osmolyte and heat shock proteins (Graziano, 2012).

To conclude, these exposed evidence further support the hypothesis that TMAO accumulation is related to high hydrostatic pressure, which remains the only environment factor that is linear with depth (P. H. Yancey et al., 2004).

4.3 Higher concentrations of TMAO in seawater-adapted eels

To date, no experiment has yet attempted to assess TMAO levels related to capture depth (and thus pressure) in eels. However, the eel is a very attractive species because it can dive up to 2000 m deep, as seen earlier. As it encounters more and more POPs during its life cycle, it is highly likely that pressure also inhibits its metabolism of pollutants during migration. Even if there is still no evidence of the effects of pressure on this metabolism or on TMAO synthesis in eels, it would be highly possible that the eel also suffers from these highly pressurized environmental conditions. If our assumptions are confirmed, it would be interesting to compare the TMAO levels in yellow eels with those in silver eels. This may allow to define the modifications related to TMAO synthesis occurring during the silvering process of European eels.

In 1987, Toshiko et al. conducted an experiment on eels (*Anguilla japonica*) in order to determine the different levels of TMAO when eels are reared in fresh water (FW) or seawater (SW) environments. They measured TMAO concentrations as well as TMA monooxygenase levels in muscle, kidney and liver tissues. FW and SW groups were each divided into two groups: 5 eels were reared in the “Ringer’s group” and the 5 others were reared in the “TMA group” where eels accepted intraperitoneally injection (into the peritoneum/body cavity) of 3.5% TMA HCl solution three times a day (Toshiko et al., 1988).

TMAO and TMA were determined by gas liquid chromatography GLC as described by Tokunaga et al. (Tokunaga, Lida, & Miwa, 1977). Table 4 includes TMA and TMAO levels in tissues of eels after reared in fresh water (FW) and seawater (SW) environments (TMAO levels are noted TMO in table 4). Concretely, all tested tissues of fish injected with TMA solution had higher TMA levels than those of fish injected with the Ringer's solution which seems normal given that eels can directly assimilate TMA from their environment. Then, little differences were observed in TMAO levels in tissues (noted TMO in table 4) when eels were reared with Ringer's solution. Most important, the accumulation of TMAO in seawater (SW) fish injected with TMA solution was considerably higher than that found in freshwater (FW) fish also injected with TMA solution. Indeed, TMAO muscle level of SW fish is 16 times higher than found in FW fish.

Moreover, there is a strong likelihood that TMAO accumulation caused by the SW adaptation could have been endogenous because fish that were not injected with TMA solution (but ringer's solution) showed higher TMAO levels in SW than FW (Toshiko et al., 1988).

Tissue	FW				SW			
	TMA	Ringer TMO	TMA	TMO	TMA	Ringer TMO	TMA	TMO
Muscle	—	0.2 ± 0.1	±	0.3 ± 0.1	—	0.3 ± 0.1	±	4.8 ± 0.3
Liver	—	0.3 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	—	0.8 ± 0.4	±	18.4 ± 1.2
Kidney	—	0.6 ± 0.1	0.3 ± 0.1	0.8 ± 0.1	0.1 ± 0.1	0.9 ± 0.1	0.3 ± 0.1	10.1 ± 1.9

Table 4: Trimethylamine (TMA) and Trimethylamine N-oxide (TMO) concentrations in tissues of eel injected with Ringer's and TMA solutions in FW and SW environments (mg/100g) (Toshiko et al., 1988)

Finally, we can say that TMAO concentrations in all tissues were significantly higher when fish were reared in SW environment rather than in the FW. This observation may be explained by the fact that, the enzymes levels (TMA monooxygenase) were also extremely elevated in the liver and kidney in SW eels (Table 5).

Tissue	FW				SW			
	Ringer		TMA		Ringer		TMA	
	A	B	A	B	A	B	A	B
Muscle	0	0	0	0	0.01	0.14	0.02	0.28
Liver	0.06	0.75	0.42	5.61	13.46	138.62	26.46	285.75
Kidney	0	0	0.16	2.32	2.18	28.06	2.98	35.86

Table 5: TMA-monooxygenase levels in eels after injection of Ringer's and TMA solutions in FW and SW environments A: $\mu\text{mol/hr/g}$ tissue, B: nmol/hr/mg protein (Toshiko et al., 1988)

From this study, it can be concluded that the silvering process undergone by eels would also modify the ability of TMAO accumulation to adapt them to the depths they will encounter during their spawning migration.

5. Our Hypothesis

A lot of questions remain unanswered due to the lack of experiments made with eels under high hydrostatic pressure. However, based on the above information, we suspect that similarly as in other fish species exposed to HHP, the HHP to which migrating eels are exposed may affect the xenobiotic metabolism released upon body lipids utilization. High pressurized conditions encountered by eels when reaching the deep sea, could repress expression levels of proteins involved in detoxification processes. This would in turn increase the circulation levels of POPs, which could enhance lipid mobilization, and thus reducing the chance for eels to complete their migration and reproduce successfully.

Besides, we would like to investigate the possible role of increased TMAO levels in both the resistance to HHP and the regulation of POPs metabolizing pathways. HHP-related increased TMAO production by FMO₃ in eel liver cells may interfere with phase 1 detoxification processes. This could be related to interactions of TMAO with AhR and HSP90 thus affecting their molecular interactions with xenobiotics leading to decreased activation of phase 1 detoxification process. TMAO could also inhibit HSP90's induction as well as the activation of AhR response through the AhR-Xenobiotic complex.

6. Scientific Strategy

The initial plan of this master thesis was to work on fresh liver cells of non-migrating yellow eels exposed to 3-methylcholanthrene in hyperbaric chambers mimicking deep-sea pressure.

The aim was to evaluate the levels of TMAO produced in the liver both under high pressure (30MPa) and atmospheric pressure (0.1MPa). Thus, we wanted to prove the increase of TMAO synthesis under high hydrostatic pressure in eels.

The purpose of the second experiment would have been to measure the expression of 5 main genes involved in the detoxification process of the eel in liver: CYP1A, AhR, HSP70, HSP90 and FMO₃. The goal was to compare the expression levels of genes under pressure with those at atmospheric pressure. This would have allowed to certify the hypothesis of the inhibitory role of pressure on pollutant metabolism.

Unfortunately, the recent circumstances related to COVID-19 crisis and safety measures put in place have prevented the planned experiments from being carried out. Nevertheless, a proposal for material and methods is developed in the following chapter and contains the necessary information for the handlings initially planned.

CHAPTER 5: PROPOSAL OF A METHODOLOGY AND MATERIALS

1. Animals

1.1 Origin

Eels will be supplied by a fish farm and will, possibly be at the yellow stage. About 20 fish will be sufficient to carry out the study. The sex of the eel is not important in principle but should be the same for all eels in the experiment so that this factor does not interfere with the results.

1.2 Aquaculture parameters

Before being euthanized, eels will remain in aquariums where their living conditions must be optimal to ensure their survival. According to the Food and Agriculture organization of the United Nations, following essential parameters must be respected for the welfare of eels in aquaculture (FAO, n.d.).

Water quality: Eels need pure water in abundance with enough amount of oxygen.

Optimal growing temperature: 24-26°C.

Food intake: food formulated as evaporated-pressed or extruded dough mainly made of insect larvae, crustaceans and fish. Feed every 3 days.

2. Products and reagents

Products

1% Penstrep Medium

3-methylcholanthrene (25 μ M)

ATP Assay Kit

Aurum total RNA Mini Kit

Chloroform

Choline

Cytotoxicity Detection Kit (LDH)

Ethanol 70%

Fetal bovine serum (FBS)

Hanks' Balanced Salt Solution (HBSS) with agarose 1%

iScript cDNA Synthesis Kit

Leibovitz L-15 medium (with L-glutamine)

Low Stringency Wash Buffer

Penicillin/Streptomycin

Syber Green

Trimethylamine

Trizol

Providers (optional)

Invitrogen

Sigma-Aldrich

Perkin Elmer

Biorad

VWR Chemicals

Sigma-Aldrich

Roche Applied Science

Optional

Life Technologies

Invitrogen

Biorad

Life Technologies

Sigma-Aldrich

Life Technologies

Life Technologies

Sigma-Aldrich

Life Technologies

3. Sampling Preparation and Precision Cut Liver Slices (PCLS)

3.1 TMA, choline and carnitine

Choline, carnitine and TMA, are TMAO precursors. Those precursors should be administered to fish in order to stimulate the production of TMAO in the liver. They must be administered before the eels are euthanized (Bilinski, 1964). Thus, they may be added in the diet or simply injected in a single dose by intravenous and intramuscular way. Eels must then be killed 24 hours later.

3.2 Liver extraction

After fish are euthanized, the liver is immediately extracted (Figure 21). The liver is put on ice in a saline solution (Hanks' Balance Salt Solution HBSS).

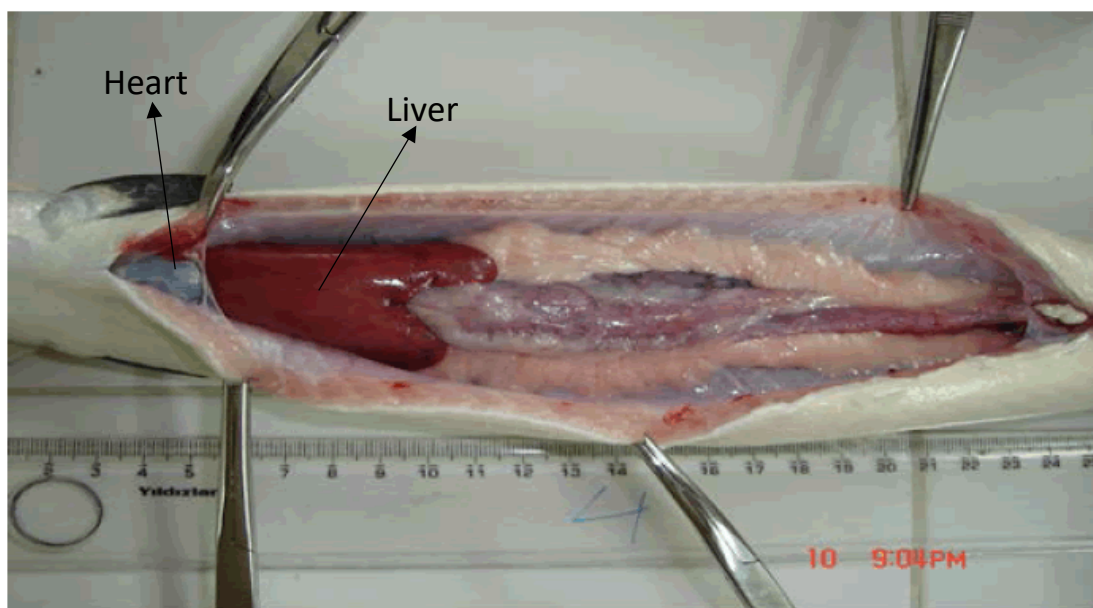


Figure 21: Female eel's liver (Rad, 2013)

3.3 Coating

A solution of 1% agarose in HBSS is prepared and heated to above 65°C. The solution must then be cooled in plastic molds. When the temperature reaches 26°C, each tissue must be placed in plastic molds.

The agarose solution will polymerize for 5 minutes at room temperature and then 5 minutes on ice. Once the gels are formed, they must be covered with cold HBSS to ensure the feeding of the tissues through the gels.

3.4 Slicing

Slicing will be done via the Semi-Automatic Vibrating Microtome. Once the gel has been removed from the mold, it must be glued to the removable plate of the vibratome vat. Slicing is carried out using a vibrating blade microtome (Leica V1000S) .

The parameters may vary depending on the handling circumstances but the recommended data are as follows:

- Frequency of 80 hertz
- Speed and amplitude of the blade respectively 0.25mm and 0.8mm
- Cutting thickness is ideally 100 μm

The microtome tank must be filled with HBSS and a refrigeration system to maintain the temperature at 25°C.

4. Slice culture

4.1 Exposure of slices to PAH-model 3-MC

After slicing, each slice must be placed individually in a well of a 24-well plates containing culture medium contaminated with PAH-model 3-methylcholanthrene. The culture medium is composed of 89% of Leibovitz medium (L-15 medium [+] L-glutamine [+] L-amino acids), 10% of FBS (Fetal Bovine Serum) and 1% of Penstrep medium (Penicillin 10 000U/ml & Streptomycin 10 000 $\mu\text{g/ml}$). Plates must be incubated with 3-MC (25 μM) for 6 hours at 0.1 MPa and 4°C (Lemaire et al., 2012).

4.2 Culture of slices in hyperbaric conditions

Exposure of Precision Cut Liver slices to high hydrostatic pressure will be performed in custom-designed 500-mL hyperbaric chambers from Autoclave (Rantigny, France). Chambers must be filled with demineralized water and maintained at 4°C by cold water circulation in the chamber's jacket (Figure 22).

First incubation of PCLS must be with or without 3-MC (25 μ M) and performed and agitated in the 24-well plates for 6h at 0.1MPa (atmospheric pressure).

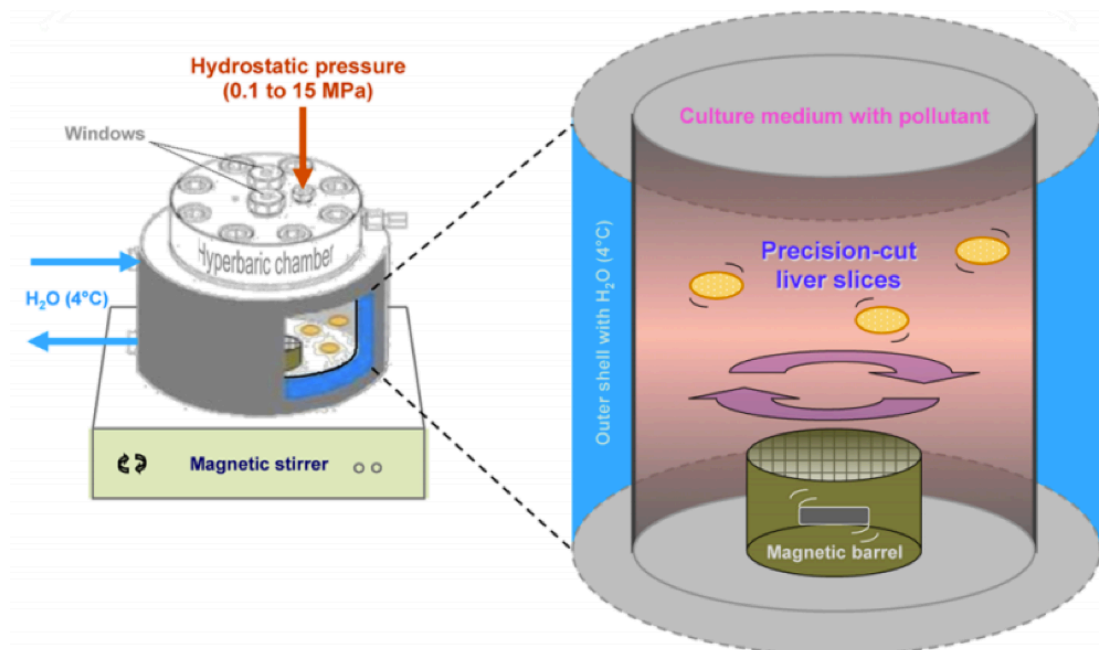


Figure 22: Procedure of hyperbaric exposure of European eel liver slices (Lemaire et al., 2012)

For the pressurized slices, hyperbaric chamber must be filled with oxygen-saturated L15+ media containing 3-MC. Bubbling of pure oxygen must ensure the equally high oxygen concentration in each cylinder. Magnetic stirrers will allow a continuous homogenization of the media during the exposure period. In the hyperbaric chamber, each PCLS must be placed on the 15-cm² section of fiberglass grid to allow them to be continuously bathed in the media during incubation. The chosen dose of 3-MC must significantly increase CYP1A transcript level in PCLS of eels incubated for 6-15h in these hyperbaric chambers (Lemaire et al., 2012).

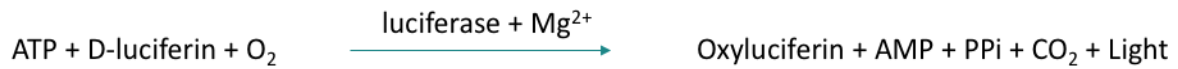
5. Slice survival check

5.1 ATP assay

a) Method

Cell viability will be estimated by the ATP slice content reported by the quantity of proteins. ATP dosage is done via the *ATPassay Kit* 1step Luminescence Assay System. The measurement

is based on the production of light from the reaction of ATP with D-luciferin and luciferase, the light emitted is then proportional to the concentration of ATP in the slices.



b) Protocol

From the 10mM ATP stock solution, a standard range from 0.025 μM to 0.8 μM is prepared in a buffer consisting of 90% of HClO_4 (2%) and 10% of the neutralizer mixture. 10mL buffer is added to the substrate in the container. Afterwards, 100 μL of ATP standard, blank or sample is placed on a 96-well black microplate and 60 μL of substrate is added.

The plate must then be shaken for 2 minutes at 700rpm before being read by a luminometer at room temperature. The results obtained are expressed in RUL (relative unit of light).

5.2 LDH assay

a) Method

Lactate dehydrogenase (LDH) is a cytosolic enzyme present in many different cell types. Plasma membrane damage releases LDH into the cell culture media. LDH catalyzes the conversion of lactate to pyruvate via NAD^+ reduction to NADH. Diaphorase then uses NADH to reduce a tetrazolium salt (INT) to a red formazan product that can be measured at 490nm (Figure 23). The level of formazan formation is directly proportional to the amount of LDH released into the medium, which is indicative of cell cytotoxicity.

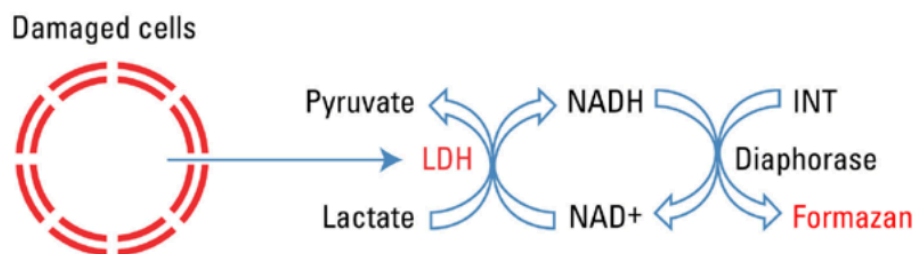


Figure 23: Schematic LDH cytotoxicity assay mechanism (Thermo-Scientific, 2016)

b) Protocol

1. Prepare the “blanks” for media containing PIC, DMSO and Triton-X (PIC = FBS medium high gluc).
2. Centrifugation of the media sampled at 250g (5000rpm – Micro centaur MSE) for 10 minutes.
3. Remove supernatants and measure the quantity of supernatants.
4. Collect 50 µL of the supernatants “blanks” and put them in wells of a 96-well plate.
5. Add 50 µL of milli-q water in the wells.
6. Prepare the “reaction mix” for the LDH test.
7. Add 100 µL of the “reaction mix” in the wells.
8. Incubate for 14minutes at room temperature, out of light.
9. Read the measurements with a spectrophotometer.

6. TMAO measurement

After slices have been exposed to high hydrostatic pressure, the first analysis is the measurement of the TMAO content levels in both exposed and unexposed slices to compare the effects of pressure on TMAO synthesis in eels.

The two TMAO quantification methods that can be used in this case are (1) Wekell and Barnett method using ferrous sulfate and EDTA and (2) Liquid chromatography-mass spectrometry method (LC-MS/MS).

6.1 Wekell and Barnett Method (1991)

The principle of this method is the following: TMAO content in tissues is first reduced to TMA with 1.0 mL iron reagent (Fe-EDTA). The TMA must then be extracted in toluene and react with a picric acid reagent. A spectrophotometric analysis may then quantify the concentration of TMA picrate products, which must be compared to the TMAO standards treated identically. TMAO contents of the tissues can be calculated based on all dilutions made in the process (Downing, Wallace, & Yancey, 2018; Wekell & Barnett, 1991).

6.2 LC-MS/MS method

This method can be separated into two parts: sample extraction method and LC-MS/MS instrument method development.

The sample extraction may be performed using protein precipitation (PPT) or liquid-liquid extraction (LLE). The instrument method development consists in the physical separation

capabilities of liquid chromatography (HPLC) combined with the mass analysis of mass spectrometry (MS). Liquid chromatography will separate mixtures with multiple components, mass spectrometry will provide structural identity of the individual components with high molecular specificity (Z. Wang et al., 2014).

7. Gene expression: reverse transcription quantitative PCR analysis (RT-qPCR)

In order to assess the expression levels of the genes of interest (in this case CYP1A, AhR, HSP70, HSP90 and FMO₃) a reverse transcription quantitative Polymerase Chain Reaction should be carried out. This method of analysis consists in the quantification of RNA transcripts by reverse-transcribing them into cDNA (complementary DNA). This cDNA must then be quantified by real-time qPCR in order to determine the expression rates of interest genes.

7.1 RNA extraction

Material

- Kit Aurumn total RNA mini, Biorad
- Warning : all elements of the kit are RNase and DNase free -> only open under a laminar flow hood turned on and cleaned.
 - DNase I (-80°C room, -20°C column freezer)
 - DNase dilution solution
 - Low Stringency Wash Buffer
 - High Stringency Wash Buffer
 - Elution buffer
 - Tubes Collection tubes, elution tubes
 - RNA extraction columns
- RNase and DNase free tips: 1000 µL, 200 µL
- RNase and DNase free tubes: 0.2 mL, 1.5 mL, 2 mL
- Micropipettes P200, P1000
- Ice tray
- Ethanol 70% (aliquot)
- Chloroform (aliquot)
- Trizol (cold room)
- Syringes 1.1 and 0.7 mm (sterile)

7.2 RNA quantification

Material

- RNA Aliquots (on ice)
- Pipette 2 µL or 10 µL
- Autoclave tips or DNA/RNA, DNase/RNase free
- Water DEPC (aliquot)

7.3 Reverse transcription

Material

- iScript cDNA Synthesis Kit, Biorad - 1 year stability
Warning : all elements of the kit are RNase and DNase free -> only open under a laminar flow hood turned on and cleaned
 - o Reverse transcriptase (-80°C room, -20°C column freezer)
 - o Reaction mix (room -80°C, column freezer -20°C)
- Water DEPC (ambient temperature)
- RNase and DNase free tips
- RNase and DNase free tubes: 0.2 mL

7.4 Quantitative Polymerase Chain Reaction (qPCR)

Material

- qPCR microplates
- Transparent qPCR film and brown plate for sealing microplates
- Syber Green (room -80°C, column freezer -20°C)
- Primers (room -80°C, column freezer -20°C)
- ADN c (-80°C room, -20°C column freezer)
- Water DEPC (ambient temperature)

7.5 Real-time qPCR primers design

Primers are essential parameters in RT-qPCR. Within the context of this work, the main genes whose expression is of interest are CYP4501A, HSP70, HSP90, AhR and FMO₃. By comparing the potential different expression levels of those genes under pressure, we could confirm the inhibitory influence of pressure on pollutant metabolism in eels.

a) Primers design

Based on the sequences of the genes of interest in the European eel, it is not complicated to obtain primers. However, it turns out that these genes have rarely been sequenced in eels. It was therefore not easy to find the sequences on the basis of which the primers could be designed.

Thus, based on sequences from *Anguilla anguilla* (European eel) and *Anguilla japonica* (Japanese eel) we were able to design the primers via the Primer Blast program. Further, a BLAST search was performed to confirm the specificity of the primers.

Gene	Sequence
HSP70	Forward 5'-TCAACCCAGATGAAGCAGTG-3'
	Reverse 5'-GCAGCAGATCCTGAACATTG-3'
HSP90	Forward 5'-ACCATTGCCAAGTCAGGAAC-3'
	Reverse 5'-ACTGCTCATCGTCATTGTGC-3'
CYP1A (Pair 1)	Forward 5'-AGACACCGCACTGAATGGAT-3'
	Reverse 5'-CGGGGCTAAAAGAGGAAGGG-3'
CYP1A (Pair 2)	Forward 5'-GGTGCCAACCCACACCTTAG-3'
	Reverse 5'-TCCCATTCAAGACCACCACG-3'
AhR	Forward 5'-TGCAGGCTCTCAATGGCTTTGT-3'
	Reverse 5'-AGGAAGCCAGAGGAGTTGTCAA-3'

Table 6: Sequences of the HSP70, HSP90, CYP1A and AhR primers

We decided to design 2 pairs of primers for CYP1A to increase the chances of obtaining representative results for the expression of this gene, which plays essential role in our research. The primers of FMO₃ were not designed due to a lack of time and due to the general confusion of which were the actual genes involved in TMA oxidation.

To ensure a representative and efficient qPCR, it was also planned to use primers for 3 reference genes previously used in a reliable and conclusive scientific trial on European eels in 2017 (Politis et al., 2017). In that experiment, they used the following 3 reference genes that we would have used for our qPCR:

- Elongation factor 1-alpha : ef1a
- Ribosomal 18s RNA : 18s
- Ribosomal 40s RNA : 40s

Genes	Sequences
Ef1a	Forward 5'-CTGAAGCCTGGTATGGTGGT-3'
	Reverse 5'-CATGGTGCATTTCCACAGAC-3'
18s	Forward 5'-AGAGCAGGGGAAGTGA-3'
	Reverse 5'-ACCTGGCTGTATTTGCCATC-3'
40s	Forward 5'-TGACCGATGATGAGGTTGAG-3'
	Reverse 5'-GTTTGTGTCCAGACCGTTG-3'

Table 7: Sequences of genes references (Politis et al., 2017)

Still, it is important to specify that these primers were not used for PCR because we were not able to accomplish the experiment and thus, the RT-qPCR. It is thus not certified that they are accurate.

Moreover, we did not proceed to primers validation as it should have been done before ordering the primers. This step is intended to ensure the proper functionality of the sequences chosen as primers. This step should therefore necessarily be done before any future use of these primers in qPCR.

CONCLUSION

Throughout this bibliographical research, we tried to provide elements of response in order to understand the impacts of pressure and TMAO on the pollutant detoxification process in European eels.

Based on the ability of eels to dive up to 2000 meters deep, we have outlined the impact that high hydrostatic pressure can have on protein structures and fish cell membranes. By the infiltration of water on proteins, pressure is able to denaturate, dissociate and separate some protein aggregations. Thus affecting enzymatic reactions, pressure can affect catabolic reactions. However, some pressure-adapted species revealed the occurrence of two adaptive mechanisms.

First, by amino-acids substitutions thus increasing protein stability and reducing volume changes. Besides, a more general stabilizing action involving increasing trimethylamine N-oxide (TMAO) levels was proposed. These pressure-adapted species were also shown to maintain permeability, compressibility and fluidity of their membranes, required for proper function. This was done by modifying their fatty acid profile to reach a higher unsaturated to saturated fatty acid ratio. Eels, on the other hand are capable of adapting in the same way as deep-sea species.

Further, we wanted to investigate the impact of such high pressures on the eel detoxification process based on experiments carried out on other marine species. The first was conducted on a deep-sea species *Coryphaenoides rupestris*. After being exposed to 3-MC pollutant and pressures going up to 15MPa, a significant decrease in CYP1A expression levels was indicated compared to the levels at atmospheric pressure (0.1MPa). Same results were obtained for chaperones HSP70 and HSP90. Another experiment carried out in the same way at same pressures on shallow species *Dichentrarchus labrax*, revealed the same results. Besides the repression of CYP1A, HSP70 and HSP90 transcript levels, stable levels of antioxidant enzymes CAT and GPX were observed at high pressure excluding the hypothesis of a global repression of transcription. Both experiments thus clearly indicated a general inhibitory effect of high pressure on the expression levels of HSP70, HSP90 and CYP1A.

In order to identify by which mechanism both adapted and non-adapted species could be affected by pressure, concentrations of different osmolytes in fish were analyzed and it turned out that TMAO osmolyte levels increased linearly with depth in a large range of fish. So, we gathered various experiments that measured TMAO levels in several teleost species as TMAO measurements in deep-sea eels has never been done before. Elevated levels of that osmolyte were thus found in deep-sea cods, different grenadiers species and anemones.

This thesis therefore lead us to assume that there would be an increased production of TMAO in eels reaching high depths and interfering with phase 1 detoxification process. This could be related to interactions between TMAO and AhR leading to a decreased response of AhR through its complex with xenobiotics. High TMAO levels could then be explained by an induced production of FMO₃ enzyme regulated by high hydrostatic pressure.

Perspectives of this work would be to be able to prove the effects of high hydrostatic pressure on the European eel's hepatic activity by measuring transcript levels of CYP1A, HSP70, HSP90, FMO₃ and AhR. High levels of FMO₃ could partially explain the induction of TMAO synthesis. Finally, to correctly confirm our hypotheses, it would require to measure TMAO levels in pressurized eel liver cells to confirm linear correlation of its synthesis with increased pressure.

If our assumptions were confirmed, if high pressure was increasing TMAO synthesis thus interfering with phase 1 detoxification process, what would be the consequences for migratory eels ? High pressure would be at the origin of high concentrations of persistent pollutants in marine organisms affecting lipid reserves. If, due to low energy reserves, they are not able to complete their migration and thus reproduce, they will die. This experiment would thus help to explain the current decline in European eels.

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The impacts of pressure and trimethylamine N-oxide on pollutant metabolism in European eels

Presented by Rosalie Donck

The aim of this master thesis was to assess the role of trimethylamine N-oxide (TMAO) in the inhibitory effect of pressure on pollutant metabolism in European eels.

By measuring TMAO levels in liver slices exposed to pressures going up to 30MPa, we first wanted to prove correlation between increasing pressure and TMAO levels in eel liver cells. Further, we wanted to compare transcript levels of associated genes implicated in pollutant metabolism under high pressure (30MPa) with those remaining at atmospheric pressure (0.1MPa). This would have allowed us to evaluate whether or not pressure inhibits the metabolism of pollutants in the liver of the European eel. As safety measures related to the COVID-19 crisis prevented us from carrying out the planned experiments, this thesis explains our intention and the importance of this work.

First, the complete life cycle as well as the different life stages of the eel have made it possible to understand the importance of its spawning migration towards the Sargasso Sea, announcing the end of its life but simultaneously the beginning of that of its offspring. Over years, wild eel stocks have been gradually declining to a point where they are now classified as “Critically endangered species”. Among the different lethal obstacles involved in this decline, POPs are largely responsible. Indeed, scientific reports have shown that pollutants in high concentrations in the bloodstream are able to cause disturbances in the reproductive, immune, endocrine and nervous systems. They are also able to induce lipolysis thus depleting energy reserves needed for migration and so, endangering European eel’s life. After questioning which factor was preventing eels from getting rid of those persistent pollutants, high hydrostatic pressure appeared to be overwhelmingly implicated.

The second part of this work has therefore focused on the effects of pressure on global fish, and more precisely, pollutant metabolism. As pressure experiences had not yet been done on eels, we relied on results from other fish species. These data demonstrated the inhibitory effect of pressure on the expression of genes involved in metabolic reactions of pollutants. Thus, CYP1A, HSP70 and HSP90 expression levels decreased drastically after being exposed to high pressure (15MPa representative 1500 m depth) and pollutant (3-MC 25 μ M) for 6 and 15 hours in both pressure and non-pressure adapted species.

Consequently, third part of the work focused on the potential implication of the osmolyte TMAO in the inhibitory effect of pressure. It was demonstrated that accumulated TMAO levels were significantly higher in deep-sea fish, making it possible to establish a linear relationship between TMAO concentrations and depth (and thus pressure). These experiments conducted on other species than eels have led to the postulate that HHP induces the production of FMO₃ in fish liver cells and that the increased production of TMAO may interfere with phase 1 detoxification process thus affecting the migration of European eels.

In conclusion, only by carrying out the planned eel experiments will we be able to validate the hypothesis of the involvement of TMAO in the pressure inhibition of detoxification process in European eels. These experiments would thus help to explain the current decline in European eels.