Universidade de Aveiro Departamento de Biologia
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João Diogo Formiga Rodrigues da Silva

Efeitos do ozono em *Scophthalmus maximus* cultivado em sistema fechado

Effects of ozone in *Scophthalmus maximus* raised in a closed system

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Marinha, realizada sob a orientação científica do Prof. Doutor Mário Guilherme Garcês Pacheco, Professor Auxiliar do Departamento de Biologia da Universidade de Aveiro.

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“For all at last returns to the sea -- to Oceanus, the ocean river, like the ever-flowing stream of time, the beginning and the end.”

Rachel Carson

Palavras-chave

Ozono, *Scophthalmus maximus*, genotoxicidade, efeitos fisiológicos, aquacultura

Resumo

O tratamento de água por ozonização tem demonstrado ser uma ferramenta útil na remoção de resíduos sólidos e estabilização da qualidade da água em sistemas de recirculação, assim como no controle de doenças, pelo que a sua relevância no contexto da piscicultura intensiva é consensualmente assumida. Contudo, as suas consequências a nível bioquímico, fisiológico e citogenético em peixes são ainda pouco conhecidas. Assim, o presente trabalho teve como objectivo investigar os efeitos da exposição a ozono em pregado (*Scophthalmus maximus)* através da avaliação do seu potencial genotóxico (teste de anomalias nucleares eritrociticas - ANE), de indicadores de stress (níveis plasmáticos de cortisol, glucose e lactato), de alterações nos parâmetros hematológicos (concentração de hemoglobina - Hb, contagem de eritrócitos e hemoglobina corpuscular média - HCM) e índices de condição fisiológica (factor de condição de Fulton – K, índice hepatossomático – IHS).

Indivíduos com um peso médio de aproximadamente 75.0±1.41g foram submetidos a 6 horas diárias de exposição a água tratada com ozono (0.15 mg L^{-1}) durante 3 dias consecutivos. Os peixes foram amostrados nos dias 1, 2 e 3, assim como 1 (R1) e 7 (R7) dias após o tratamento, de modo a avaliar a eventual de recuperação. Um grupo controlo foi mantido nas mesmas condições experimentais, mas sem exposição ao ozono. O teste t foi utilizado para avaliar a significância estatística das diferenças entre os grupos tratado e controlo a cada momento de amostragem.

Foi detectada uma indução de ANE ao longo da exposição, indicando dano genético. Adicionalmente, este efeito clastogénico prolongou-se para além do período de exposição até ao dia R7. A concentração de glucose plasmática aumentou significativamente apenas no período pós-exposição (R1 e R7). Relativamente ao lactato plasmático, foram registados recorrentemente níveis mais baixos nos animais expostos, apesar dessa descida ter sido estatisticamente significativa apenas no primeiro dia de exposição e no dia R1. O cortisol plasmático aumentou significativamente apenas no primeiro dia de exposição, após o que se manteve inalterado até ao final da experiência. A concentração de Hb aumentou significativamente nos dias 1 e 3, assim como em R1, onde atingiu o nível máximo. Do modo semelhante, o nº de eritrócitos aumentou significativamente nos dias 2 e 3, mostrando um efeito prolongado até ao dia R1. Não se registaram alterações ao longo da experiência na HCM, assim como no factor K e IHS.

Os resultados apontam claramente para uma condição de stress induzida pelo ozono, expressa num aumento inicial de cortisol plasmático e um aumento tardio da glicemia (período pós-exposição). A redução de lactato plasmático observada pode constituir um aumento compensatório do metabolismo aeróbico dos peixes expostos, o que está de acordo com o aparente aumento do transporte de oxigénio, expresso nos aumentos de Hb e nº de eritrócitos. Os custos metabólicos associados aos processos de reparação do ADN e desintoxicação do ozono e/ou seus subprodutos, assim como o desvio de energia para manutenção do metabolismo, podem ter repercussões negativas na taxa de crescimento do pregado.

Globalmente, ficou demonstrado que os pregados juvenis não foram capazes de se adaptar completamente à exposição a água tratada com ozono em condições realistas, pelo que se sugere a interferência com a saúde dos peixes. Os presentes resultados contribuem para um conhecimento biológico da toxicidade do ozono e para o estabelecimento de margens de segurança na aquicultura em sistemas de recirculação.

Apesar de o presente estudo ter sido desenhado para avaliar o impacto do ozono no contexto dos sistemas de recirculação em aquicultura, o risco para as populações aquáticas selvagens associado a descargas de efluentes (municipais e industriais) tratados com ozono não pode ser subestimado. Nesse sentido, assume particular importância o potencial genotóxico demonstrado, dada a relação causal já demonstrada em relação a malformações e lesões neoplásicas.

Keywords Ozone, *Scophthalmus maximus*, genotoxicity, physiological effects, aquaculture

Abstract

Ozonation is proven useful in recirculating systems promoting the removal of solid matter, the stabilization of water quality and disease control, being thus consensually assumed that it could find a relevant place in the intensive fish culture. Nevertheless, its biochemical, physiological and cytogenetic effects on fish are still largely unknown. Hence, this research investigated the effects of ozone exposure in turbot (*Scophthalmus maximus)* by assessing its genotoxic potential (erythrocytic nuclear abnormalities assay - ENA), general stress indicators (plasma cortisol, glucose and lactate), alterations on hematological parameters (hemoglobin concentration - Hb, red blood cell count - RBC, and mean cell hemoglobin - MCH) and physiological state indices (Fulton's condition factor – K, hepatossomatic index - HSI).

Turbot specimens with an average weight of approximately 75.0±1.41g were subjected to a daily 6-hr ozone (0.15 mg L^{-1}) exposure, repeated for 3 consecutive days. In order to assess the potential recovery after ozone treatment, fish were also analyzed on 1- (R1) and 7-day (R7) post-treatment. A control group was kept under the same experimental conditions but without ozone exposure. The t-test was used to assess the statistical significance of differences between ozone-treated and control groups in each sampling moment.

A significant induction on ENA frequency was recurrently detected along the exposure period, signalling genetic damage. Moreover, this clastogenic effect was prolonged beyond the exposure period up to day R7. Plasmatic concentrations of glucose increased significantly only on the post-treatment period (R1 and R7) and no statistically significance were observed during the ozone exposure. Concerning plasma lactate concentration, lower levels were regularly found in ozone treated fish in relation to the control, even though statistically significant differences were recorded only on the first day of treatment and on R1. Plasmatic levels of cortisol revealed a significant elevation on ozone group following 1 day exposure and afterwards no significant alterations were recorded up to the end of the experiment. The Hb concentration was significantly increased on days 1 and 3, as well as on day R1 where it reached the maximum level. Similarly, RBC were significantly increased on days 2 and 3, showing a prolonged effect on day R1. No significant alterations were observed along the experiment on MCH levels, as well as on K factor and HSI.

The results clearly pointed out an ozone-induced stressful condition, expressed by an early plasma cortisol increase and a late hyperglycemia (post-treatment period). The lactate-lowering effect observed may constitute a compensatory increase of the aerobic capacity of fish, which is in line with the apparent improvement in oxygen transport expressed by increased Hb and RBC. The metabolic costs associated with DNA repair and detoxification of ozone and/or ozone by-products, as well as an increased expenditure of energy to sustain fish metabolism, allowing less energy for growth, can have negative repercussions on turbot growth performance.

Taking into account the overall data, it was demonstrated that juvenile turbot were not able to fully adapt to ozonated water under realistic conditions (considering the tested ozone levels and the exposure duration) and thus, the interference with fish health is hypothesized.

The present findings contribute to a biological based knowledge of ozone toxicity and to the establishment of safety margins in aquaculture practices adopted in recirculation systems, promoting sustainability and fish welfare.

Though the present study was designed to assess the impacted of ozonated water in the context of recirculation aquaculture systems (RASs), the risk to wild aquatic populations resulting from discharges of ozone primary-treated effluents (municipal and industrial) cannot be overlooked. In this regard, the genotoxic potential demonstrated can assume particular importance due to causal linkage with malformations and neoplastic lesions.

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Chapter I

INTRODUCTION

I – INTRODUCTION

1. General Considerations on Aquaculture

1.1 Overview on Fisheries and Aquaculture

While there is no consensus regarding to what extent, it is unquestionable that the world's fish resources are severely damaged. Human-driven erosion of marine biodiversity has recently been projected to lead to the collapse of all currently fished taxa by 2048 (Tal et al., 2009).

Determining the quantity of fish in the oceans, their capacity to endure fishing pressures and ecological damages is extremely complex. To make matters worse, fishing is not the only issue distressing the marine environment, as there are others, such as pollution, habitat devastation, and environmental changes.

At the end of 2004, the world fishing fleet consisted of about 4 million units, of which 1.3 million were decked vessels of various types, tonnage and power, and 2.7 million were undecked. Global capture production in 2004 reached 95.0 million tonnes, an increase of 5 percent in comparison with 2003 (FAO, 2006).

In 2005, as in previous years, around one-quarter of the stock groups were underexploited or moderately exploited (3 % and 20 %, respectively) and could perhaps produce more. About half of the stocks (52 %) were fully exploited and therefore producing catches that were at or close to their maximum sustainable limits, with no room for further expansion. The other one-quarter were either overexploited, depleted or recovering from depletion (17 %, 7 % and 1 %, respectively) and thus were yielding less than their maximum potential owing to excess fishing pressure exerted in the past, with no possibilities in the short or medium term of further expansion and with an increased risk of further declines and need for rebuilding (FAO, 2006). Unfortunately, it is widely agreed that such levels of production are not sustainable and in consequence aquaculture presents itself as the logical and unavoidable solution.

Capture fisheries and aquaculture supplied the world with about 106 million tonnes of food fish in 2004, providing an apparent per capita supply of 16.6 kg (live weight equivalent), which is the highest on record. Of this total, aquaculture accounted for 43 %

(FAO, 2006). Although there was a decrease in the involvement of capture fisheries to human consumption, aquaculture increased its contribution.

Aquaculture continues to grow more rapidly than all other animal food-producing sectors, with an average annual growth rate for the world of 8.8 percent per year since 1970, compared with only 1.2 percent for capture fisheries and 2.8 percent for terrestrial farmed meat production systems (FAO, 2006).

Production from aquaculture has greatly outpaced population growth, with per capita supply from aquaculture increasing from 0.7 kg in 1970 to 7.1 kg in 2004, representing an average annual growth rate of 7.1 percent. World aquaculture (food fish and aquatic plants) has grown significantly during the past half-century. From a production of below 1 million tonnes in the early 1950s, production in 2004 was reported to have risen to 59.4 million tonnes, with a value of US$70.3 billion (FAO, 2006).

1.2 Intensive Farming and Recirculating Aquaculture Systems (RASs)

Responding to the continuous decline in fishery harvests and in an effort to meet seafood consumption, aquaculture showed necessity to improve in efficiency and productivity. The future of aquaculture largely depends on our ability to reduce the risk of environmental interactions and impacts while expanding fish production.

In intensive farming a growing fish population must have the capacity to absorb nutrients and produce waste, throughout the growth process, matched to the full capacity of the container in which they are housed. Preferably, several populations are cultured simultaneous, with each at a different stage in the growth process. In this manner, relatively constant and efficient use is made of all tanks at all times and the fish harvest is semi-continuous. However, limited suitable water supplies and stringent control of wastewater and nutrient discharges from raceway facilities prompted the demand for recirculating systems. These have been employed in the last two decades for applications in intensive aquaculture systems (Tango and Gagnon, 2003) as they are one of the future platforms that offer a sustainable method for farming marine and fresh water fish.

Water reuse increases the mass of fish that can be produced on a limited water resource and allows for more control over the culture environment. The water treatment loop of recirculating systems allows a 100 fold reduction in top-up water needs

(d'Orbcastel et al., 2008). Moreover, because of intensive water reuse, systems that recirculate water, have effluents that are relatively low in volume, but contain proportionally higher nutrient and organic levels, which can be treated for discharge more readily (on a total mass basis) than other aquaculture effluents (Brazil et al., 2002).

The ability of RASs to effectively manage, collect and treat nutrient wastes that accumulate during the fish growth, is a key factor in their future development as mainstream environmentally sound fish production systems (Tal et al., 2009).

The use of recirculating systems to achieve environmental control is not without drawbacks. Reusing the majority of water each day requires processes that remove both dissolved and particulate matter to maintain water quality. Focus is often placed on ammonia removal within a biofilter and on processes that remove settleable and filterable solids. However, as production intensifies and water reuse increases, the removal of nitrite, certain dissolved organics termed refractory organics and colloidal solids left by conventional filtering becomes increasingly important (Brazil et al., 2002).

Current methods for the disinfection of seawater, which clean water supplies and/or avoid blooms of potentially pathogenic microorganisms, include treatments with antibiotics, ozone, filtration, heat, and ultraviolet (UV) irradiation (Whipple and Rohovec, 1994; Pascho et al., 1995; Chang et al., 1998; Liltved and Cripps, 1999; Munro et al., 1999; Frerichs et al., 2000). Ozonation and/or UV irradiation are the most common methods and can be used to treat and sometimes disinfect recirculated water before it returns to the fish culture tanks (Brazil, 1996; Bullock et al., 1997; Summerfelt and Hochheimer, 1997; Christensen et al., 2000; Krumins et al., 2001a,b; Summerfelt, 2003; Summerfelt et al., 2004; Sharrer et al., 2005; Sharrer and Summerfelt, 2007).

2. Ozone Application on RASs – Usefulness and Limitations

2.1 Ozone as a Disinfectant Agent

Ozone is popularly used to disinfect water supplies and industrial effluents such as those from textiles, pharmaceutical, pulp and paper as well as from the aquaculture systems (Tango and Gagnon, 2003). It is a powerful oxidizing agent that has seen wide use

in aquaculture applications, particularly in RASs, for achieving both disinfection and water quality improvements (Rosenthal, 1981; Owsley, 1991; Cryer, 1992; Wedemeyer, 1996; Summerfelt and Hochheimer, 1997).

Summarizing, ozone is added to aquaculture system waters to:

- Impart water quality improvements by oxidizing larger and relatively complex organic molecules and thereby creating smaller and more biodegradable molecules.
- Break apart refractory organic molecules, which can reduce the color of water.
- Oxidize nitrite to nitrate.
- Oxidize ammonia.
- Enhance fine solids removal by changing particle size (i.e., microflocculating fine particulate matter) and surface properties, which can make particles easier to settle, filter, or float (Reckhow et al., 1993).
- Inactive fish pathogens (even if simply by improving water quality) (Summerfelt, 2003)

Despite these apparent advantages, ozone does not completely reduce the total heterotrophic bacterial counts. Additionally, the ozone demand created by the natural organic matter (NOM) and nitrite in the RAS has barred disinfection of the process water, because the ozone doses applied have to be relatively low and the dissolved ozone reacts so fast that a residual cannot be maintained to provide sufficient ozone concentration and contact time for disinfection (Bullock et al., 1997).

During ozonation NOM is oxidized to form more biodegradable organic compounds, referred to as ozonation by-products (OBP). Low-molecular weight compounds, such as aldehydes (Weinberg et al., 1993) and carboxylic acids (Gagnon et al., 1997) have been the main organic OBP identified, whereas bromate and other brominated compounds, the major inorganic OBP (Tango and Gagnon, 2003).

2.2 Ozone Toxicity

One of the major obstacles of the increasing usage of ozone in aquaculture is the lack of relevant risk assessment in culture conditions. For marine systems, questions have arisen concerning the potential toxicity of OBP. Despite the apparent advantages of ozonation, safety borders and biological basis of ozone toxicity should be assessed (Ritola et al., 2000).

Although ozone has a rapid reaction rate and few harmful reaction products, it is toxic to aquatic life at low levels (Wedemeyer et al., 1979; Langlais et al., 1991). Ozone gas showed also to be toxic to humans. Standards set by the federal Occupational Health and Safety Administration only allow for a maximum single exposure level of 0.3 ppm for less than 10 min duration and of 0.1 ppm on a time-weighted average for an 8-h period (Occupational Health and Safety Administration, 1993). Furthermore, recent epidemiological studies have shown that increased ozone exposure is associated with a risk of lung cancer (Abbey et al., 1995; Lawrence et al., 1998).

Animal studies have also demonstrated that ozone exposure can induce lung tumors (Hassett et al., 1985; Last et al., 1987; Herbert et al., 1996). However, the exact carcinogenic mechanisms of ozone remain less clear (Cheng et al., 2003).

In adult fish, the use of ozone requires particular care as it is the cause of death, even at low concentrations, following the alteration of the branchial epithelium and the outermost layers of the epidermis (Rosenlund, 1975; Wedemeyer et al., 1979; Paller and Heidinger, 1980; Richardson et al., 1983).

According to Wedemeyer et al. (1979), the maximum safe level of chronic ozone exposure for salmonids is 0.002 mg/l. A compilation of results from several studies indicates that most fish exposed to ozone concentrations greater than 0.008 - 0.06 mg/l will develop severe gill damage that can result in serum osmolality imbalances and can kill fish immediately or leave them more susceptible to microbial infections (Bullock et al., 1997).

Ozone is a very unstable molecule and, after injection into raw water in RASs, it decomposes very rapidly as it reacts with NOM and naturally occurring bromide, and

iodide producing harmful OBP, namely bromate. Unfortunately, the production conditions and toxicity towards aquatic animals of these OBP are not well understood.

In oxidative stress, pro-oxidant forces overwhelm the antioxidant defense system of animals. This has been detected after exposure to certain xenobiotics including ozone (Mustafa, 1990). Reactive oxygen species (ROS) and other pro-oxidants are continually detoxified and removed in cells by antioxidant defense systems comprising both antioxidant enzymes and small molecular weight free radical scavengers. If antioxidant defenses are effective in detoxifying ROS, then no harmful consequence results to the tissues. However, if the ROS attack is severe, then, antioxidant defense systems may be overwhelmed, resulting in inhibition of antioxidant enzymes and oxidative damage to lipid, protein, DNA and other key molecules. Such processes may in turn provoke alterations in molecular and membrane structures and functions leading to cell and tissue damage (Ritola et al., 2002). According to Mustafa (1990), ozone-induced ROS are capable of causing oxidative stress in mammals, which is reflected in peroxidation of lipids, loss of functional groups and enzyme activities and alteration in membrane permeability. Therefore, it is probable that similar processes could occur in fish as well (Ritola et al., 2000).

Bromate is known as an oxidative mutagen and a potent genotoxic carcinogen in laboratory animals, inducing renal and thyroid follicular cell tumors in rats (Kurokawa et al., 1986 a,b; DeAngelo et al., 1998) and renal tumors in mice (DeAngelo et al., 1998). There is a significant body of work that is highly suggestive that bromate causes DNA damage secondary to oxidative stress from intracellular bromate within the kidney cells where tumors arise (Sai et al., 1991; Umemura et al., 1998; Richardson et al., 2007). Moreover, bromate is classified as carcinogenic to human health by the IARC (International Agency for the Research on Cancer) and the USEPA (United States Environmental Protection Agency) and is a known toxin to fish and other aquatic life, causing respiratory and osmoregulatory dysfunction (Grguric et al., 1994).

Some bromate compounds might be clastogens (i.e., break chromosomes), and previous studies have shown that potassium bromate induced chromosomal aberrations in mammalian cells *in vitro* (Richardson et al., 2007). Its clastogenicity has also been confirmed *in vivo*, with studies in mice and rats showing the induction of micronuclei in bone marrow (IARC, 1999; Richardson et al., 2007). Despite the relative abundance of investigations carried out on ozone exposure leading to bromate and ROS toxicity, there is

a scarcity of studies on OBP effects in fish and especially in turbot as an economically important species frequently raised in RASs. Furthermore, adding to genotoxicity, ozone and its OBP may also be able to induce endocrine and metabolic disturbances, these virtually undocumented, given that xenobiotics can have either direct adverse effects on the endocrine glands and tissues, or their effects can be indirect through alterations of homeostasis and activities of non-endocrine organs (Hontela, 1998).

Aquatic contaminants adversely affect fish endocrine system. Thus, alterations on specific hormonal functions may constitute important stress indicators (Hontela, 1997). Cortisol, a corticoid synthesized by the interrenal cells of the teleost kidney, plays a major role in the physiological response to stress (Oliveira et al., 2007). Plasma lactate, glucose and cortisol are widely used as stress indicators but no research was made concerning cultured turbot exposed to ozone, pointing out the need of investigation on this context. Similarly hematological parameters such as hemoglobin quantification and red blood cell (RBC) count can also account for the physiological state of fish. According to Roche and Bogé (1996), any environmental disturbance can be considered to be a potential source of stress, and can theoretically be detected by changes of hormone or substrate concentrations in plasma, or by changes in erythrocytes parameters.

3. Turbot Characterization

3.1 Distribution

The turbot, *Scophthalmus maximus* L., also called *Psetta maxima* (Scophthalmidae, Pleuronectiformes), is naturally distributed in European waters, from Northeast Atlantic to the Arctic Circle (30º to 70ºN; 23ºW- 42ºE). It occurs in the Baltic and in the Mediterranean, as well as in the Black Sea, where a subspecies *Psetta maxima maeotica* has been described. It also exists in the Southeast Pacific Ocean (Chile) and in China, where it has been introduced for farming. Wild populations inhabit along all European coasts to North West Africa (Morocco), where it is also farmed. Though this species is not considered endangered, it declines in wild catches and some genetic evidence suggest the existence of historical population reductions for European turbot.

3.2 Biology

The turbot is a predator species that lives on sandy, rocky or mixed bottoms; it is common in brackish waters. When juvenile, its diet is based on crustaceans: Malacostraca and Decapoda. Adults feed mainly on other bottom-living fishes (sand eels, gobies, etc.), and, to a lesser extent, on crustaceans and bivalves. The turbot exhibits one of the most important growth rates observed in flatfish (around 30 cm every 3 years).

The spawning season occurs between April and August in Mediterranean populations and between May and August in Atlantic areas. Females reach maturity at 3 years old (around 46 cm length) and males at 2 years old (around 30 cm long). Fecundity is generally over 5 million eggs. Their eggs are pelagic, smooth and spherical, of 1.1 mm diameter and an oil globule of 0.18 mm.

3.3 Aquaculture Production

Aquaculture of turbot first started in Scotland in the 1970s. At the beginning of the 1990s the technological development of juvenile production allowed to expand considerably the production of aquaculture turbot and the number of farms. Turbot is cultured in Spain, France and Portugal but also in Denmark, Germany, Iceland, Ireland, Italy, Norway and Wales. European production has now stabilized around 5 000 tonnes per year. Spain is undoubtedly the world leader of adult turbot production (50% of the total production). Nowadays, fishery and aquaculture share almost equally the market (Danancher and Garcia-Vasquez, 2007). Turbot is identified as one of the most valued marine aquaculture species, with several characteristics that make it an interesting species for commercial growers and European consumers (Foss et al., 2007). These characteristics include: high growth rate and high feed conversion efficiency (Imsland et al., 1995, 1996), high tolerance to stress and handling operations (van Ham, 2003), moderate water quality requirements (Person-Le Ruyet et al., 2003), high adaptability to a range of environmental conditions (Imsland and Jonassen, 2002) and few disease problems (Mulcahy, 2002).

Turbot is almost exclusively reared in land-based intensive production systems (Foss et al., 2007) but can also be grown out in flat-bottomed metal cages (cages floating or

submerged at various levels in the sea). Despite reduced production costs, culture at sea remains less used (Danancher and Garcia-Vasquez, 2007) seeing that accessibility to coastal areas is increasingly limited as tourism and recreational activities develop. Environmentally, nutrient emissions to aquatic ecosystems are of great public concern and must be carefully monitored. In this context, some systems of marine fish production, including for turbot, are moving towards land-based farming, with technological equipment for water re-circulation (Aubin et al., 2006).

4. Thesis Objectives

Micronucleus (MN) assay is one of the most commonly used genotoxicity indicator as it is a simple method to evaluate genetic damage because it enables the detection of chromosome loss and breakage (Fenech, 2000). Micronuclei are found in dividing cells containing chromosome breaks and/or chromosomes unable to travel to the spindle poles during mitosis (Fenech, 2000; Stoiber et al., 2004). They can result from an interaction of chemical, physical or biological agents with nongenomic structures such as the mitotic spindle. Recently, other authors have interpreted other nuclear abnormalities as resulting from analogous damage (Ayllón and Garcia-Vaquez, 2000; Serrano-Garcia and Montero-Montoya, 2001), creating the basis for the erythrocytic nuclear abnormalities (ENA) assay. Thus, nuclear abnormalities such as kidney shaped, lobed, segmented and notched nuclei (Carrasco et al., 1990) along with micronuclei can be counted to assess the exposure to genotoxic contaminants (Marques et al., 2008). ENA assay has been successfully applied to different fish species exposed to various classes of environmental genotoxins (Guilherme et al., 2008).

The evaluation of blood chemistry parameters in animals is a routine and important tool in clinical practices, providing essential information on the physiological status of the animal, and therefore helping the biologist to make proper decisions. Application of blood chemistry monitoring in aquaculture is promising since it has been shown that changes in some parameters correlate to water quality, infectious disease or toxicant exposure (Chen et al., 2003).

The use of plasma cortisol as an indicator of physiological status has been well documented in numerous fish studies (reviewed by Hontela, 1997). Cortisol is involved in energy metabolism regulation, anti-inflammatory response as well as immune competence (Hontela, 1997; Wendelaar Bonga, 1997). Alterations in plasma cortisol, as well as on glucose and lactate levels can reflect endocrine alterations, reducing fish physiological competence and survival (Oliveira et al., 2008). Recent *in vivo* studies demonstrated that some environmental pollutants could act as endocrine disrupters in fish (Van der Kraak et al., 1992), and one of the endocrine targets of xenobiotics seems to be the hypothalamo–pituitary–interrenal (HPI) axis (Hontela, 1998). Acute stress, such as handling and confinement (Iwama et al., 1976), exposure to low pH, heavy metals (Hontela et al., 1995), chlorinated resin acids (Kennedy et al., 1995) and polycyclic aromatic hydrocarbons (PAHs) (Thomas and Rice, 1987) induced a plasma cortisol increase in several fish species.

Changes in carbohydrate metabolism measured as plasmatic glucose and lactate can also be used as general stress indicators in fish (Santos and Pacheco, 1996; Pacheco and Santos, 2001).

With the exception of mammals, the red blood cells (RBC) of vertebrates are nucleated and possess mitochondria as well as the other cellular organelles that exist in normal somatic cells (Wang et al. 1999). Both RBC and hemoglobin (Hb) can be used as stress indicators and in stressful circumstances, an increase in oxygen uptake can occur driving to an increase in RBC count as well as Hb. According to Wang et al. (1999) several studies on salmonids have shown that whole blood oxygen consumption in vitro increases approximately 2-fold within minutes after adrenergic stimulation.

Condition factor and hepatosomatic index (HSI) are calculated as indirect indices of physiological state. Fulton's condition (K) factor is widely used in fisheries and general fish biology studies. This factor is calculated from the relationship between the weight of a fish and its length, with the intention of describing the "condition" of that individual (Nash et al., 2006). HSI can be a significant predictor of energy reserves (lipid, protein, glycogen and total energy).

The well-defined biological meaning and suitability of the previously mentioned parameters for analyzing the health status of fish as well as the lack of information on

ozone toxicity to farmed fish provided the rationale for the present investigation. Hence, this research was designed to assess the ozone effects to juvenile turbot (*Scophthalmus maximus*), under realistic farming conditions, evaluating the genotoxic potential (ENA assay), hematological alterations (Hb levels, RBC count and mean cell hemoglobin - MCH), general stress indicators (plasma cortisol, glucose and lactate) and physiological state indices (K factor and HSI). These parameters were determined in fish subjected to a daily 6-hr ozone exposure, repeated for 3 consecutive days. In order to assess the potential recovery after ozone treatment, fish were also analyzed on 1- and 7-day post-treatment.

The increasing scale of production in intensive aquaculture systems demands accurate and efficient tools to screen and monitor the health status of fish during rearing. In this perspective, it was intended to contribute to the establishment of the safety margins for the use of ozone in RASs, improving aquaculture practices.

Chapter II

MATERIAL AND METHODS

II – MATERIAL AND METHODS

1. Fish and Holding Conditions

Juvenile turbot (*Scophthalmus maximus*), weighing 75.0±1.41g and measuring 18.4±0,87cm, were supplied by Aquacria Piscícolas, SA, Torreira (Portugal). Prior to the experiment described below, fish were acclimatized to the experimental tanks/conditions for two weeks. During acclimatization and experimental periods, fish were kept in 80-L plastic tanks with an effective volume of 40 L of seawater (21 g/L salinity), in a recirculating system, where water parameters were maintained as follows: 17.5±0.3 ºC temperature, 8.3±0.3 mg L^{-1} dissolved oxygen, 0.06±0.08 mg L^{-1} nitrite and 0.13±0.12 mg L^{-1} ammonia. Flow rate was maintained at 0.4 L min^{-1}. Fish were held in an initial density of 14.8 Kg/m^2, under a 09:13 light:dark photoperiod and fed by hand with a commercial dry food (Skretting, Norway), twice a day (10h a.m. and 17h p.m.) to visual satiation. On the days of exposure fish were fed only in the afternoon.

2. Experimental Design and Sampling

The experiment was carried out using triplicate groups of 40 turbot. The number of fish per tank was defined in order to maintain an adequate density up to the end of the experiment, taking into account the *S. maximus* requirements and the successive fish subtraction along the experiment. Fish, kept in the previously described conditions, were subjected to daily 6-hr ozone (0.15 mg L^{-1} measured using DPD colorimetric method measured as Br_2) exposure, starting at 8h a.m., and repeated for 3 consecutive days. In order to assess the potential post-treatment recovery, fish were also collected on day 4 (1 day recovery – R1) and 10 (7 days recovery – R7). A control group was kept under the same experimental conditions but without ozone exposure.

Ozone gas, produced by a commercial ozone generator (Ozonia, Switzerland), was mixed with the seawater using an injector connected to the water circulation system. Ozone concentration in the tanks was monitored at 1-h intervals and the potency of the generator adjusted to provide the desired ozone level.

On sampling dates, four turbot were collected from each replicate tank (control and ozone-treated groups) and measured (to the nearest millimeter) and weighted (to the nearest milligram). Fish were dissected, blood samples were collected with heparinised Pasteur pipettes from the cardinal vein and smears immediately prepared. After that, liver was carefully excised and weighted (to the nearest milligram). After sampling, fish were killed by decapitation.

3. Analytical Procedures

3.1 Scoring Genotoxic Damage

Genotoxicity was tested using the erythrocytic nuclear abnormalities (ENA) assay, carried out in mature peripheral erythrocytes according to the procedures of Schmid (1976), Carrasco et al. (1990) and Smith (1990) as adapted by Pacheco and Santos (1996). Briefly, one blood smear per animal was fixed with methanol during 10 min and stained with Giemsa (5%) during 30 min. From each smear, 1000 erythrocytes were scored under 1000x magnification to determine the frequency of the following nuclear lesions categories: micronuclei (MN), lobed nuclei (L), segmented nuclei (S), kidney shaped nuclei (K) and notched nuclei (N). Blebbed and lobed nuclei were considered in a single category - lobed nuclei - and not differentially scored as suggested by other authors (Guilherme et al., 2008) due to some ambiguity in their distinction. The results were expressed as the mean value (‰) of the sum (MN+L+S+K+N) for all the lesions observed.

3.2 Stress Indicators

These biochemical parameters were determined in plasma obtained by blood centrifugation at 12.000 rpm for 10 min. Plasma samples were stored in polyethylene Eppendorf test tubes at -80 ºC until analyses.

The cortisol quantification was performed using a Gentaur (Belgium) kit. Briefly, cortisol in the sample competes with horseradish peroxidase-cortisol for binding onto the limited number of anti-cortisol sites in the microplate. Cortisol concentration, measured spectrophotometrically (450 nm), is calculated based on a series of standards and the color intensity is inversely proportional to the cortisol concentration in the sample.

Plasma glucose was measured spectrophotometrically (340 nm) according to the method modified from Banauch et al. (1975) based on the quantification of NADH after a glucose oxidation catalysed by the glucose dehydrogenase. The quantity of NADH formed is proportional to the glucose concentration.

Plasma lactate levels were determined spectrophotometrically (340 nm) according to the method modified from Noll (1977) using lactate dehydrogenase (LDH), alanine aminotransferase (ALT) and NAD, measuring the NADH appearance.

3.3 Hematology

Hemoglobin (Hb) was determined by the colorimetric cyanomethemoglobin method using the Drabkin's reagent. The intensity of the color is proportional to hemoglobin concentration and was measured spectrophotometrically at 540 nm.

Red blood cell (RBC) count was carried out using a hemacytometer chamber. Both RBC count and concentration of Hb were immediately estimated after blood sampling.

Mean cell hemoglobin (MCH), representing the absolute amount of hemoglobin in the each red blood cell in the sample, was calculated as follows and expressed as picograms (pg) per cell:

$$\text{MCH (pg)} = [\text{Hb (g/dL)} \times 10] / [\text{RBC count (millions/}\mu\text{L)}]$$

3.4 Physiological state indices

The hepatosomatic index (HSI) was calculated by the following formula:

$$\text{HSI (\%)} = (\text{Liver weight (g)} / \text{Total body weight (g)}) \times 100$$

The Fulton's condition factor (K) was calculated from the relationship:

$$K = \text{Total body weight (g)} / \text{Total length}^3 \text{ (cm)}$$

4. Statistical Analysis

SigmaStat software (SPSS Inc.) was used for statistical analyses. All results are expressed as mean ± standard deviation (SD). Data were first tested for normality and homogeneity of variance to meet statistical demands. The t-test was used to assess the statistical significance of differences between ozone-treated and control groups in each sampling moment. Statistical significance level was set to $P < 0.05$.

Chapter III

RESULTS

III – RESULTS

1. Genotoxicity Assessment

The different categories of erythrocytic nuclear abnormalities that constitute the basis of the ENA assay are presented in figure 1, in parallel with the normal nuclear shape.

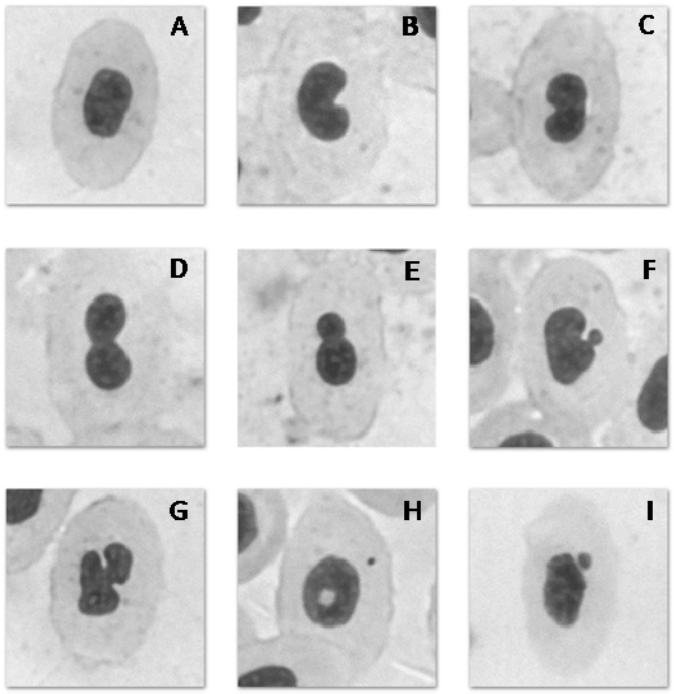

Figure 1 - Mature erythrocytes of turbot (*Scophthalmus maximus*) with nuclear normal shape (A) and nuclear abnormalities: kidney-shaped (B and C), segmented (D and E), lobed (F and G), notched (H) and micronuclei (I). Giemsa stain; 2200 x amplification.

The results of the ENA assay regarded as the sum of all the individual lesions scored are depicted in figure 2. Thus, a statistically significant increase in ENA frequency was observed throughout the experiment in ozone-treated group when compared with the corresponding control group. The maximum ENA level was observed following 3 days exposure, also corresponding to the highest increment degree (around 191 %). Nevertheless, a temporal pattern of variation was not clear and no signals of recovery were perceptible on the first day post-treatment (R1) showing approximately 157 % increase, thought a slight decline on ENA frequency is discernible on the seventh day post-treatment (R7). However, ENA level in ozone exposed turbot in R7 is still significantly higher than the respective control.

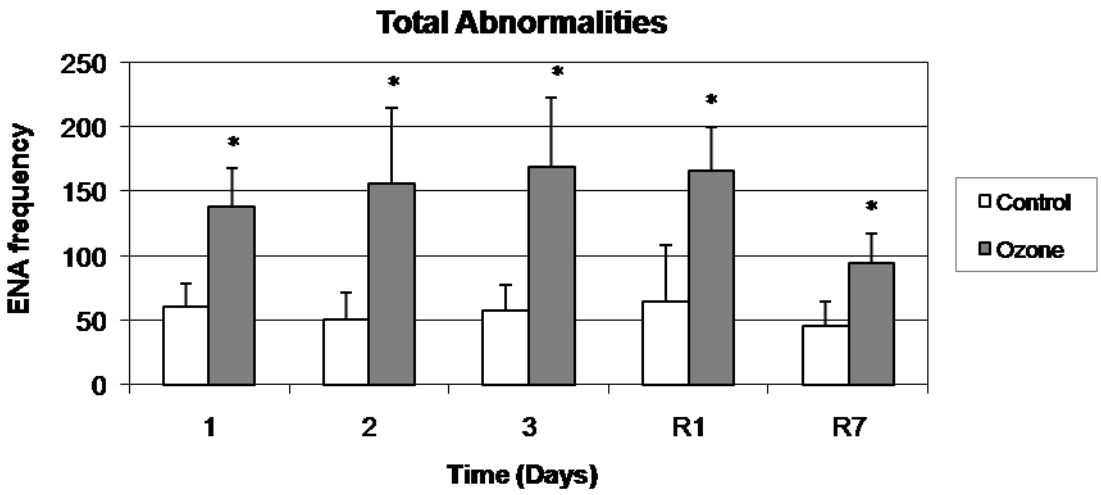

Figure 2 - Mean frequency (‰) of total nuclear abnormalities (ENA) in mature peripheral erythrocytes of turbot (*Scophthalmus maximus*) subjected to daily 6-hr ozone exposure for 3 consecutive days, plus 1-day (R1) and 7-day (R7) recovery. The asterisk (*) denotes statistically significant differences from the control group ($P < 0.05$). Error bars represent the standard deviation.

Table 1 shows the frequency of each lesion individually. In addition, the sum of all the nuclear abnormalities except micronuclei (K+L+S+N), as well as lobed nuclei + micronuclei (L+MN) are also presented.

Both kidney shaped and lobed nuclei displayed significantly increased frequencies in ozone group at all the sampling times, including at R1 and R7. While kidney shaped frequency showed a time-related increase up to day 3 followed by a less pronounced increase on R1 and R7, lobed nuclei reached the maximum level on R1.

Though less frequent than the previous categories, segmented nuclei increased significantly in treated turbot for all the sampling moments, excluding on R7. In this case, the peak in frequency was observed on day 2, followed by a time-related decrease from day 3 to R7 where it was no more different from the control group. Notched nuclei increased significantly on day 2, keeping significantly higher than the control during the recovery period (R1 and R7). Differently, MN frequency significantly increased only on day 2 and maintained somewhat regular and at the control level throughout exposure and recovery periods. A similar pattern of response can be seen in segmented, notched and micronuclei concerning the point in time where the maximum frequency was reached (day 2).

Lobed nuclei presented recurrently along the experiment the highest frequency among the scored nuclear lesions, thereby having the greater contribution to the total or sub-totals estimated. Contrarily, due to their scarcity MN have little influence in the combined data, i.e. in total abnormalities the sub-total L+MN.

Both sub-totals showed significant increases for ozone exposed group throughout the experiment, including on recovery period. Nonetheless, the maximum value was observed on day 3 for K+L+S+N and on R1 for L+MN.

Table 1 – Mean frequency (‰) of each nuclear abnormality category (±SD) in mature peripheral erythrocytes of turbot (*Scophthalmus maximus*) subjected to daily 6-hr ozone exposure for 3 consecutive days, plus 1-day (R1) and 7-day (R7) recovery. The asterisk (*) denotes significant difference from the control group ($P < 0.05$).

Time (Days)	Condiction	Kidney shaped (K)	Lobed (L)	Segmented (S)	Notched (N)	Micronuclei (MN)	Sub-total K+L+S+N	Sub-total L+MN	Total
1	Control	18,83 ± 7,85	30,67 ± 9,73	9,00 ± 5,01	2,08 ± 1,38	0,00 ± 0,00	60,58 ± 18,03	30,67 ± 9,73	60,58 ± 18,03
	Ozone	37,92 ± 11,07*	74,00 ± 17,99*	22,08 ± 11,15*	3,92 ± 2,97	0,25 ± 0,62	137,92 ± 29,30*	74,25 ± 18,11*	138,17 ± 29,69*
2	Control	17,58 ± 10,91	22,83 ± 9,48	9,92 ± 4,46	0,67 ± 1,07	0,00 ± 0,00	51,00 ± 20,07	22,83 ± 9,48	51,00 ± 20,07
	Ozone	40,33 ± 12,63*	85,58 ± 45,20*	22,42 ± 10,89*	6,67 ± 3,47*	0,75 ± 1,06*	155,00 ± 58,45*	86,33 ± 45,36*	155,75 ± 58,59*
3	Control	19,67 ± 6,79	27,08 ± 12,57	8,17 ± 4,37	2,83 ± 2,41	0,08 ± 0,29	57,75 ± 19,12	27,17 ± 12,71	57,83 ± 19,22
	Ozone	40,58 ± 13,77*	103,17 ± 37,49*	19,17 ± 8,74*	4,92 ± 2,81	0,33 ± 0,65	167,83 ± 53,79*	103,50 ± 37,61*	168,17 ± 53,93*
R1	Control	17,25 ± 6,37	38,33 ± 38,88	8,00 ± 3,59	0,67 ± 0,98	0,17 ± 0,39	64,25 ± 43,05	38,50 ± 39,24	64,42 ± 43,41
	Ozone	27,50 ± 13,07*	115,83 ± 28,41*	16,25 ± 6,72*	5,17 ± 2,55*	0,58 ± 0,90	164,75 ± 34,00*	116,42 ± 28,22*	165,33 ± 33,74*
R7	Control	12,08 ± 3,87	24,92 ± 11,77	7,58 ± 5,63	1,00 ± 1,21	0,00 ± 0,00	45,58 ± 18,90	24,92 ± 11,77	45,58 ± 18,90
	Ozone	18,33 ± 7,28*	63,00 ± 17,72*	8,58 ± 4,66	3,75 ± 1,76*	0,25 ± 0,45	93,67 ± 23,37*	63,25 ± 17,81*	93,92 ± 24,46*

2. Stress Indicators

According to figure 3a, plasmatic concentrations of glucose increased significantly only on the post-treatment period (R1 and R7) and no statistically significant differences were observed during the ozone exposure notwithstanding on day 3 the glucose concentration appeared substantially high in the ozone group. Moreover, the fish hyperglycaemic response seems to be accentuated from R1 to R7, as the plasma glucose rise was around 75 % and 118 %, respectively.

Concerning plasma lactate concentration, lower levels were regularly found in ozone treated fish in relation to the control, even though statistically significant differences were recorded only on the first day of treatment and on R1 (Figure 3b). Taking into consideration these significant alterations, it is important to highlight that the decrease extent was higher in R1 (approximately 62 %) when compared to the first day of exposure (approximately 50 %).

Plasmatic levels of cortisol revealed a significant elevation on ozone group following 1 day exposure (Figure 3c), representing an 1828 times increment in comparison with the control level. Afterwards no significant alterations were recorded up to the end of the experiment, though a considerable cortisol increase was discernible on R7.

3. Hematology

The Hb concentration was significantly increased in ozone exposed turbot on days 1 and 3, as well as after 1 day recovery (R1) where it reached the maximum value (increase percentage around 42 %) (Figure 4a). After 7 days recovery (R7), no difference was found on Hb levels between control and treated groups.

Ozone treated fish revealed significant increases in RBC count on days 2 and 3, followed by a prolonged effect on R1 (Figure 4b). Similarly to Hb response, RBC count in ozone group completely recovered to the control level on R7 (Figure 4b).

There were no significant alterations on MCH in the course of both exposure and recovery periods as observed in figure 4c.

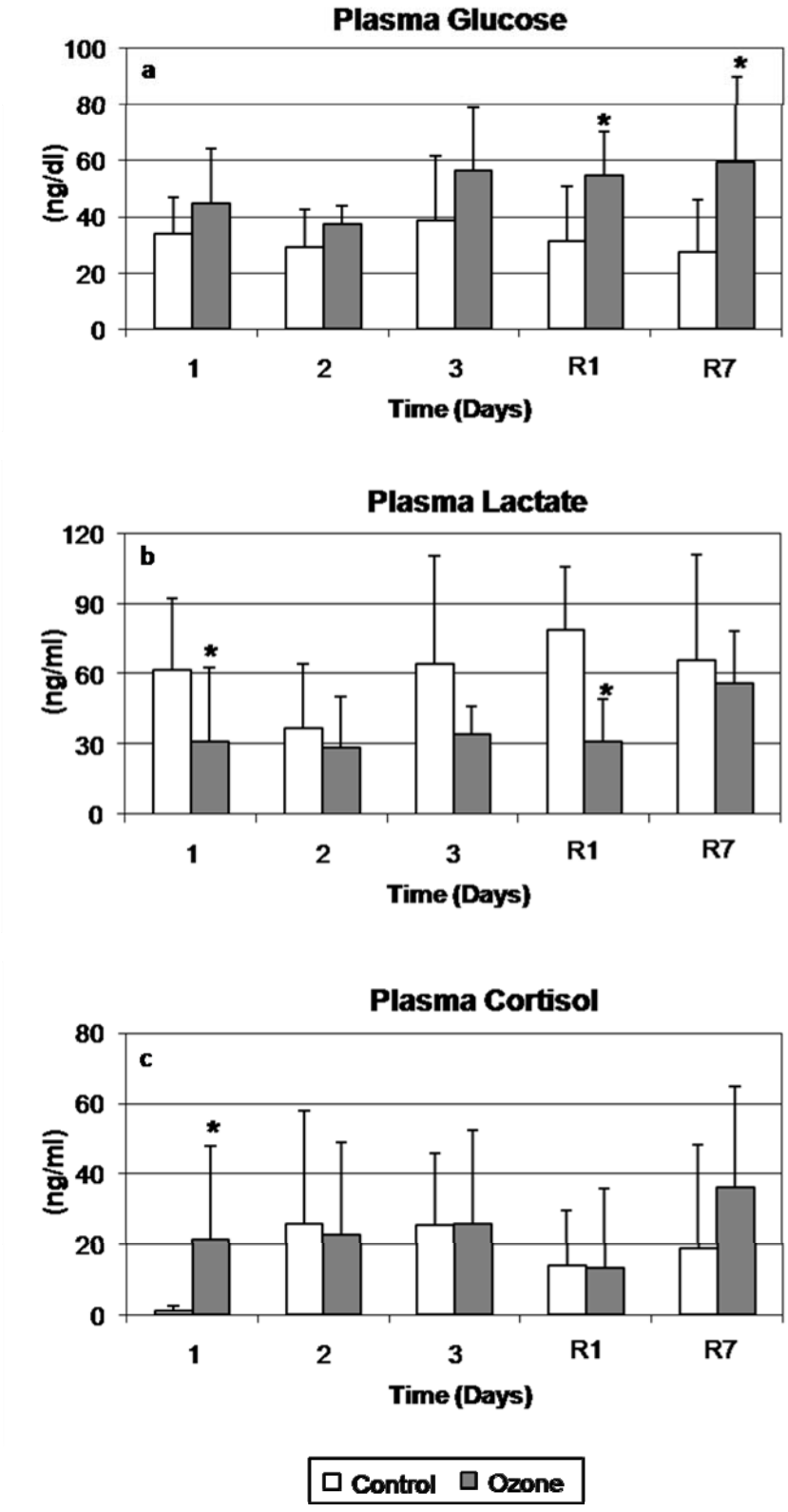

Figure 3 - Mean concentrations of plasma glucose (a), lactate (b) and cortisol (c) in turbot (*Scophthalmus maximus*) subjected to daily 6-hr ozone exposure for 3 consecutive days, plus 1-day (R1) and 7-day (R7) recovery. The asterisk (*) denotes statistically significant differences from the control group ($P < 0.05$). Error bars represent the standard deviation.

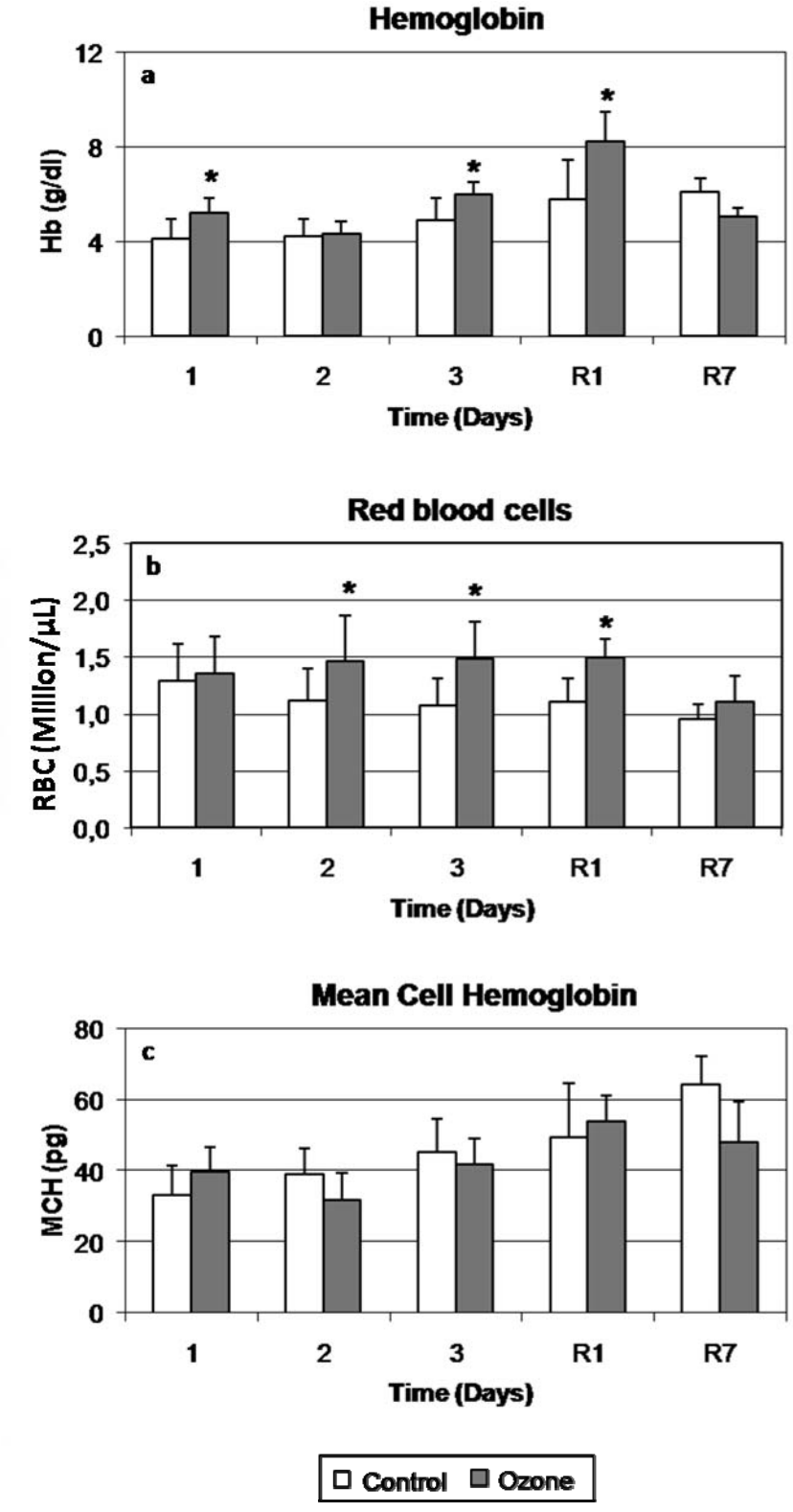

Figure 4 - Mean concentration of hemoglobin (a), red blood cells count (RBC) (b) and mean cell hemoglobin (MHC) (c) in turbot (*Scophthalmus maximus*) subjected to daily 6-hr ozone exposure for 3 consecutive days, plus 1-day (R1) and 7-day (R7) recovery. The asterisk (*) denotes statistically significant differences from the control group ($P < 0.05$). Error bars represent the standard deviation.

4. Physiological State Indices

According to figure 5a, there were no statistically significant alterations on Fulton's condition factor (K). In addition, no variation pattern could be detected.

Similarly, in relation to hepatosomatic index (HSI) there were no significant alterations in the course of both exposure and recovery periods, as observed in figure 5b.

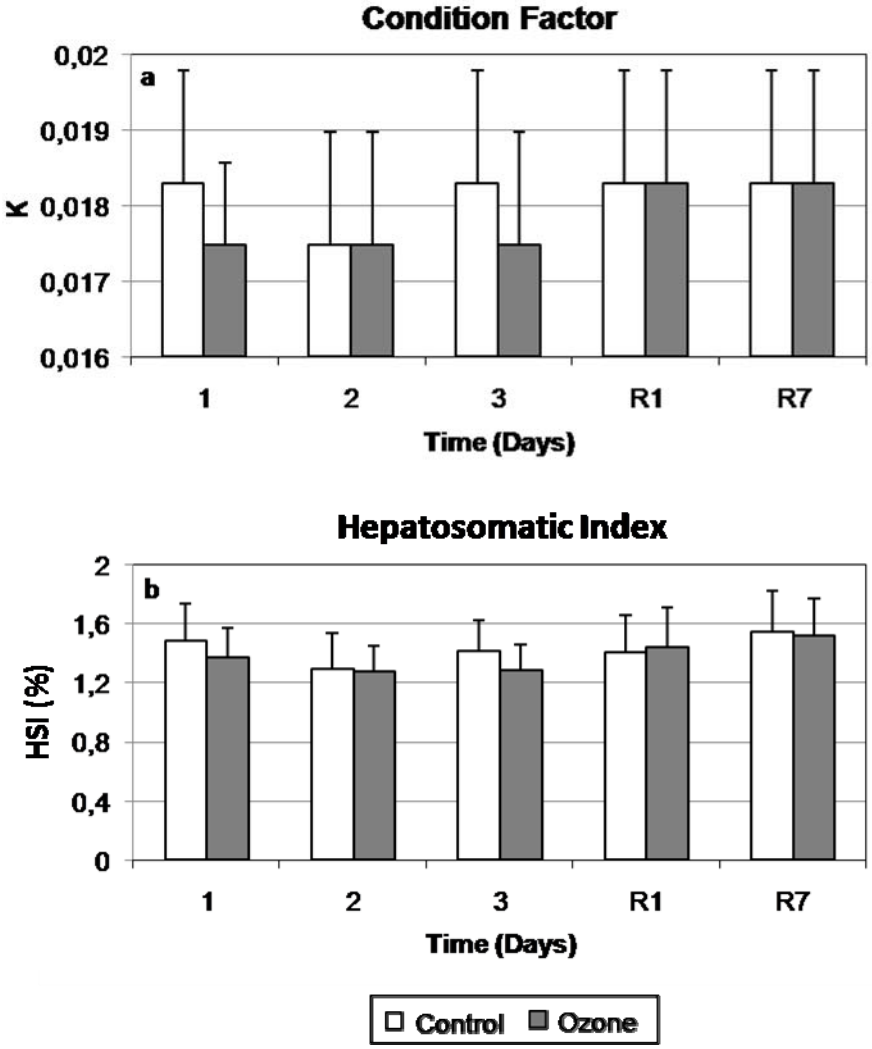

Figure 5 - Fulton's condition factor (K) (a) and hepatosomatic index (HSI) (b) in turbot (*Scophthalmus maximus*) subjected to daily 6-hr ozone exposure for 3 consecutive days, plus 1-day (R1) and 7-day (R7) recovery. Error bars represent the standard deviation.
No mortality occurred during the experiment in both control and exposed groups.

Chapter IV

DISCUSSION

IV – DISCUSSION

The vast majority of existing literature on ozone-induced toxicity, genotoxicity, and carcinogenicity concerns mammal systems due to the inherent apprehension caused in the context of human health. Ozone biological effects are mainly attributed to its ability to cause oxidation or peroxidation of biomolecules directly and/or via free radical reactions (Mustafa, 1990).

To the authors' knowledge, limited information is available on the effects of ozone or its byproducts on fish, namely concerning genetic damage and physiological alterations, which highlights the novelty of the present study. Consequently, the present discussion will be done taking into consideration the available literature including studies with non-fish systems.

Both cytogenetic and hemato-biochemical parameters were assessed taking blood as a key target tissue on fish exposure to ozonated water, due to its function as a vehicle between gill - the main uptake pathway - and other target organs. In addition, blood can also reflect the status of other tissues/organs. In this direction, a study carried out by Kilemade et al. (2004), using comet assay, demonstrated that turbot blood is a suitable predictor of DNA damage in the whole organism. Furthermore, changes in the biochemical blood profile mirror changes in metabolism and biochemical processes of the organism.

The tested ozone level and the exposure duration were selected on a realistic basis, taking into account the aquaculture practices in RASs, and particularly in turbot (*Scophthalmus maximus*) farming.

1. Genetic Damage

DNA damage is constantly induced and repaired, leading to a steady-state process. Thus, genotoxicity observation indicates that the equilibrium has been shifted towards DNA damage accumulation, either through an increase in the number of DNA-damaging events or a decrease in DNA repair (Vock et al., 1998). The exposure of living organisms to certain xenobiotics may generate reactive metabolites that can interact with DNA forming adducts, and/or reactive oxygen species (ROS) (redox cycling routes) capable of inducing single or double DNA strand breaks (Oliveira et al., 2007).

Genetic damage have been evaluated by different methodologies, namely by the DNA alkaline unwinding assay (Rao et al., 1996; Maria et al., 2002) and single cell gel electrophoresis (known as Comet assay) (Çavaş and Könen, 2008), that demonstrated its suitability on genotoxicity assessment in fish. Nevertheless, these methodologies fail to account for DNA excision processes.

In this perspective, micronucleus (MN) assay can constitute a suitable complementary indicator. Micronucleus, caused either by a clastogenic action or by abnormal spindle formation, is only expressed in dividing cells, providing a convenient and reliable index of both chromosome breakage (for which double-strand breaks is a prerequisite) and chromosome loss (Fenech, 2000). Additionally, single-strand breaks can be converted to double-strand breaks if the replication machinery leaves an interrupted daughter strand. It is assumed that other nuclear abnormalities can provide an additional and complementary measure of chromosome damage and rearrangement (Fenech, 2000), which can be scored together with MN and expressed as total nuclear abnormalities frequency, being the basis for the erythrocytic nuclear abnormalities (ENA) assay (Pacheco and Santos, 1996). Although the mechanisms underlying the formation of nuclear abnormalities have not been fully explained, several studies indicate that they are induced in response to exposure to genotoxic agents (Çavaş and Ergene-Gözükara, 2005). Moreover, according to Serrano-Garcia and Montero-Montoya (2001), budding cell nuclei, which include lobed nuclei and bi-nucleated cells, have a similar origin as MN.

As pointed out by different authors (Flyunt et al., 2002; Cataldo, 2006), the action of ozone on DNA and RNA molecules have important implications in the disinfection mechanism exerted by ozone in water treatment. In fact, both bacteria and viruses result damaged and inactivated because the oxidizing action of ozone is directed toward the nucleic acids of these organisms (Theruvathu et al., 2001). Surprisingly, the effects of ozonated water on non-target organisms, namely fish raised in RASs, remain almost completely unknown. To the best of our knowledge, this is the first study on ozone genotoxicity in fish, keeping in view the assessment of the accuracy of aquaculture practices as well as the risk to biota associated to ozonated water/effluent discharges on the aquatic environment.

Using an *in vitro* ozone exposure system, Fukunaga et al. (1999) showed the ozone ability to penetrate through the membrane of rainbow trout (*Oncorhynchus mykiss*) RBC, causing oxidative stress inside the cell induced by ROS, namely ozone-derived H_2O_2 and $OH^{\bullet}$. Therefore, the oxidation of DNA mediated by ROS is a likely mechanism for ozone genotoxicity.

On the other hand, the ozone by-products (OBP) are also of concern. The genotoxicity and carcinogenicity of disinfection by-products formed when disinfectants, including ozone, react with naturally occurring organic matter, anthropogenic contaminants, bromide, and iodide during the production of drinking water were criteriously reviewed by Richardson and coworkers (Richardson et al., 2007). When a powerful oxidant such as ozone is introduced into seawater, it reacts with a wide array of compounds, and new oxidants are formed; some of them may also react with biomolecules altering its functional properties (Grotmol et al., 2003). It is well documented that

OBP, such as bromate, are of great concern since it have been shown to be a carcinogen in laboratory animals (Kurokawa et al., 1986; DeAngelo et al., 1998; Delker et al., 2006). Bromate is produced primarily by ozone disinfection when source waters contain high levels (>50 mg/L) of natural bromide. There is also a significant body of work on mammal systems that is highly suggestive that bromate causes DNA damage secondary to oxidative stress from intracellular bromate (Sai et al., 1991, 1992a, b, 1994; Umemura et al., 1998), presenting it as a potent oxidative mutagen.

Concerning the current results, the ENA assay showed the existence of a notably higher frequency of nuclear abnormalities in erythrocytes of ozone exposed *S. maximus* when compared to unexposed fish. The appearance of nuclear lesions can be determinately affected by a variety of factors such as erythropoiesis, time required for maturation and the lifespan of the erythrocytes (Udroiu, 2006). Considering that the ENA frequency did not return to the control level after 7 days without ozone exposure, it is evident a prolonged effect and the permanence of genotoxic conditions into the fish body resulting from the balance between the previous factors.

Analyzing each lesion individually, it must be noted that all categories were able to detect the effect of the ozonated water, though different patterns could be observed, namely as a function of the time. Thus, the frequency of kidney shaped, lobed and notched nuclei didn't recover up to the control level in the post-treatment period. Differently, the frequency of segmented and MN showed the ability to better recover after the end of the genotoxic stimulus. However, the responsiveness of segmented and MN is not comparable since the last type of lesion showed to be the less responsive among all the categories scored. Overall, the results advise the use of total abnormalities frequency, rather than a single type of lesion individually, in order to improve the suitability of the assessment.

The clastogenicity currently observed in ozone exposed turbot is in agreement with a study carried out on salmon sperm where cells subjected in vitro to ozonolysis showed high DNA degradation (Cataldo, 2006). In addition, the results also agree with previous studies demonstrating potassium bromate as a clastogen in human cell culture systems through sister chromatid exchange (SCE), chromosomal aberrations and MN tests (Ballmaier and Epe, 2006; Kaya and Topaktaş, 2007), as well as *in vivo* induction of MN in rats (Sai et al., 1992b). Nonetheless, it is not possible to determine if the effects observed in the present turbot exposure are related to an effect of ozone (directly or via ROS overproduction) or to any by-product (e.g. brominated species).

In an attempt to evaluate the implication of this genetic damage on fish fitness and subsequent repercussion of aquaculture productivity, it can be hypothesized a negative impact on fish growth. In this direction, it was previously demonstrated in salmonid larvae (brook trout, rainbow trout) that a reduced growth can result from the costs for repairing DNA damage involving an energetically expensive nucleotide excision repair (Olson and Mitchell, 2006). On the other hand, it was demonstrated that, in the presence of a prolonged elevation of ENA frequency, fish spleen can respond by increasing the rate of erythrocytes removal (Pacheco and Santos, 2002). This splenic response can affect the hematological dynamics and fish metabolism as the organism tend to compensate cell elimination with and increased erythropoiesis. So far, a linkage between an elevated frequency of erythrocytes with abnormal nuclear morphology and a physiological dysfunction, namely in terms of gas transport capacities, was not demonstrated. Nevertheless, the mentioned changes in the metabolism, taken together, could markedly decrease the portion of energy allocated to growth in fish exposed to genotoxic agents.

In a further perspective, the risk to wild fish populations inhabiting aquatic ecosystems receptors of ozone treated effluents cannot be ignored due to the known association between the occurrence of genetic damage and the development of neoplastic lesions.

2. Stress Indicators

In teleost fish, the endocrine response to stress is composed of the brain-sympathetic-chromaffin (BSC) and hypothalamic–pituitary–interrenal (HPI) axes responses (Wendelaar Bonga, 1997). The HPI response to acute exposures to stressors leads to an increase in glucocorticoid levels, of which cortisol, produced in the interrenal cells of the head kidney (homologous to mammalian adrenal tissue), is the most important. In this context, plasma cortisol is an excellent indicator of functional alterations in the HPI axis (Hontela, 2005) and thus a useful stress biomarker.

Fish carbohydrate metabolism may also be altered through exposure to a variety of stressors, being regarded as secondary acute stress responses, modulated by the previous endocrine pathways. In this direction, a typical stress response includes plasma glucose and lactate increase (Hontela et al., 1996; Santos and Pacheco, 1996; Sancho et al., 1997). These responses have an adaptive value since they augment the availability of energy substrates necessary for homeostasis maintenance (Donaldson et al., 1984). Taking together, plasmatic concentrations of cortisol, glucose and lactate are considered among the most important stress indicators in fish.

The plasma cortisol elevation observed in turbot following 6-hr exposure (day 1) to ozonated water can be regarded as a characteristic stress reaction (primary stress response). However, this effect showed to be reversible as cortisol rapidly return to control levels (on day 2), pointing out cortisol increase as an early alarm reaction. This is in agreement with Mommsen et al. (1999) who stated that under acute stress situations, plasmatic cortisol levels tend to increase within a minute to hour time frame, being followed by a gradual decrease to pre-treatment levels within a day or so, depending upon subsequent maintenance conditions. Anyway, despite the substantial evidence for elevated plasma cortisol in fish acutely subjected to a wide range of stressors (Vijayan et al., 1997; Teles et al., 2005, 2007; Oliveira et al., 2007), effects of longer and low-level exposures are still poorly understood. According to Hontela (1998), it has not been clearly established that a decrease in plasma cortisol after long exposure, following an initial cortisol peak, reflects a complete acclimation to the chemical stressor or an exhaustion of the hormonal secretion systems. Considering the short duration of the cortisol increase stage, the present results strengthen the hypothesis of turbot acclimation in relation to the HPI axis response to ozonated water exposure.

Plasma glucose increase is a classic response of fish to cope with stress. Hence, it seems surprisingly the absence of significant plasma glucose elevations during the exposure period coupled with the occurrence of a hyperglycemic response only on the post-treatment period. This pattern of response seems to indicate a physiologic adjustment during post-treatment phase, probably as a response to a blocking in this secondary stress response during the ozone exposure. Therefore, turbot seems to exhibit a delayed mobilization of glycogen reserves and a carbohydrate metabolism modification, possibly through a higher turnover and use of glucose by tissues challenged by ozone and/or its by-products.

The rise of plasma lactate concentration is also a common manifestation of stress in fish as an outcome of the intense anaerobic muscular activity (Van-Raaij et al., 1996). Therefore, most authors report hyperlactacidemia in various fish species following acute exposure to different stressors (Sancho et al., 1997; Teles et al., 2005). On the contrary, a lactate-lowering effect of ozonated water was presently observed in exposed turbot (day 1 and R1). This effect may be indicative of a decrease in lactate production and/or an increase in lactate removal. Lusková et al. (2002) attributed lactate decrease induced by the pesticide diazinon in *Cyprinus carpio* to a reduction in the glycolytic process due to the lower metabolic rate. It was also demonstrated in mammal systems that a lactate-lowering effect may be related to a greater flux of pyruvate from the glycolytic pathway to the tricarboxylic acid cycle, which results in a decrease in lactate production (Mercier et al., 1994). Accordingly, a compensatory increase of the aerobic capacity of fish was observed in response to cold acclimation (Guderley, 1990). This explanation gains plausibility in

relation to the reduced metabolic rate by analyzing the results of Hb and RBC count (discussed below) that are suggestive of increased oxygen availability.

Van-Raaij et al. (1996) stated that hyperglycaemia is probably a result of liver glycogenolysis stimulated by catecholamins and of gluconeogenesis stimulation by cortisol. Analyzing in parallel glucose and cortisol responses of turbot, it seems improbable a cortisol-induced gluconeogenesis as no cortisol elevation was found concomitantly or closely preceding the glucose rise. Hence, it is reinforced the statement of Van Der Boon et al. (1991) that cortisol influence on fish carbohydrate metabolism is not very comprehensive, since glucose is not the most important fuel in fish energy metabolism. In fish, other mechanisms besides interrenal cortisol release may be controlling glucose availability; according to Vijayan et al. (1997), under acute stress, catecholamines could be rapidly released resulting in increased glycogenolysis. Thus, though not evaluated in the present study, catecholamins seems to be on the basis of the measured hyperglycaemia. On the other hand, the concomitance of lactate decrease and glucose increase in R1 raises a doubt about the use of lactate on liver gluconeogenesis processes as stated by Oliveira et al. (2008). Contrarily to the present findings, Chen et al. (2003) found no changes in blood chemistry, including plasma glucose, in Nile tilapia (*Oreochromis niloticus*) after ozone treatment.

Chronic elevation of cortisol level is found to change feeding behavior, carbohydrate metabolism and growth of fish (de Oliveira Fernandes and Volpato, 1993; Gregory and Wood, 1999). Furthermore, the elevation of plasma cortisol can decrease the growth hormone levels (Pickering et al., 1991). On the other hand, chronic stress and consequent cortisol increase is an effective suppressor of immunity, and can lead to decreased immune competence in fish. Previous studies have demonstrated that stress reduces lymphocyte proliferation and impairs antibody responses (Prophete et al., 2006), ultimately leading to an enhanced susceptibility to microbial agents of the water environment. Therefore, taking into account the short duration of plasma cortisol rise detected in the current study, the potential for ozonated water to increase the susceptibility to diseases and reduce growth performance in turbot is not likely on the basis of cortisol effects. However, bearing in mind the plasma glucose increase as a prolonged effect observed along 7 days recovery, the occurrence of a depletion of energy reserves in turbot should be strongly considered.

3. Hematology

The ability of an organism to acquire O_2 from its environment is key to survival and play an important role in dictating organism' adaptation competence to unfavorable conditions. As a response to the ozone exposure, turbot carried out physiological and hematological adjustments involving an improvement of gas transport capacity. This alteration aims to ensure an appropriate O_2 supply to the different tissues, including aerobic muscles, in order to maintain the capacity for continued swimming following glycolytic exhaustion of the white muscle. According to Milligan and Wood (1987), this effect can be related to catecholamines released into the circulation after stressful circumstances. These alterations on hematological status and associated modifications to the respiratory cascade can influence the energy metabolism profile (as discussed above). Nonetheless, the return of Hb concentration and RBC count to the control levels after 7 days recovery was not accompanied by the regularization of plasma glucose concentration.

The increased Hb concentration measured in the blood of exposed turbot seems to be attributable to a higher number of RBC, since its count was concomitantly increased on day 3 and R1 and no alterations were found on MCH levels. Thus, the results are indicative of an improved erythropoiesis rate induced by the ozone exposure, which is in agreement with Wedemeyer et al. (1979) who found increased hematocrit in rainbow trout (*Oncorhynchus mykiss*) exposed to ozone.

Previous studies established that the primary target of ozone toxicity for fish was not the gill but rather RBC (Fukunaga et al., 1991, 1992a). In a later *in vitro* study with *O. mykiss* RBC (Fukunaga et al., 1999), it was demonstrated that ozone exposure induces cell membrane lipid peroxidation and hemolysis. Moreover, it was verified the role of H_2O_2 generation in mediating RBC damage as a consequence of ozone diffusion inside of the cell. However, this hemolytic action of ozone was not confirmed by current results as RBC count was significantly increased in ozone-treated turbot.

4. Physiological State Indices

Condition factor (K) and hepatosomatic index (HSI) are indirect measures of energy status. Though they can be affected by a number of factors (e.g. sex, seasons, environmental conditions, stress, and availability of food), K factor and HSI can provide valuable information concerning the health and general well-being of fish.

In the present study, none of the estimated physiological state indices showed significant alterations. According to the present K factor values, the exposure to ozonated water did not affect

feed uptake and conversion. Similarly, the unaltered HSI revealed no signs of hypertrophy (increase in size) or atrophy (decrease in size) of the liver. Assuming that the observed plasma glucose increases are related to an incremented mobilization of energy substrata reserves (glycogen or others) stored in the liver, it would be expectable a decrease on HSI. The lack of a correspondence between metabolic alterations and HSI values is probably related with the short duration of the performed experiment, also pointing out some limitations of this index as an indicator of physiological state. This is in agreement with Chellappa et al. (1995) who stated that HSI is a poor predictor of energy reserves. The HSI values found for turbot are among the normal values (1 – 2%) defined for teleost fish (Munshi and Dutta, 1996).

Despite evidences provided by the above discussed parameters towards the occurrence of genotoxicity and hemato-biochemical alterations and subsequent negative impact on turbot growth, K factor did not confirm that possibility. However, to accurately assess the influence of ozone exposure on fish growth, altering K factor, longer exposures need to be carried out.

Chapter V

FINAL REMARKS

V – FINAL REMARKS

The present study revealed that the exposure of juvenile turbot (*Scophthalmus maximus*) to ozone for 6 h/day for three consecutive days promote hematological, biochemical and physiological alterations as well as genotoxic effects.

A significant induction on erythrocytic nuclear abnormalities (ENA) frequency was detected as a measure of genetic damage. Moreover, this clastogenic effect was prolonged beyond the exposure period. Further research is required to elucidate the mechanisms involved on DNA damage induced by ozonated water on fish, namely assessing antioxidant defenses and peroxidative damage.

The results clearly point out an ozone-induced stressful condition, expressed by an early plasma cortisol increase and a late hyperglycemia (post-treatment period). The lactate-lowering effect observed may constitute a compensatory increase of the aerobic capacity of fish, which is in line with the apparent improvement in oxygen transport expressed by increased hemoglobin level and red blood cells count. Nevertheless, the establishment of a consistent relation between plasma cortisol, glucose and lactate seems difficult.

The metabolic costs associated with DNA repair and detoxification of ozone and/or ozone by-products, as well as an increased expenditure of energy to sustain fish metabolism, allowing less energy for growth, can have negative repercussions on turbot growth performance.

Taking into account the overall data, it was demonstrated that juvenile turbot are not able to fully adapt to ozonated water, under realistic conditions considering the tested ozone levels and the exposure duration, and the interference with fish health is hypothesized.

The present findings contribute to a biological based knowledge of ozone toxicity and to the establishment of safety margins in aquaculture practices adopted in recirculation systems, promoting sustainability and fish welfare.

Though the present study was designed to assess the impacted of ozonated water in the context of recirculation aquaculture systems (RASs), the risks to wild aquatic populations resulting from discharges of ozone primary-treated effluents (municipal and industrial) cannot be overlooked. In this regard, the genotoxic potential demonstrated can assume particular importance due to causal linkage with malformations and neoplastic lesions.

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