

DOKUZ EYLÜL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

**MULTI-BIOMARKER RESPONSES OF HEAVY
METALS IN FISH SPECIES FROM İZMİR BAY**

by
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İZMİR

MULTI-BIOMARKER RESPONSES OF HEAVY METALS IN FISH SPECIES FROM İZMİR BAY

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**by
Mustafa BİLGİN**

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Ph.D. THESIS EXAMINATION RESULT FORM

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MULTI-BIOMARKER RESPONSES OF HEAVY METALS IN FISH SPECIES FROM İZMİR BAY

ABSTRACT

Biomarkers (superoxide dismutase, catalase, malondialdehyde and metallothionein) were investigated to evaluate the bioaccumulation of metals in the organs of fish species (*Sparus aurata*, *Chelon labrosus*, *Diplodus vulgaris*) in the İzmir Bay. The fish were collected from the Inner and Outer Bays in 3 periods, in November 2019, November 2020 and May 2021. Metal analyzes were carried out with Atomic Absorption Spectrometer and biomarker analyzes were made with a microplate reader. Depending on the metal and biomarker measurements found in the organs, the levels in the liver and gills tissues were observed to be higher than in the muscle tissues. According to statistical results, significant differences were found between tissues for all metal and biomarker parameters. In addition, it was determined that biomarkers responded significant answers for metal bioaccumulations. Although the metal levels determined according to the health risk assessment were below the threshold limit values, the As levels for the lifetime cancer risk were determined within the limits that should be considered. This study revealed that the combined use of biomarkers and metal concentrations can be an important tool for investigating the health status of marine ecosystems. In conclusion, this thesis will provide robust findings for its related study field.

Keywords: Catalase, superoxide dismutase, malondialdehyde, metallothionein, metal pollution, *Sparus aurata*, *Chelon labrosus*, *Diplodus vulgaris*, İzmir Bay, health risks

İZMİR KÖRFEZİ'NDEKİ BALIK TÜRLERİNDE AĞIR METALLERE KARŞI ÇOKLU BİYOIŞARETÇİ TEPKİLERİ

ÖZ

İzmir Körfezi'nde balık türlerinin (*Sparus aurata*, *Chelon labrosus*, *Diplodus vulgaris*) organlarındaki metallerin biyobirikimini değerlendirmek için biyoişaretçiler (süperoksit dismutaz, katalaz, malondialdehit ve metallotiyonein) araştırılmıştır. Balıklar İç ve Dış Körfezlerden Kasım 2019, Kasım 2020 ve Mayıs 2021 olmak üzere 3 örnekleme döneminde toplanmıştır. Metal analizleri Atomik Absorpsiyon Spektrometresi ile, biyoişaretçi analizleri ise mikropilok okuyucu ile yapılmıştır. Organlarda bulunan metal ve biyoişaretçi ölçümlerine bağlı olarak karaciğer ve solungaç dokularındaki seviyelerin kas dokularına göre daha yüksek olduğu gözlemlendi. İstatistiksel sonuçlara göre, tüm metal ve biyoişaretçi parametreleri için dokular arasında önemli farklılıklar bulundu. Ayrıca biyoişaretçilerin metal biyobirikimleri için anlamlı cevaplar verdiği belirlendi. Sağlık risk değerlendirmesine göre belirlenen metal seviyeleri eşik sınır değerlerin altında olmakla birlikte, yaşam boyu kanser riski için As seviyeleri dikkat edilmesi gereken limitlerde tespit edildi. Bu çalışma, biyoişaretçilerin ve metal konsantrasyonlarının birlikte kullanımının deniz ekosistemlerinin sağlık durumunu araştırmak için önemli bir araç olabileceğini ortaya koydu. Sonuç olarak, bu tez, daha sonraki araştırmalarda ilgili çalışma alanı için sağlam bulgular sağlayacaktır.

Anahtar kelimeler: Katalaz, süperoksit dismutaz, malondialdehit, metallotiyonein, metal kirliliği, *Sparus aurata*, *Chelon labrosus*, *Diplodus vulgaris*, İzmir Körfezi, sağlık riskleri

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CHAPTER 1

INTRODUCTION

1.1 Introduction

Many metabolic reactions in living cells provide to the formation of free radicals. Also, heavy metals, pesticides, radioactive substances and contaminated water are caused forming free radicals in organisms. Aerobic organisms have enhanced antioxidant defense to control the free radical formation and to prevent the harmful effects of these molecules. In some cases, the antioxidant defense systems cannot completely prevent the effect of free radicals and “oxidative stress” occurs.

Antioxidant enzymes (such as superoxide dismutase, catalase, glutathione peroxidase) have ability to capture and stabilize free radicals. Primary antioxidants react with existing radicals to form harmless forms and prevent the formation of new free radicals. At the same time, living cells contain enzymes that constitute the defense system against oxidative damage. Determining only the chemical concentrations on the organisms do not reveal the in vivo effects of the pollutants. In order for these chemical analyses to be significant, it is also necessary to research for biomarkers that indicate organisms are exposed to toxic substances.

Using biomarkers and biological effects for observing marine pollution has become increasingly important in recent years. Biomarkers (such as metallothionein, malondialdehyde, antioxidant enzymes) are the indicators that chemical pollutants make some changes at the cellular or molecular level in cells (Nesto, Romano, Moschino, Mauri & Da Ros, 2007) Biomarkers act as early warning systems because of environmental changes in the organism that react to the resulting free radicals (Gérard-Monnier et al., 1998; Viarengo, Lowe, Bolognesi, Fabbri & Koehler, 2007).

Natural sources have been under influence of chemicals released by several anthropogenic activities for decades. Many inorganic and organic pollutants have reached the environment. In 1960s, scientific communities had been realized the

potential threat to the terrestrial and/or aquatic environments (van der Oost, Beyer & Vermeulen, 2003).

It's known that, İzmir Bay is heavily polluted by anthropogenic and industrial activities. Marine organisms can considerably accumulate pollutants such as metals. Heavy metals with toxic effects can reach humans through the food chain. The main of this work is the study of the relationships between metals and biomarkers and its utility as metallic pollution biomarkers. In this context, the heavy metal concentrations and enzyme activities of biomarkers in fish species from İzmir Bay were researched in this thesis.

The aims of this thesis are:

- Metal concentrations and biomarker enzyme activities dataset for fish species were formed in especially Inner and Outer Bays where the extensive fishing occurs.
- Metal concentrations were determined in fish species in İzmir Bay. These concentrations were compared with the limits established by specific authorities and whether they carry a potential risk to human health.
- Biomarker measurements were performed to monitor the effects of inorganic pollutants on the organisms in İzmir Bay.
- Relationships that occur combined using of metal pollutions and biomarker measurements were evaluated.
- Temporal, spatial, tissues and species-specific variations on metal concentrations and biomarker enzyme were monitored.

1.2 Previous Studies

Many studies have been carried out about metal pollution and their impacts on marine environment for decades (Ghedira et al., 2010; He et al., 2012; Solé et al., 2010). However, there are less studies about biomarkers in this study area. Here are

some examples of pollutant related biomarker studies using organisms from İzmir and Türkiye:

Arinç, Sen & Bozcaarmutlu (2000) had been investigated cytochrome P4501A isozyme, which is associated with 7-ethoxyresorufin (EROD) in fish species to evaluate organic pollutants. According to the results, similar trends had been observed with increased biomarker results and elevated concentrations of pollutants for the contaminated sites.

Bozcaarmutlu, Sapmaz, Aygun & Arinç (2009) researched pollution status by using biomarkers on fish species. Results expressed that mullets from polluted areas have been showed 6-9 times higher EROD activity, 2-4 times higher glutathione-S-transferase, and 2-3 times higher catalase activity in comparison with reference site. Determined polyaromatic hydrocarbon levels had been supported biomarker results.

Kucuksezgin et al. (2008) had been investigated possible relationships between biomarkers and trace metals in *Mytilus galloprovincialis* from Inner and Middle Bays of İzmir Bay. Metal levels had been found higher in Inner Bay. Also, superoxide dismutase and trace metals had showed positive correlation while glutathione peroxidase and metals have been showed negative correlations.

Uluturhan et al., (2019) had been investigated seasonal variations of biomarkers in relationship with metals and pesticides pollution in bivalve species (*Mytilus galloprovincialis* and *Tapes decussatus*) from Homa Lagoon. Higher levels of metals had been reported on hepatopancreas tissues to compare with soft tissues. For *M. galloprovincialis*, highest levels of biomarkers had been found in January and for *T. decussatus* highest levels of AChE and CAT activities were detected in November. Species-specific physiologies and environmental conditions of bivalve species might have been affected seasonal variations of pollutants and biomarkers.

Çakal Arslan, Boyacioğlu, Parlak, Katalay & Karaaslan (2015) researched biological damages related to pollution in fish species from Aliğa Bay. Micronuclei,

a genotoxicological biomarker, had been utilized for the assessment. Samples were collected from polluted and relatively low polluted areas from the Bay. Result demonstrated that micronuclei frequencies had been found at higher levels in polluted areas. Therefore, this publishing had been demonstrated that micronuclei might be used on pollution monitoring studies.



CHAPTER 2

TRACE METALS

2.1 Metals and Metalloids

Metals are the materials that electron donor, high capability of electrical conductivity, and malleability. Atmospheric distributions of metals have been evaluated by using structural properties and environmental factors (Khlifi & Hamza-Chaffai, 2010). Metals and metalloids are natural members of the Earth's crust and their cycling in the environment has been occurred since the formation of the planet. From past to present, utilization of metal has increased globally. Therefore, this massive usage of metals causes vital variations on both terrestrial and aquatic environment. Metal inputs in the ecosystems can be divided as two partitions. The first group is the anthropogenic inputs which are the major contributors of the metal pollution. Waste dumps, manures, mining facilities, metal-based industrial organizations, metal runoffs, and smelting can be counted for this category. Another main route for the metal pollution is natural occurrences which are soil erosion, weathering, volcanic activities, metal corrosion, sediment resuspension, and evaporation (Briffa, Sinagra & Blundell, 2020).

Metals are robust structures that they are not biologically degradable. Organisms are tending to store, detoxify or excrete the metals. Apart from the toxic metals, some metals are essential, and organisms needed them that's why we called them "essential metals". However, even the essential metals could be as toxic forms in cases of excessive bioaccumulations (Tchounwou, Yedjou, Patlolla & Sutton, 2012).

In literature, metals which have a specific density of 5 g/cm³ are described as "heavy metals" and they have a potential to make harmful circumstances on both living organisms and environment. These metals may be toxic in their lower concentrations and the heavy metals may cause adverse effects in short (acute) or long (chronic) time period when they could reach to the humans by biomagnification (Jaishankar, Tseten, Anbalagan, Mathew & Beeregowda, 2014).

Metalloids can also cause toxicological effects due to their tendency to form covalent bonds. This situation provides metalloids reacting organic compounds in the environment and it can make toxic consequences (Briffa et al., 2020).

2.2 Metal Pollution

Discarding inorganic pollutants from natural and anthropogenic sources to the atmosphere, soils and water causes metal pollution (Briffa et al., 2020). In modern years, metal pollution is one of the major threats on the environmental pollution. Rapidly increasing human-based activities and natural processes make pollution pressures on waterbeds. Increased pollution and industrialization over the years provides metal pollution serious concern worldwide (Mahboob et al., 2022; Sahu & Basti, 2021).

After the entrance of the metals, they usually accumulate on sediments. In case of continuous and increasing metal inputs, it leads to an elevation in the concentration of metal-based pollutants in the sediment. Contaminated sediments are having close relationship with organisms, especially the benthic biota. Therefore, metal transportation from sediment to organisms occurs and bioaccumulation reveals (Ansari, Marr & Tariq, 2004). Metal pollution can be reached even conservative areas such as corals and tropical wetlands (Chuan & Yunus, 2019; Tracy, Weil & Harvell, 2020).

Many studies have been carried out to reduce and remove the hazardous materials from the environment. Conventional methods have been utilized for it. Organic amendments are used to improve the quality of soil that owing metals. Producing of biochar, the organic-based synthetic coals, are made contribution on the remediation of metal from contaminated areas with regulating physical characteristics. Press muds biofertilizers and composts, side product that contain nutrients, are used as remediation agent to remove metals in accumulated sediments. Also, microorganisms have been involved in transforming toxic materials onto non-toxic versions. In current years new methodologies have been developed for advancing environment to remove pollutants.

Genetic manipulations, using proteomics (large scale studies of proteins), biosensors, and waste treatment, and bioreactors can be defined in this category (Pandey & Singh, 2018).

Biomonitoring studies in pollutant environment have the vital roles at this point. Organisms are having strong relationship with their local environment all the time. Therefore, using organisms provide analysts measurable parameters as bioindicators to evaluate pollution status of the environment and one of the most trustable tools are biomarkers due to their sensitive and rapid analyze techniques and acting as early warning system on the detection of chemical stresses (Mussali-Galante, Tovar-Sánchez, Valverde & Rojas Del Castillo, 2013).

2.3 Metal Bioaccumulation

Wang (2016) defined bioaccumulation as: “Bioaccumulation is defined as the increase of contaminant concentrations in aquatic organisms following uptake from the ambient environmental medium.”

Bioaccumulation points out the way of pollutants (metals) entering the food chain from the sources. Also, bioaccumulation is related to elevated concentration of bioavailable metals in organisms. To avoid harmful effects of metal exposure onto both aquatic organisms and humans, the understanding the dynamic processes of metal bioaccumulation is an important part of the marine biomonitoring. Metal bioaccumulation is increased on food chain. Greater organisms accumulate more pollutants, therefore biomagnification occurs (Blowes, Ptacek, Jambor & Weisener, 2003) (Figure 2.1).

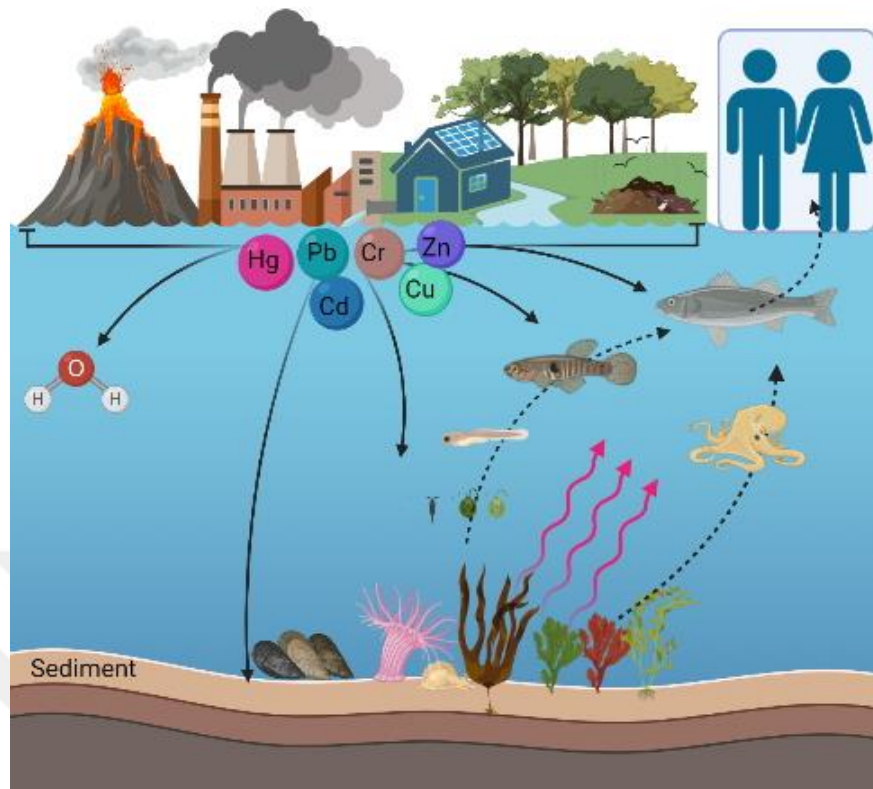


Figure 2.1 Schematic model of the pathway of metals on bioaccumulation and biomagnification processes in the marine environment.

The bioaccumulation pathway of the pollutants may be stemmed from seawater, suspended particles and the sedimentary zones through the food web. The bioaccumulation rates depend on bioavailability of the metals for chemical, biological and/or environmental factors. Storing or discharging on the organisms are related to the ability of the organism. Absorption sources of pollutants were mentioned as solution, suspended particles, sediments, and food (Bryan, 1979).

Natural and human originated ways are the main sources of metal bioaccumulation. Their toxicity on environment and human health would make it one of the major concerns. Many processes have been carried out (electrochemical treatment, precipitation, filtration, ion exchange, oxidation, and reduction) in order to reduce metal load from wastewater systems to prevent reaching them wetlands (A. Mishra & Malik, 2013).

Metal bioaccumulation is generally related with environmental factors (such as salinity, temperature, pH), amount of metal loadings in the wetland, exposure time, and the way of uptake. Besides, the age of organisms, living habits and dietary the of fish are the distinguished parameters, too. Fish distributed along the polluted aquatic environment tend to carry out bioaccumulation in their bodies. Fish species can accumulate metals as soluble or particulate form from the water. Soluble metals contain labile and non-labile fractions. Especially, labile ones are the most dangerous types (Jezierska & Witeska, 2006).

In literature, metal ions showed variations on the bioaccumulation in organs. Liver, gills and kidneys were generally having the majority of the metal loads in the organism. Metal bioaccumulation in these organs confirms that main route of the metal ions is the dietary uptake. Considerable degree of accumulations was also detected on many organs such as brain, gonads, and bones. In muscle tissues, relatively low concentrations were found (Jezierska & Witeska, 2006).

Liver is the most efficient organ for detoxification and liver include many enzymes related to metals like metallothioneins. Therefore, liver contains high levels of metals (Cretì, Trinchella & Scudeiro, 2010). In addition, metal ions were firstly deposited at gills through the passive diffusion, therefore higher concentrations in gills can be related with this natural phenomenon (da Silva et al., 2021). Increased levels of metal bioaccumulation at livers, gills, and muscle tissues of fish species are under the influenced of feeding habits, lipid contents, swimming activity, and fish physiology (Vetsis, Kalantzi, Pergantis, Kokokiris & Karakassis, 2021).

The reason of higher concentrations on livers and gills than the muscle; liver tissues responded more for the antioxidant enzymes and gills responded to biomarker of effects (Gusso-Choueri et al., 2015). In addition, gills have thinnest epithelium and pollutants could be exposed through the respiration. Also, liver can concentrate metals and other pollutants to the considerable degree (Farombi, Adelowo & Ajimoko, 2007).

CHAPTER 3

BIOMARKERS

3.1 What Are The Biomarkers?

Peakall (1994) defined biomarkers as: “a change in a biological response (ranging from molecular through cellular and physiological responses to behavioral changes) which can be related to exposure to or toxic effects of environmental chemicals”.

Authorities have been categorized biomarkers into the three subgroups (National Research Committee [NRC], 1987; World Health Organization[WHO], 1993).

- Biomarkers of **exposure**: covering detection and determination of toxic materials, its metabolites or target molecule/cell.
- Biomarkers of **effect**: detectable or recognized health disease associated with measurable biochemical, physiological, or different impacts within organisms.
- Biomarkers of **susceptibility**: changes in receptors that under influence on the exposure of pollutants which alter the susceptibility of an organism.

Biomarkers can be used not only determine the environmental levels, but also give an opinion that biological effects of pollutants. Therefore, crucial knowledges could be gained about organisms which affected by toxic compounds and stress parameters by screening multi-biomarker measurements (McCarthy, Halbrook & Shugart, 1991).

Stegeman et al. (2018) had stated that six criteria are available to evaluate positive and negative sides of fish biomarkers for gaining meaningful information.

- i. Biomarker assay should be easily quantified and affordable.
- ii. The response of biomarker should reflect to pollutants, its exposure and side effects to establish early warning system.
- iii. Quantified baseline biomarker data should be expressed the difference between natural occurrence and contaminated changes.

- iv. The reasons of confounding factors to biomarker studies should be maintained.
- v. Underlying mechanism of the relationships between biomarker response and pollutant exposure should be well expressed.
- vi. Relationship between biomarker's toxicological difference and responses to the whole body should be established.

The primary reason for the concentration of pollutants in the marine environment is the increase on inputs of domestic or industrial pollutants in the ecosystem. Thereby, environmental monitoring studies are needed to identify the negative effects of pollutants in the environment and natural resources, to assess and to manage these risks (Cajaraville, Bebianno & Blasco, 2000).

Environmental biomonitoring programs have been focused on analytical measurements of chemical or physical factors. Chemical analyses had based on nutrient levels, physicochemical parameters (e.g. temperature, salinity) or concentrations of chemical compounds in water. It is well known that they are strong indicators but they only give information about environmental conditions, not biological and metabolic effects on organisms (Lam, 2009).

In particular, the impact of pollutants showing high toxicity on marine organisms has started to attract the attention of researchers. In the first studies, the economic costs of analytical processes were high and the results of these expensive analyzes are generally determined below the determination limit. After that, studies were evolved to about detecting the pollution effects that will occur in the marine environment in the early period. The indicators obtained because of these studies are called as biomarkers. Biomarkers are measurable biological reactions in a biological system resulting from exposure to pollutants. Bioindicator responses detected in cells, body fluids, tissues or organs; it is limited to chemical, molecular or physical changes, and these changes are indicative of the effects of pollutants. (Lam & Gray, 2003).

Previous studies explained that oxidative stress biomarkers can be used effectively in the effect of pollution level on organisms (Di Giulio, R.T., Washburn, P. C., Wenning, R. J., Winston, G. W. & Jewell, 1989).

Oxidative stress is a phenomenon that influences on production or accumulation of various oxygen-based radicals (Reactive Oxygen Species, in short ROS) such as superoxide, singlet oxygen, peroxides that can be harmful to protein, and lipid compounds and DNA in the cells. Antioxidant enzyme systems can regulate the organisms to prevent harmful effects of ROS production. Catalase (CAT) is one of these antioxidants that counteracts the toxic effects of ROS (Lionetto et al., 2003). The mechanism of oxidative stress in the enzymatic defense system were schemed at Figure 3.1.

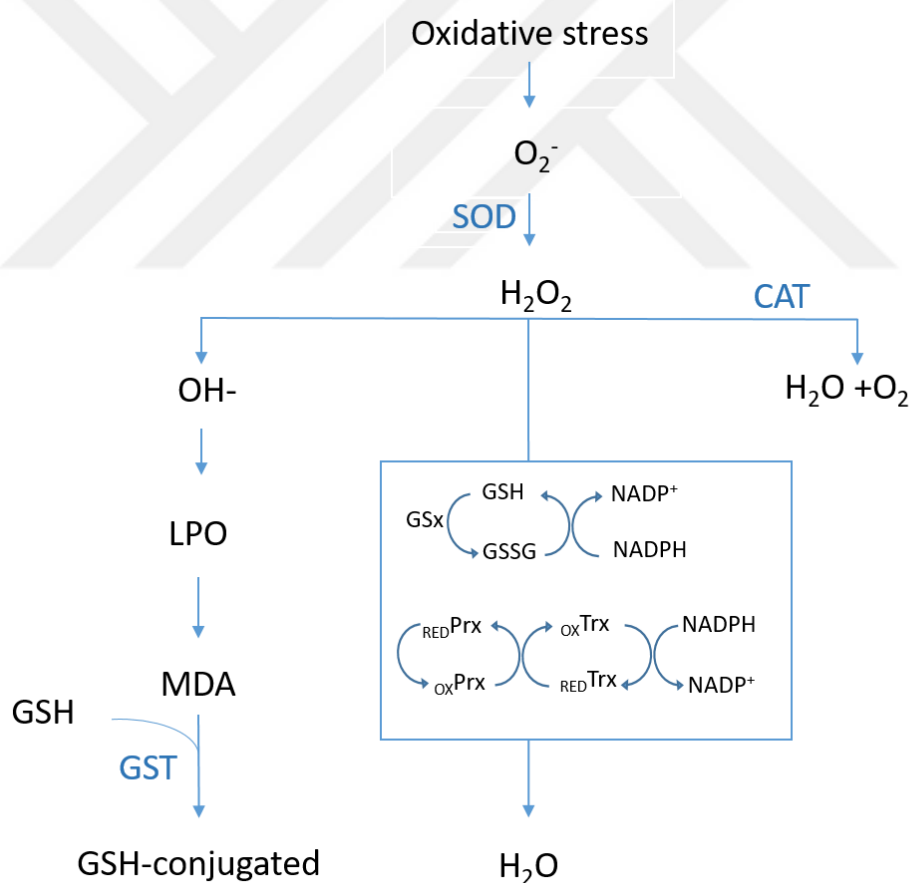


Figure 3.1 Antioxidant enzyme defence system. Regenerated from Hsueh et al., (2022). CAT: catalase; SOD: superoxide dismutase; GPx: glutathione peroxidase; GST: glutathione S-transferase; PRx: peroxiredoxin; TRx: thioredoxin; MDA: malondialdehyde; LPO: lipid peroxidation; RED: reduced; OX: oxidized.

3.2 Classifications of Biomarkers

Viarengo et al. (2007) were classified environmental usage of biomarkers into three main groups.

- **Stress biomarkers:** These biomarkers are rapidly activated in the face of the effects of toxic chemicals on the organism and used like as early warning tool. Lysosomal membrane stability, antioxidant enzymes (catalase, superoxide dismutase and glutathione peroxidase) and lipofuscin lysosomal contents and neutral lipid accumulations are included in this class.
- **Genotoxicological biomarkers:** Biomarkers in this class are toxicological markers at the indicating the damage. Micronucleus test, DNA damage and chromosomal damage can count in this group.
- **Exposure biomarkers:** Biomarkers expressing the products formed as a result of the reactions occurring in the organism due to the pollutants they are exposed to. We can include biomarkers such as metallothioneins (MT), cholinesterase activity, oxygenases and 7-ethoxirezorufine-O-Deethylase (EROD) activity, malondialdehyde (MDA) content (Viarengo et al., 2007).

In the concept of this thesis, CAT and SOD were included the stress biomarkers, however, MDA and MT were included exposure biomarkers.

3.2.1 Catalase

CAT (EC 1.11.1.6) is an enzyme that contains heme proteins. Main role of CAT in the cells is conversion of hydrogen peroxide (H_2O_2) to molecular oxygen (O_2) and water (H_2O) (Lesser, 2006). CAT mechanisms are found in all known aerobic cells. Higher amounts of their concentrations are existing in the erythrocytes to eliminate H_2O_2 during the process of autoxidation of oxyhemoglobin to methemoglobin (Everse, 2013).

CAT has 240 kDa weight. Also, it has a tetrameric protein that includes four subunits. CAT presents almost every living organism to utilize oxygen. Fe and Mn are the main cofactors for CAT. An abundance of CAT is considerably high in the cells. They can decompose millions of H_2O_2 molecules within a second (Ighodaro & Akinloye, 2018).

Besides these, CAT were involved in many industrial processes on food and textile applications. CAT acts as H_2O_2 removal agent for sterilization or bleaching (X. Liu & Kokare, 2017).

3.2.2 Superoxide Dismutase (SOD)

In the enzymatic response system, superoxide dismutase (SOD; EC1.15.1.1) is one of the vital antioxidant enzymes in the almost all aerobic organisms. SOD is defined as partitioning of superoxide (O_2^-) into the peroxides (H_2O_2) and molecular oxygen (O_2) (McCord & Fridovich, 1969). SOD is only antioxidant enzyme that the ability of scavenging O_2^- anions by converting free radicals. For this reason SOD can also use in many medical applications (F. Wang & Zhang, 2015).

SOD molecules are involved a metal atom as cofactor. Main types of SOD are Mn-SOD and Cu/Zn-SOD. These molecules present in both eukaryote and prokaryote organisms (Zeinali, Homaei & Kamrani, 2015). Apart from these, Fe-SOD can occur at protozoans, chloroplasts of algae, and prokaryotes (Wang, Wu, Zhang, Chen & Zhou, 2013).

According to the previous studies, raising of SOD activity points out increasing oxidative stress tolerance in the organisms. This ability makes SOD one of the environmental screening biomarker (Tanwir et al., 2021).

SOD has also taken a role on therapeutic interventions. SOD is used as inhibitor of SOD-induced DNA strand breaks on cancer therapy. In addition, SOD is involved in

oxidative stress related pathologic treatment programs due to its ability of being anti-inflammatory agent on lipid peroxidation inhibition (Prasad et al., 2018)

3.2.3 Malondialdehyde (MDA)

Malondialdehyde is one of the three-carbon aldehyde compound which is highly reactive. Due to occurring naturally in the cells, MDA is defined a biomarker for oxidative stress. MDA is generally available as enol form. Lipid peroxidation is continuously process in sub-cellular levels which includes several chain reactions of oxidative degradation of lipids (Dzoyem, Kuete & Eloff, 2014). MDA is a one of the most robust products that occurs in the end of the lipid peroxidation reactions in the organisms which makes it useful biomarker for assessing oxidative stress. ROS molecules are triggered lipid peroxidation (LPO) procedure that finalize MDA production. Therefore, MDA contents are fluctuated with the amount of pollutants that expose the cells (Z. Singh, Karthigesu, P. Singh & Kaur, 2014)

Since fish have too much unsaturated fatty oils in their tissues, they tend to produce LPO radicals. In the result of the oxidative stress factors, cells can be produces MDA content that they can be decomposed to the unstable forms such as ketones, aldehydes, hydrocarbon or alcohols (Mendes, Cardoso & Pestana, 2009).

LPO takes a part in the pathogenesis occurrence of several types that end in injuries and damages of the tissue with the under influences of toxic materials (Dzoyem et al., 2014).

3.2.4 Metallothionein (MT)

Metallothionein (MT) is a low molecular protein compound with approximately 6500 Da. Its composition includes thiol-containing (around 30%) cysteine residues. It has two clusters (α - and β -clusters) that occurs metal binding. Many organisms have ability to possess MT-encoding genes for both toxic (such as Hg, and Cd) and essential (such as Cu and Zn) for the forming the MT reaction. MT compounds are vital for the

organism due to their ability of detoxification of toxic metals. MTs take a role in the transport of metals such as Cu and Cd inside the body. Toxic metals do not cause harmful results for the organism only when they bond with MT molecules. Toxic metals that have not made an organometallic bond with an MT protein can have harmful effects on the organism.(M. Nordberg & G. Nordberg, 2016; Smith & Nordberg, 2015).

MT can be defined as major contributor to cytoprotection. With the binding of metals, it blocks forming ROS that originated metal uptake. Toxic metal ions interact with intracellular MTs to form complexes as the cell needed. After that, oxidative stress releases MT-binding organometallic compounds by contributing cofactors for antioxidant enzymes like Zn-SOD and Cu-SOD (Ettinger, 2017).

MT takes a part in binding toxic metals and removes the ROS particles in the cells. MT molecules connect toxic metals and then transport them from the body to remove (Shibuya et al., 2008). Using MTs as a biomarker of toxicity have done with limited success. Recently, the role of MTs in mediating adverse outcome pathways (also known as AOPs) could not full discovered (Fowler, 2016).

3.3 Utilization of Biomarkers in Aquatic Environments

The loadings of anthropogenic inputs that reaches aquatic ecosystem is dramatically increased. Therefore, inorganic and organic originated xenobiotics (such as polycyclic aromatic hydrocarbons, polycarbonated biphenyls, organochlorinated pesticides, phthalate esters, and toxic metals) could pose a threat directly to the marine life (Livingstone, 1993; Sarkar, Ray, Shrivastava & Sarker, 2006). It's known that chemical pollution is accepted as one of the major threats to the aquatic life, because of biodiversity loss. Especially coastal zones are faced to higher amounts of concentrated pollutants. Many studies claimed that this situation cause serious decrease of marine organisms' populations (Lionetto, Caricato & Giordano, 2021).

Due to their diagnostic capabilities and acting as an early warning system, using biomarkers in marine biomonitoring studies are getting more important in recent years (Livingstone, 1993). Biomarkers response the pollutants under cellular level at first, after that, occurrence come up to the tissues, organs, and eventually whole body. Furthermore, these responses are developed from individual to population and ecosystem level (Hamza-Chaffai, 2014).

Utilization of biomarkers provide us many benefits for the assessment of the environmental status in ecosystem. Biomarkers can identify the correlation between contaminant and organisms to evaluate the sub-lethal and adverse effects. They can gain information about distinguished compounds of chemicals that can assume for their uptake and bioaccumulation. They may contribute spatial and temporal comparison for the pollutants. Periodical applications of biomarkers provide analysts to predict the potential environmental pollutions in early stage and to take an action before the irreversible consequences (Sarkar et al., 2006). Biomarkers are useful tool for the environmental risk assessment because they describe the effect of stressors at physiological, sub-organismal and behavioral level. This covers immunological, cellular, physiological, and genetic determinations (Galloway, 2006).

Apart from the traditional monitoring methods, biomarkers were distinguished as affordable and robust substances. An analyst can use less costly complex chemicals by preferring for biomarker applications. Also, these determinations would be relatively rapid. These analyzes provides an advantage in taking quick action at variations in environmental parameters, as well as it can indicate that bioavailability of toxic, carcinogenic and mutagenic substances in the environment (Goksøyr et al., 1996).

Last but not the least, biomarkers can easily operate in-vivo and in-vitro cases. Some mechanisms of response can be identified at laboratory at first, before the application at the field (Sarkar et al., 2006). Measurement of biomarkers can take a part in experimental exposure tests to compare control and treated groups due to their high sensitivity capabilities (Koukouzika & Dimitriadis, 2008).

CHAPTER 4

MATERIALS AND METHODS

4.1 Selected Fish Species

4.1.1 *Sparus aurata* (Gilthead Seabream)

Domain: Eukaryota

Kingdom: Metazoa

Phylum: Chordata

Subphylum: Vertebrata

Class: Actinopterygii

Order: Perciformes

Suborder: Percoidei

Family: Sparidae

Genus: *Pagrus*

Species: *Sparus aurata*

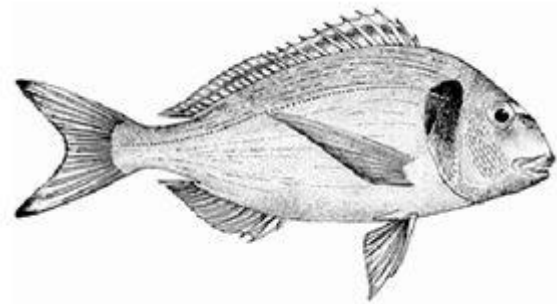


Figure 4.1 Image of *S. aurata*

Sparus aurata (Linnaeus, 1758) is one of the common demersal fish that distributed through the Mediterranean Sea and European coasts (Figure 4.1). Distribution map of the *S. aurata* can be seen in Figure 4.2. It is typically found at depths of 0–30 m, but may live up to 150 m (Bauchot & Hureau, 1990).

S. aurata, which generally spreads in tropical, subtropical, and temperate zones, lives in sandy-muddy and muddy environments where sea phenograms are found. In addition, it enters river mouths and lagoons. Generally, *S. aurata* distributes to southern coasts and the Aegean coasts in Türkiye (Saka & Fırat, 2008).

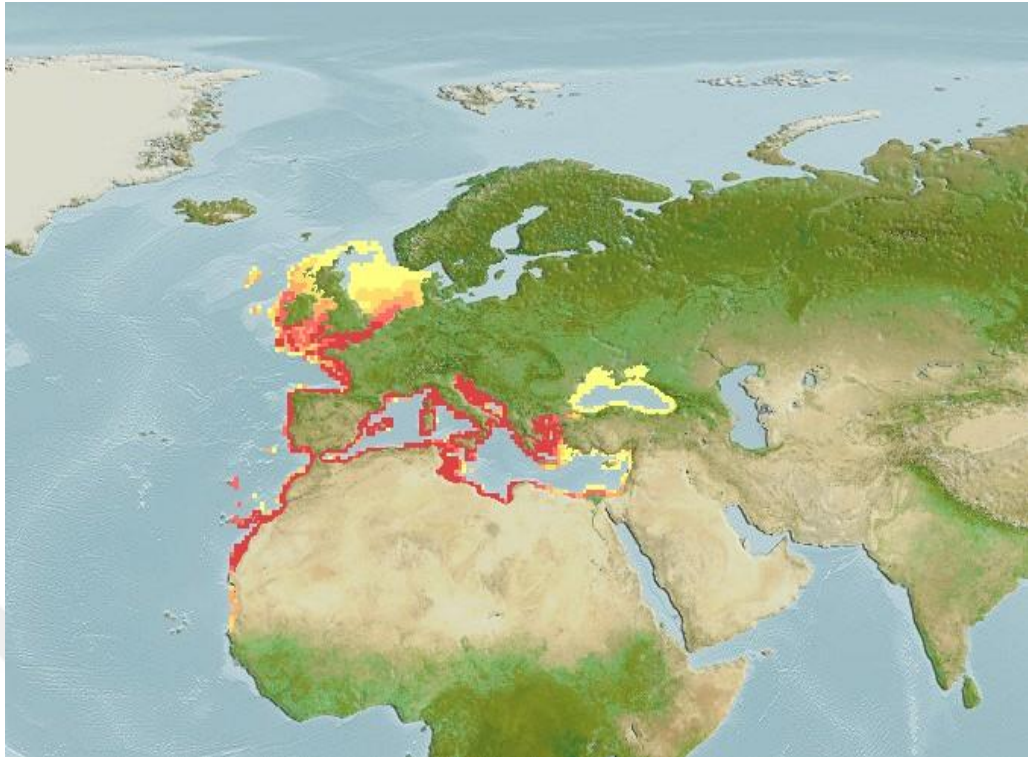


Figure 4.2 Distribution map of *S. aurata* (Aquamaps, 2019a)

The average life time of *S. aurata* is about 11 years. Duration of the larval stage lasts approximately 50 days. Sexual maturity develops in 2-3 years for females and about 2 years for males. *S. aurata* has hermaphrodite protandrous reproduction mode. Spawning season is generally occurring from October to December. Adult females give about 20000 – 80000 eggs (FAO, 2022).

Feeding habits of *S. aurata* are carnivorous and opportunistic. It feeds from the bottom sediment. Researchers reported that their individuals usually consumed molluscs and anthropods. (Hadj Taieb, Sley, Ghorbel & Jarboui, 2013).

4.1.2 *Chelon labrosus* (Thicklip Grey Mullet)

Domain: Eukaryota

Kingdom: Metazoa

Phylum: Chordata

Subphylum: Vertebrata

Class: Actinopterygii

Order: Mugiliformes

Suborder: Mugiloidei

Family: Mugilidae

Genus: *Chelon*

Species: *Chelon labrosus*

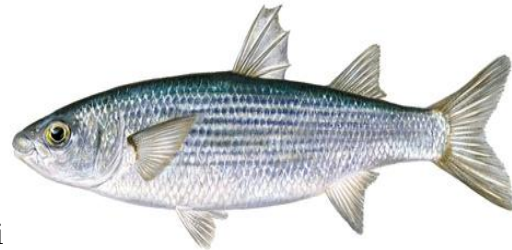


Figure 4.3 Image of *Chelon labrosus*.

Chelon labrosus (Risso, 1827) is one of the common demersal fish through the Mediterranean Sea and European coasts (Figure 4.3). Distribution map of the *C. labrosus* can be seen in Figure 4.4. It has typically average 30 cm, but 70+ cm length had been recorded in the past.

C. labrosus (thicklip grey mullet) is one of the common species of *Mugilidae* family. Its individuals generally distribute coasts and stream mouths, especially rocky and sandy bottoms. It also prefers living with larger groups (Parrino et al., 2021). Male specimens are usually smaller than females. *C. labrosus* feeding habits depend on diatoms, algae, invertebrates, and detrital pieces from benthic sediment and pelagic water column. Spawning period occurs along winter. Their larvae and eggs are pelagic (Abumourad et al., 2014).

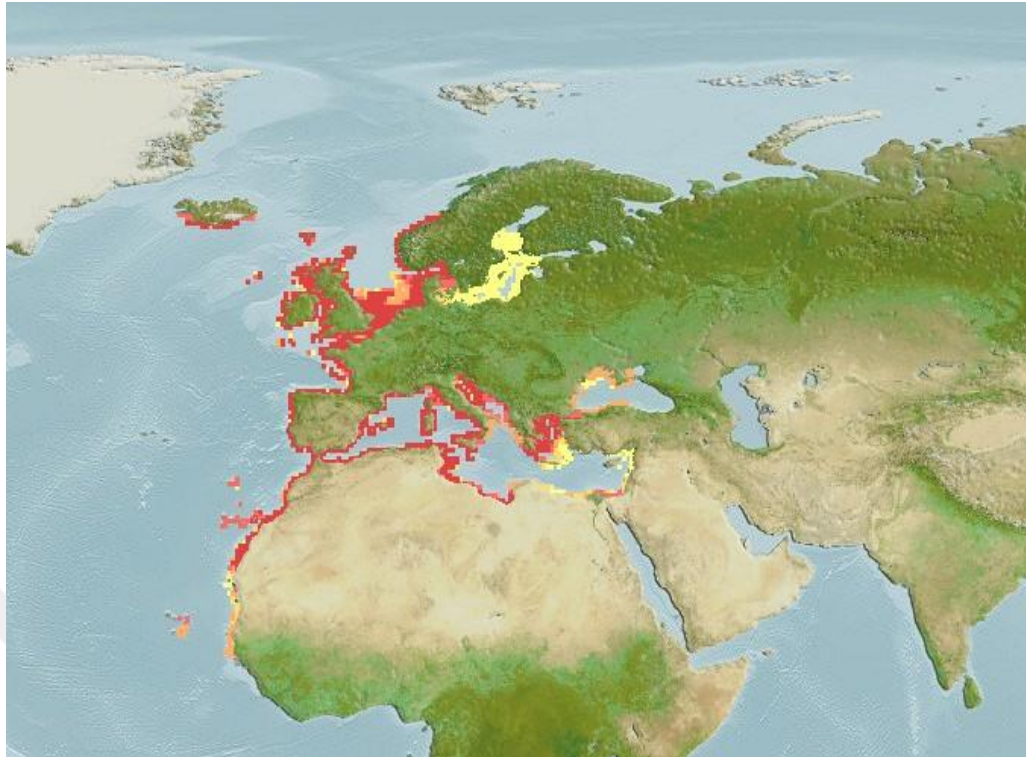


Figure 4.4 Distribution map of *C. labrosus* (Aquamaps, 2019b).

C. labrosus is also taken a place in “The IUCN Red List of Threatened Species”, their distribution is under observation (Freyhof & Kottelat, 2008).

Recently *C. labrosus* is one of the potential species for aquaculture in the Mediterranean Sea due to their suitability for producing both fish stocks and the caviars (Besbes et al., 2020).

4.1.3 *Diplodus vulgaris* (Common Two-Banded Seabream)

Domain: Eukaryota

Kingdom: Metazoa

Phylum: Chordata

Subphylum: Vertebrata

Class: Actinopterygii

Order: Perciformes

Suborder: Percoidei

Family: Sparidae

Genus: *Diplodus*

Species: *Diplodus vulgaris*



Figure 4.5 Image of *Diplodus vulgaris*.

Diplodus vulgaris (Geoffroy Saint-Hilaire, 1817) (common two-banded seabream) is a demersal marine fish that lives generally inshore waterbeds and posidonia covered areas with 0 – 90 m depth (Figure 4.5) (Cengiz, Paruğ & Kızılkaya, 2019). Specimens of *D. vulgaris* are distributed to the Mediterranean Sea, Black Sea, Western Africa, and Western Europe (Figure 4.6).

Along with *S. aurata*, it is also member of *Sparidae* family (Da Palma, 2002). Main diet of this species contains crustacea such as amphipoda, isopoda, prawns, fish, and polycheta (Osman & Mahmoud, 2009).

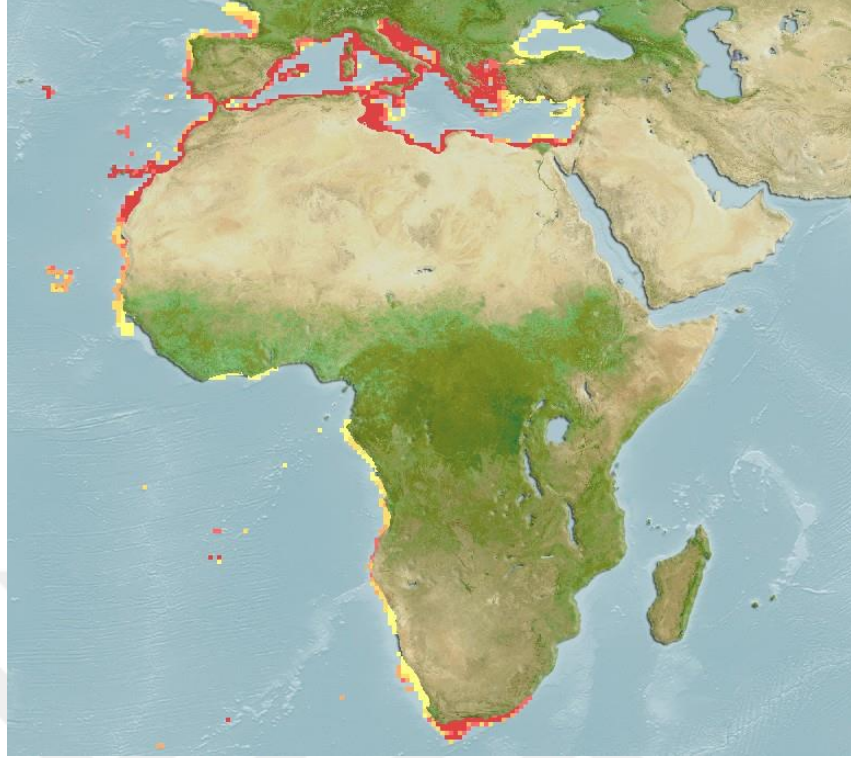


Figure 4.6 Distribution map of *D. vulgaris* (Aquamaps, 2019c).

In literature, spawning season of *D. vulgaris* were generally detected from December to March. Also, it peaks in January and February. Researchers claimed that its reproductivity depends on rudimentary hermaphroditism with possible protandry, as evidenced by the presence of specimens in sexual transition (Gonçalves & Erzini, 2000).

4.2 İzmir Bay (Study Area)

İzmir Bay is located western coast of Türkiye (Aegean Sea). İzmir Bay has L-shaped appearance from above. The leg part of “L” is approximately 20 km wide and 40 km long and the bottom part of the “L” has 5-7 km wide and 24 km long. İzmir Bay has been categorized into three subgroups, which are Inner, Middle, and Outer Bays, in respect to the their physical characteristics (Figure 4.7) (Sayın, 2003).



Figure 4.7 Satellite image of İzmir and İzmir Bay from NASA EARTHDATA

İzmir Bay's hydrographical properties depend on several different factors: atmosphere & sea interaction, Bay's variable topography, sea-level changes related to Aegean Sea, winter convection, the accumulation of waters as a result of the prevailing wind-driven circulation, antropogenic load of fresh waters that reach to Inner Bay, and water exchange with Aegean Sea (Sayın, 2003). Variation on salinity and temperature in related to discharging of Gediz River, strong evaporation and Aegean Sea. Therefore, sometimes turbulent mixing can occur eve in summer (Sayın, 2003).

Gediz River reaches to Aegean Sea at the northern side of the İzmir Bay and also it is second biggest stream in Western Anatolia. Therefore, Gediz River is the main source of the pollution for the Bay. Various industrial facilities (such as agricultural production, food processing, tanneries, paper production, textile, metal industries, and beverage manufacturing) are organized in the region (Kucuksezgin, Kontas, Altay, Uluturhan & Darılmaz, 2006).

Foça, one of the sampling area in this study, is a touristic town which is located northern side of İzmir. Also, it is next by the entrance of İzmir Bay. In addition to

tourism, Foça is a region where fishing is carrying out intensively. Various sizes of fishing boats are operating in this region. Many demersal and pelagic fish species, cephalopods, and crustaceans have been caught around Foça (Güçlüsoy & Tosunoğlu, 2019).

İnciraltı, another sampling area in this study, is located southern part of the İzmir Bay. Densely fishing has been carried out in this region. Both fish species and bivalves were caught from there. People are generally prefer consuming the seafoods from there (Aslan et al., 2018).

4.3 Conceptualization

Three different sampling periods (November 2019, November 2020 and May 2021) from Foça and İnciraltı for the selected tissues (muscle, liver, and gills) of *Sparus aurata*, *Chelon labrosus* and *Diplodus vulgaris* (only for November 2019 at Foça) were carried out to evaluate multi biomarker responses related to metal pollution. CAT, SOD, MDA and, MT (except November 2019) were selected as biomarkers for this thesis to assess biochemical effects of metals. Besides, eleven different metals and metalloids (Hg, Cd, Pb, Cr, Cu, Zn, Mn, Se, Sn, As, and V) were selected for the analyses.

Before the sampling, ethical procedures were carried out. In order to the experimental design to be carried out on a legal basis, the approval dated 03.05.2019 and numbered 13/2019 was obtained from the Multidiscipline Laboratory Animal Experiments Local Ethics Committee of Dokuz Eylül University. Also sampling permission approval were gathered from Ministry of Agriculture and Forestry, General Directorate of Nature Conservation and National Parks (27.03.2019 dated and E.1008941 numbered). The project of the thesis was funded by Dokuz Eylül University, Department of Scientific Research Projects (Project Number: 2019.KB.FEN.040).

4.3.1 Sampling and Field Works

Sampling procedures were carried out November 2019, November 2020 and May 2021 from Foça (Outer Bay) and İnciraltı (Inner Bay) (Figure 4.8). Samplings were made by fishing rods or obtained by local fishermen as alive. *S. aurata* and *C. labrosus* were sampled in all sampling periods, however, *D. vulgaris* was only sampled on November 2019 at Foça (Figure 4.9).

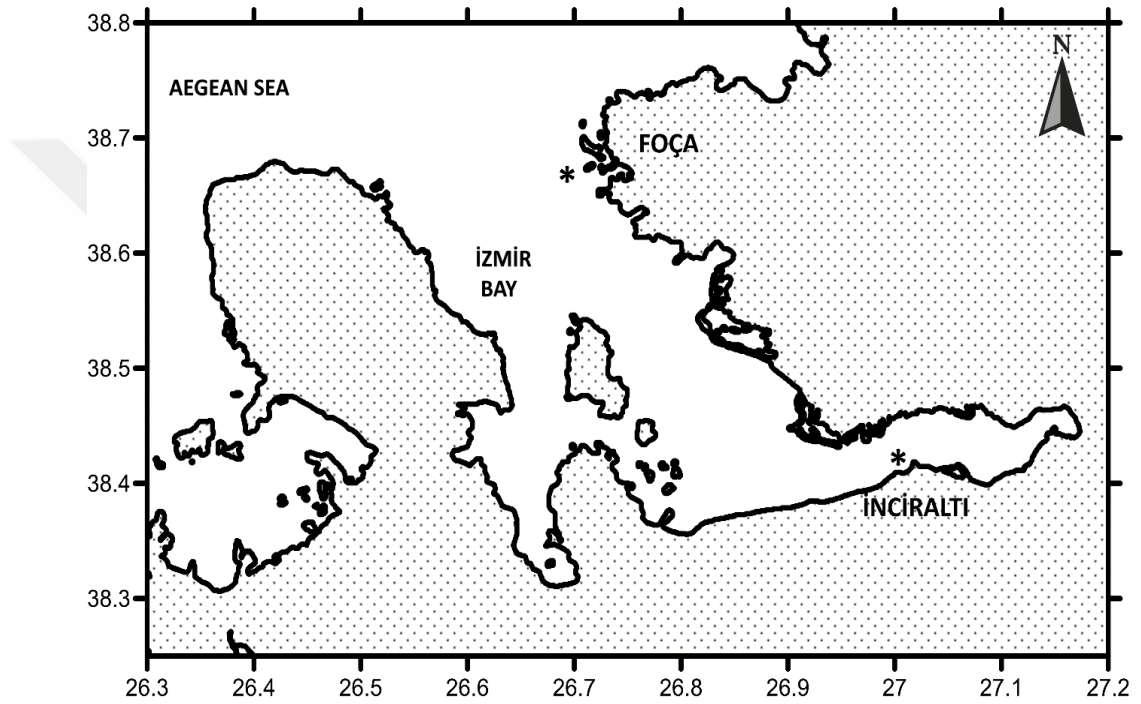


Figure 4.8 Sampling points at İzmir Bay



Figure 4.9 Collected fish samples

Physicochemical parameters (pH, salinity, temperature, conductivity, and dissolved oxygen) were measured simultaneously in-situ with portable multi parameter meter (HACH HQ40d) while fishing (Table 4.1).

Table 4.1 Physicochemical parameters (pH, salinity, temperature, conductivity, and dissolved oxygen) from sampling points

	pH	Salinity (‰)	Temp. (°C)	Cond. (mS/cm)	DO (mg/L)
Nov. 2019					
Foça	8.30	36.5	17.6	47.3	7.55
İnciraltı	8.04	36.5	16.1	45.6	6.42
Nov. 2020					
Foça	8.26	34.7	20.8	48.3	7.23
İnciraltı	8.26	34.7	17.7	45.3	5.02
May 2021					
Foça	7.91	37.8	21.0	46.2	7.57
İnciraltı	8.06	35.5	26.1	51.8	5.20

Biometric parameters (weight, fork length and total length) were measured and given Table 4.2. After that, fish specimens were immediately decapitated. Muscle, liver and gills tissues were separated. Tissue samples were divided for the metal and biomarker analyses.

Tissues for metal analysis were stored in portable deep freezers (-18 °C) in plastic bags, while samples that used in biomarker analysis were placed in cryo-tubes and stored in a liquid nitrogen tank (-196 °C) at the field. Then, samples were transported to the laboratory. Samples of metal analyses were moved to deep freezer (-26°C) and sample of biomarker analyses were moved to ultra-low temperature (ULT) freezer (-80°C).

Table 4.2 Biometric measurements from sampling periods.

	n (count)	Weight (g)	Fork Length (cm)	Total Length (cm)
Nov. 2019				
Foça				
<i>S. aurata</i>	3	254-271	24.6-25.7	26.9-28.1
<i>C. labrosus</i>	3	361-455	29.5-33.0	31.5-36.5
<i>D. vulgaris</i>	3	209-237	18.8-23.0	20.8-25.2
İnciraltı				
<i>C. labrosus</i>	8	252-272	26.5-37.0	29.0-39.0
Nov. 2020				
Foça				
<i>S. aurata</i>	5	167-279	20.5-24.5	23.0-27.5
<i>C. labrosus</i>	3	505-655	35.0-38.0	39.0-42.0
İnciraltı				
<i>S. aurata</i>	5	121-208	17.5-22.0	19.0-23.5
<i>C. labrosus</i>	5	197-787	25.0-37.5	27.5-41.0
May 2021				
Foça				
<i>S. aurata</i>	5	106-206	18.5-23.5	20.0-25.0
<i>C. labrosus</i>	5	309-364	27.5-29.5	30.0-33.5
İnciraltı				
<i>S. aurata</i>	3	134-257	20.0-26.5	21.5-29.0
<i>C. labrosus</i>	4	285-458	28.5-32.0	31.0-35.0

4.4 Metal Analyses

Firstly, tissue samples were subjected to drying using a freeze-dryer (Labconco FreeZone 4.5, KS, USA) and then, the dried samples were homogenized and weighed approximately 0.50 g in at least triplicate and digested in a microwave digestion system (Milestone MLS 1200 Mega, Sorisole (BG), Italy) in an acid mixture of 5 mL HNO₃ and 1 mL HClO₄ (Bernhard, 1976). The working procedure of microwave system for organic materials is as given in Table 4.3. The mixture with fragmented samples were adjusted to 25mL final volume and stored in HDPE bottles at room temperature with dark ambient.

Table 4.3 Working procedure of microwave digestion system for organic materials

Stage	Duration (min.)	Power (W)
1	1	250
2	1	0
3	5	250
4	5	450
5	5	650
6	5	Ventilation

Within the scope of metal analysis, mercury (Hg), cadmium (Cd), lead (Pb), chromium (Cr), copper (Cu), zinc (Zn), manganese (Mn), selenium (Se), tin (Sn), arsenic (As), and vanadium (V) analyses were carried out. Varian Atomic Absorption Spectrometer (AAS) combined systems were utilized to determine metals.

Cu, Zn, and Mn determinations were carried out with Varian AAS 280FS using flame technique, while Pb, Cd, Cr, Se, Sn, As, and V analyses were carried out with Varian AAS 280Z graphite furnace method (furnace settings can be seen at Table 4.4). Besides, Hg analyses were performed via cold vapor technique by using Varian AAS 280FS with add-on VGA-77 vapor generation module (UNEP, 1984, 1990). All chemicals using in analyses were supplied as analytical grade.

Table 4.4 Settings for analyses of metals determined on graphite furnace atomic absorption spectrometer (Varian AA280Z).

Metal	Drying			Ashing			Atomization			Cleaning	
	tramp	thold	T _f	tramp	thold	T _f	tramp	thold	T _f	thold	T _f
Cd	7.0	8.0	120	5.0	3.0	250	0.8	2.0	1800	2.0	1800
Pb	7.0	8.0	120	5.0	3.0	540	0.9	2.0	2100	0.1	2100
Cr	7.0	8.0	120	5.0	3.0	1010	0.8	2.0	2551	2.0	2551
Se	45.0	10.0	120	5.0	3.0	1000	0.8	2.0	2600	2.0	2600
Sn	45.0	10.0	120	5.0	3.0	700	1.0	2.0	2600	2.0	2600
As	45.0	10.0	120	5.0	3.0	1400	0.6	2.0	2600	2.0	2600
V	7.0	8.0	120	5.0	3.0	1400	0.9	2.0	2700	2.0	2700

For the accuracy and validity of analyses, recovery rates were set using certified standard solutions that determined as unknown sample with exact conditions other samples. The percentage of recovery rates were maintained as 100%±10 and repeated every 12 readings to verify the analyses. Recovery rates were given on Table 4.5.

Table 4.5 Recovery rates of determined metals (mean ± standard deviation)

Metals	Recovery Rate (mean, %)	Std. Dev.
Hg	99.7	3.8
Cd	100.7	4.5
Pb	102.3	4.5
Cr	100.5	4.0
Cu	96.1	5.6
Zn	98.9	2.1
Mn	97.5	3.0
Se	102.0	3.9
Sn	100.7	3.4
As	103.3	4.9
V	100.3	7.0

4.5 Biomarker Analyses

Homogenates of tissues were prepared with ISOLAB homogenizer. Centrifugation were made with Hettich refrigerated and standard centrifuges. Analytical applications of the CAT, SOD, MDA, and MT analyses were carried out with BioTek Synergy HTX multi-mode microplate reader (Figure 4.10).



Figure 4.10 Microplate reader in operating

Raw data and images of spectrum scanning were gathered by using BioTek Gen5 Data Analysis Software (version 2.06.10). All chemicals were supplied analytical and/or biological grade. All consumables were supplied as DNA and RNA-free disposable materials.

4.5.1 Determination of CAT Activity

CAT activity was determined by previously prescribed by Aebi (1984) and adapted for the microplate reader. The method was based on tracing H_2O_2 decompositions of supernatant at pH 8.0 in UV-region at 230nm wavelength.

4.5.1.1 Reagents

- pH 8.0 Tris Buffer: 50mM Tris-HCl was prepared with H_2O at the final volume of 100 mL. pH level was adjusted with adding 1M NaOH solution.
- H_2O_2 Solution: 35% H_2O_2 solution (Merck) was used for hydrogen peroxide source. Stock dilution was 11.62M. H_2O_2 diluted to 10mM with buffer for the analysis.

4.5.1.2 Sample Preparations

Approximately 100 mg tissues were homogenized with 0.3 mL pH 8.0 Tris buffer by using tissue homogenizer. Buffered samples were placed microcentrifuge tubes. Homogenized samples were centrifuged on 10000 rpm for 10 minutes at 4°C. After centrifugation, supernatants were collected.

4.5.1.3. Standards

Six standards were prepared with 10mM H₂O₂ from 0 nmol to 500 nmol with 100 nmol intervals on the microcentrifuge tubes (Figure 4.11) Serial dilutions were adjusted with pH 8.0 Tris Buffer. Due to standards were low concentrations, standard set were prepared fresh all the time. Final volumes were set 200μL in the wells.

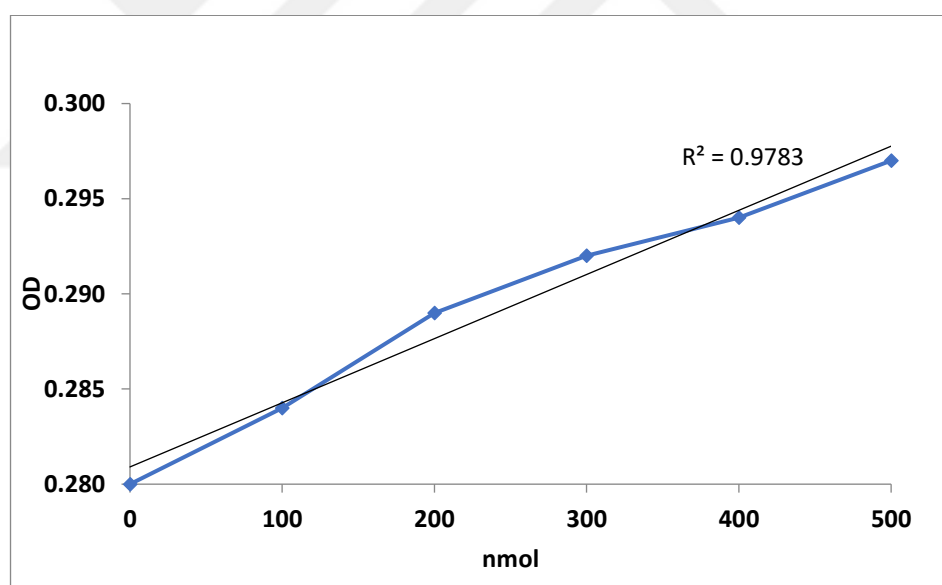


Figure 4.11 Standard curve of H₂O₂

4.5.1.4 Measurements

200 μL standard solutions were placed in UV-transparent microplate at least triplicates. 20μL samples, 80 μL Tris buffer and 100μL 10mM H₂O₂ were added the wells. Addition of H₂O₂ solution performed with multi-channel pipette. After 10

minutes of incubation, CAT activities were determined at 230nm. Kinetic readings were performed for 15 minutes with 1-minute interval (Figure 4.12).

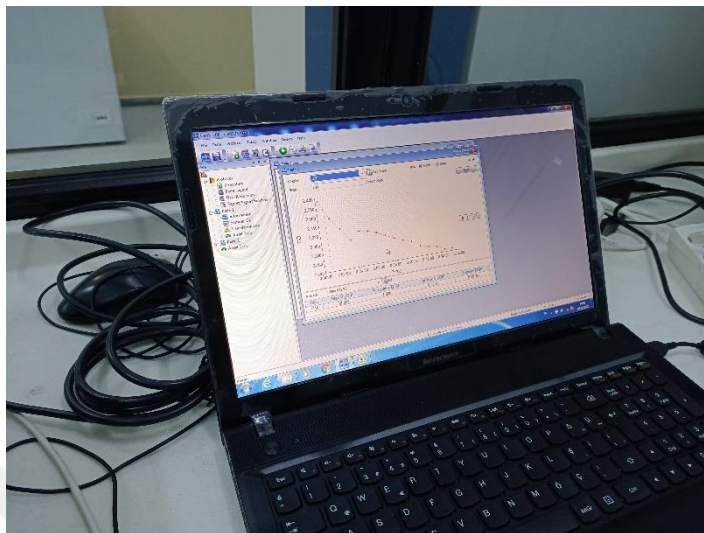


Figure 4.12 Kinetic reading session during determination of CAT activity

4.5.1.5 Quality Control

While CAT activity was being determined, spectrum scanning was performed to control whether the reaction was at the desired (UV-region) wavelength (Figure 4.13). In the kinetic reading phase, the optical density of the supernatants was expected to decrease with hydrogen peroxide exposure. In order to determine whether this downward trend is as expected, preliminary experiments were made with test samples and trend graphs were observed (Figure 4.14).

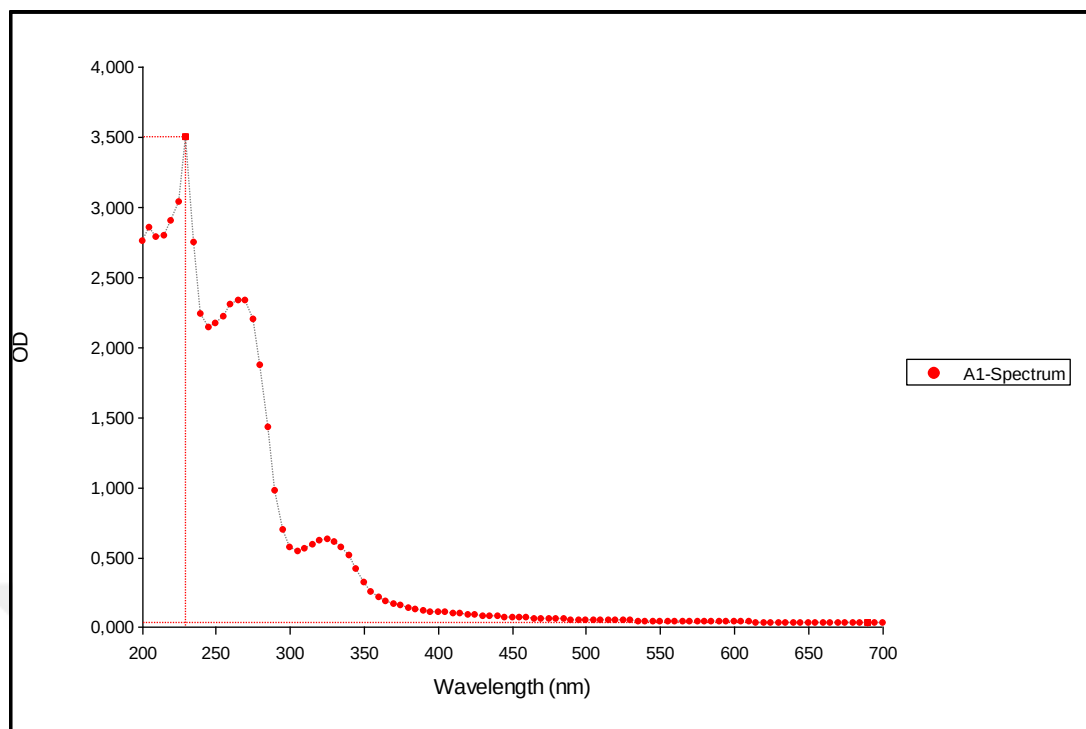


Figure 4.13 Spectrum scanning of H₂O₂. Maximum OD was observed at 230nm.

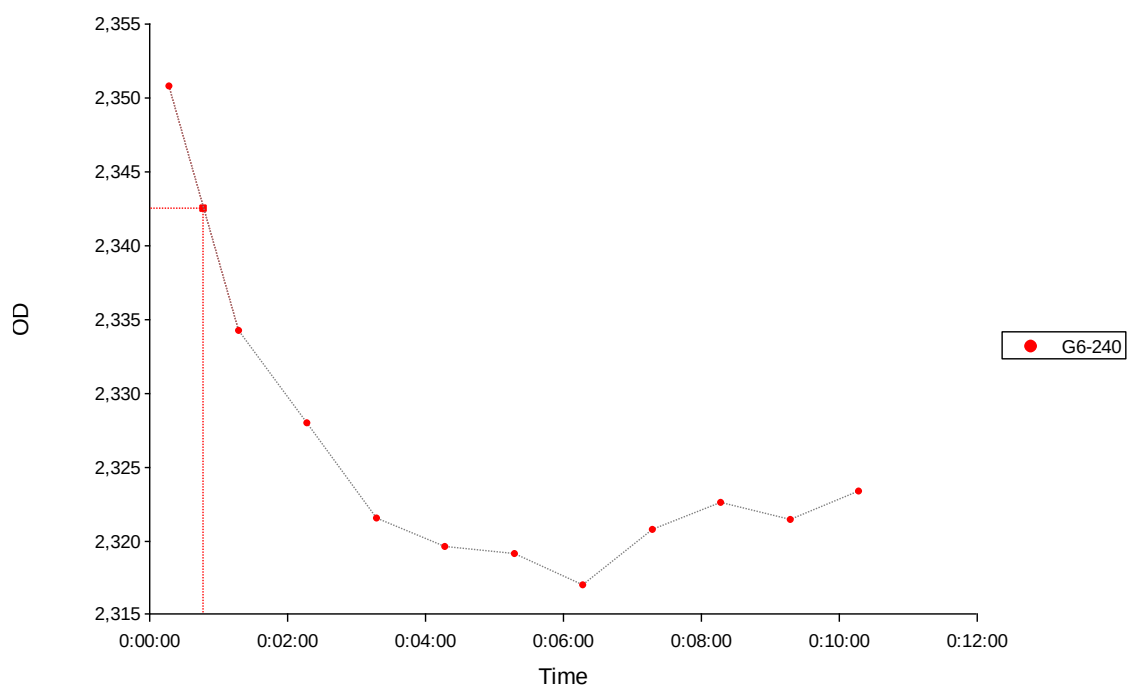


Figure 4.14 Time-dependent H₂O₂ decomposition for the tissue test sample.

4.5.1.6. Calculations

ΔOD were calculated from absorbance change during the desired reaction time ($\Delta OD_1 - \Delta OD_2$). ΔOD values were applied to the H_2O_2 standard curve to get concentration of H_2O_2 decomposed by catalase. Catalase activity can be calculated by following formula (Eq. 1.)

$$CAT \text{ Activity} = \frac{C_{H_2O_2}}{\Delta T \times V} = nmol/min/mL = mU/mL \quad (4.1)$$

C: decomposed H_2O_2 amount (nmol)

ΔT : Reaction time (min)

V: Pretreated sample volume (mL)

4.5.2 Determination of SOD Activity

SOD activity was determined by previously prescribed by Sun, Oberley & Li (1988) and adapted for the microplate reader. Determination of SOD activity was based on against blank reading in the presence of xanthine/xanthine oxidase reaction and nitroblue tetrazolium dye at 560 nm wavelength.

4.5.2.1 Reagents

- pH 7.5 Tris Buffer: 80mM Tris-HCl was prepared with H_2O and adding 93mg Na_2EDTA at the final volume of 500 mL. pH level was adjusted with adding 1M NaOH solution.
- Working Solution: This solution was included 0.3 mM Xanthine, 0.6 mM Na_2EDTA , 150 μ M nitroblue tetrazolium (NBT), 400 mM Na_2CO_3 , and 1 g/L Bovine Serum Albumin (BSA) at the final volume of 500 mL.
- Xanthine Oxidase: 167 U/L were prepared from the 5000 U/L stock.
- $CuCl_2$: 0.8 mM was prepared at the final volume of 100 mL.

4.5.2.2 Sample Preparations

Approximately 100 mg tissues were homogenized with 0.5 mL pH 8.0 Tris buffer by using tissue homogenizer. Buffered samples were placed microcentrifuge tubes. Homogenized samples were centrifuged on 10000 rpm for 30 minutes at 4°C. After centrifugation, supernatants were collected.

4.5.2.3 Measurements

Supernatants were placed to microplate cells at first. Working solution were added onto supernatants and then xanthine oxidase solution were added to the cells. After that, microplates were placed incubator at 25°C for 10 minutes. Readings were carried out at 560 nm wavelength against the blank.

4.5.2.4 Quality Control

Spectrum scanning were carried out to control reaction at desired spectral area (Figure 4.15).

4.5.2.5 Calculations

SOD activities were calculated based on inhibition rate. Percentage of inhibition rates were found according to the formula (Eq. 2). Sun et al. (1988) had been described that a unit of SOD activity inhibits NBT reduction by 50% (Eq. 3).

$$\text{Inhibition Rate (\%)} = \frac{OD_{blank} - OD_{sample}}{OD_{blank}} \times 100 \quad (4.2)$$

$$\text{SOD Activity } \left(\frac{U}{mL}\right) = \frac{\text{Inhibition Rate (\%)}}{50 \times 0.1} \quad (4.3)$$

(0.1: weight of sample)

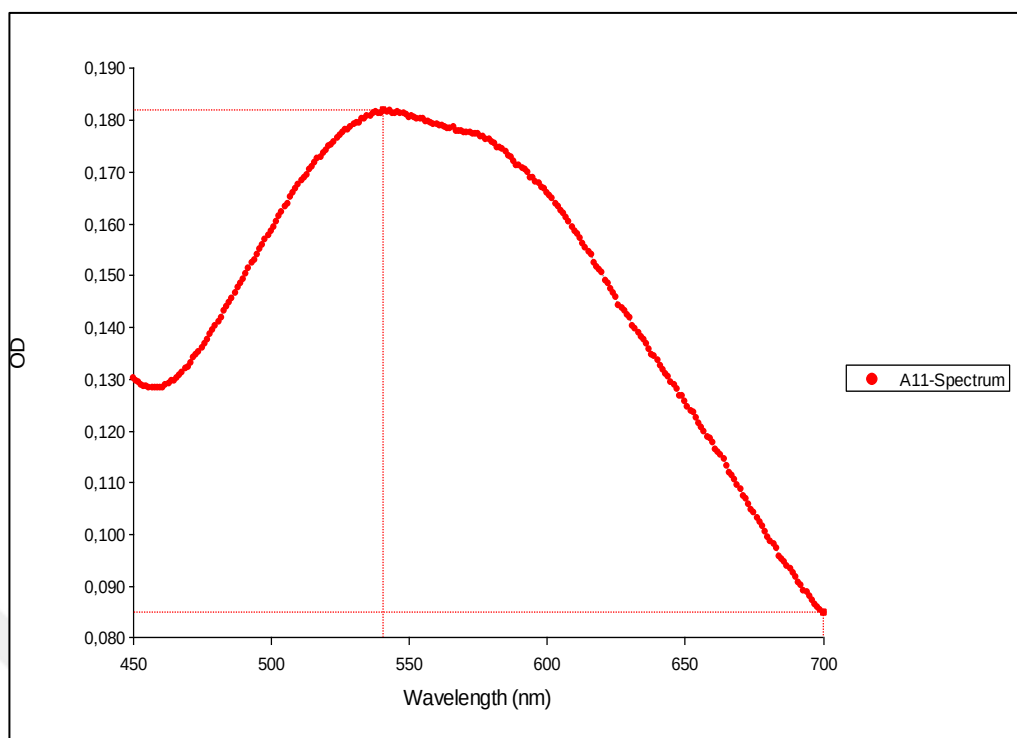


Figure 4.15 Spectral scanning of SOD reaction

4.5.3 Determination of Total Protein Quantitation

Total protein quantitation analysis was performed to calculate SOD and CAT activities as specific activities. Protein determination was measured at a wavelength of 562 nm by using the “Pierce™ BCA Protein Assay Kit” from ThermoFisher Scientific (Figure 4.16). The principal of this kit depends on Cu^{2+} reduction to Cu^{+} in the presence of bicinchoninic acid (BCA). Diluted albumin (BSA) was used for standard and curve was set 0 – 2000 $\mu\text{g/mL}$ (Figure 4.17) Total protein determinations were calculated from the curve. Quality control was carried out to confirm desired wavelength (Figure 4.18). As a result of protein determination, CAT and SOD results were calculated as mU/mg protein.

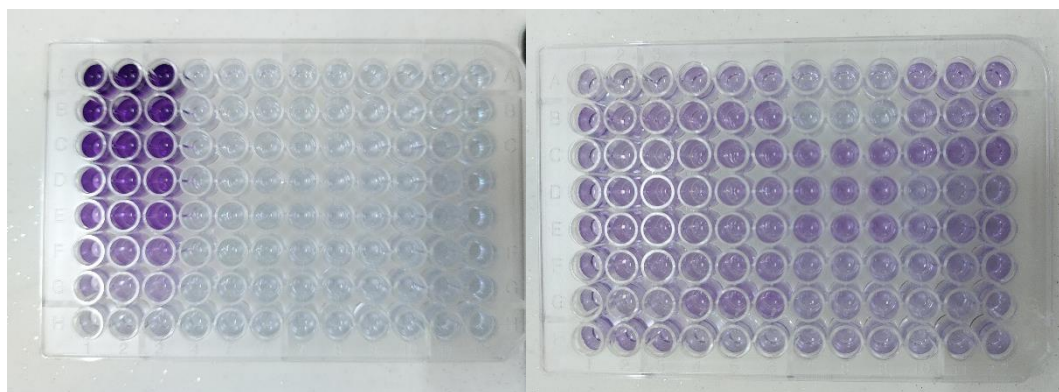


Figure 4.16 Image of protein determination standards and samples on microplates

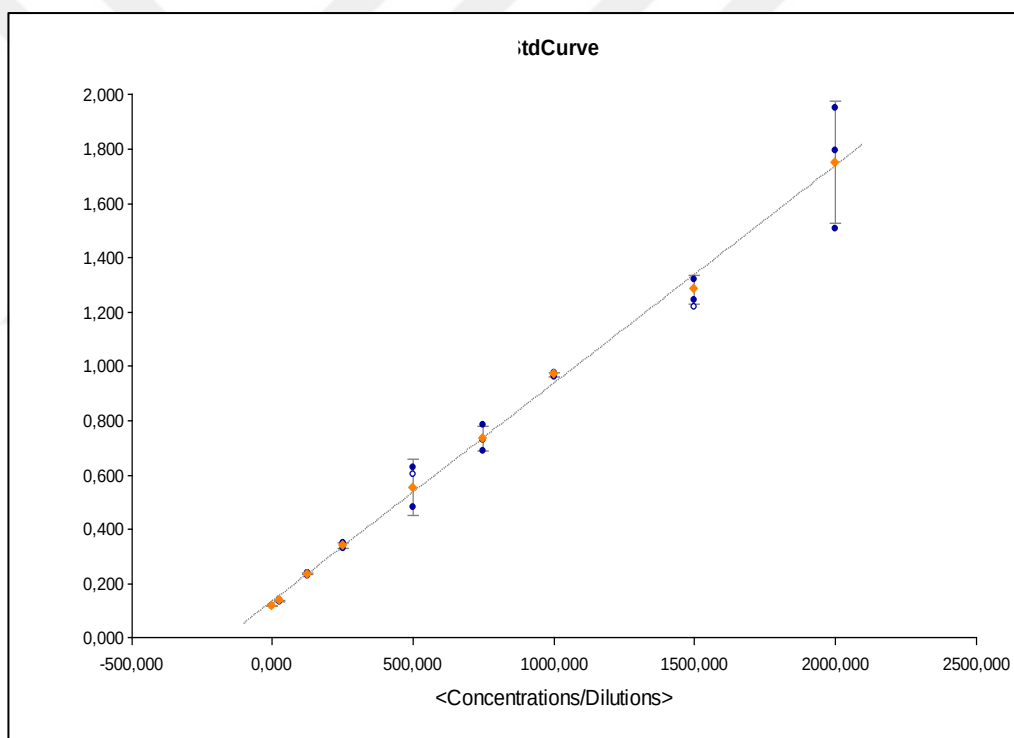


Figure 4.17 Standard curve of the BSA for determining protein quantitation.

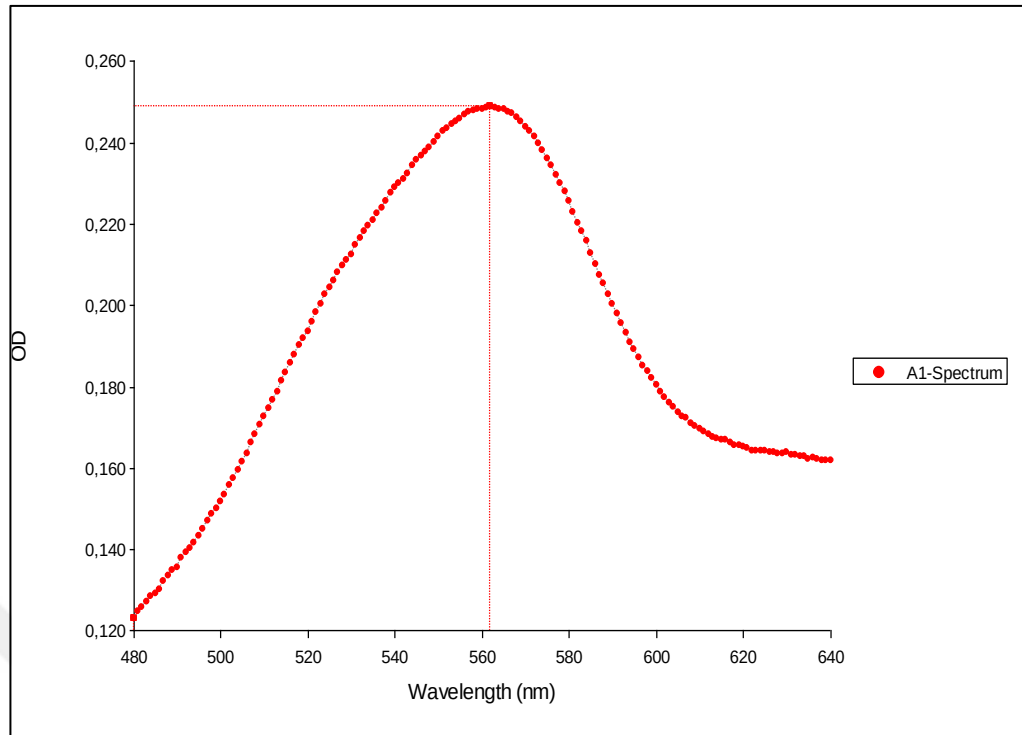


Figure 4.18 Spectrum scanning of BSA. Maximum OD was observed at 562 nm.

4.5.4 Determination of MDA Content

MDA content was determined by previously prescribed by Ohkawa, Ohishi, & Yagi (1979) and adapted on the microplate reader. The method was based on the reaction of lipid peroxides in tissue samples having reaction with thiobarbituric (TBA) acid at around pH 3.5 at 532nm wavelength.

4.5.4.1 Reagents

- pH 8.0 Tris Buffer: 50mM Tris-HCl was prepared with H₂O at the final volume of 100 mL. pH level was adjusted with adding 1M NaOH solution.
- 8.1% Sodium Dodecyl Sulfate (SDS) solution: 8.1g SDS was dissolved in 100 mL H₂O.

- 0.8% Thiobarbituric acid (TBA) solution: 0.8g TBA was dissolved in 100 mL H₂O.
- 20% Acetic Acid Solution: 100% stock solution diluted with ddH₂O to %20 into desired volume. pH level should be adjusted with NaOH solution to set between 3.00 to 3.50.
- 1,1,3,3 Tetramethoxypropan (TMP): 99% TMP solution was used for malondialdehyde source. Stock dilution was 6.01 M. TMP diluted to 1mM with ddH₂O for the standard preparation of analysis.

4.5.4.2 Sample Preparations

Firstly, 100 mg tissues were homogenized 0.3 mL pH 8.0 Tris buffer solution by using homogenizer. Homogenized samples were centrifuged on 10000 rpm for 15 minutes at 4°C. After centrifugation, supernatants were collected.

4.5.4.3 Standards

Serial standards were prepared with 1 mM TMP from 0 nmol to 5 nmol with 1 nmol intervals on the microcentrifuge tubes. Standards were treated same as samples. Standard curve can be seen at Figure 4.19.

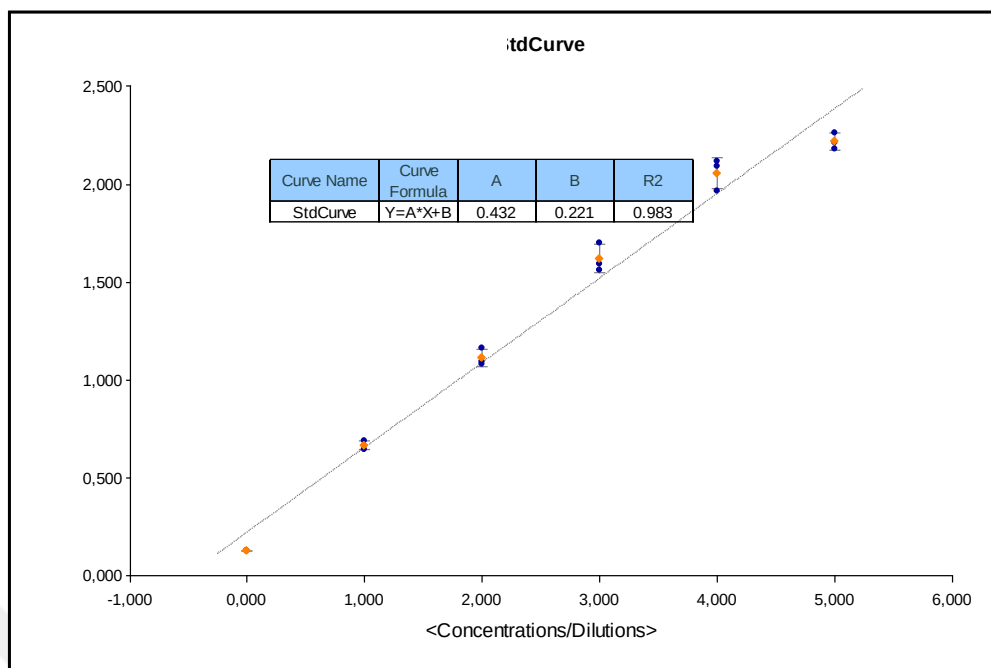


Figure 4.19 Standard curve of TMP

4.5.4.4 Development

0.1 mL of standards or samples, 0.2 mL SDS solution, 1.5 mL acetic acid solution, 1.5 mL TBA solution and 0.7 mL ddH₂O were added into the reaction vials. Vials were placed pretreated oven for 1 hour at 95°C for incubation. After the incubation, vials were cooled with tap water. At this point, increased color intensity of the standards was observed as a result of incubation (Figure 4.20). Around 1 mL suspension were placed into the centrifuged tubes. After centrifugation at 10000 rpm for 15 minutes, upper layer could be separated gently.

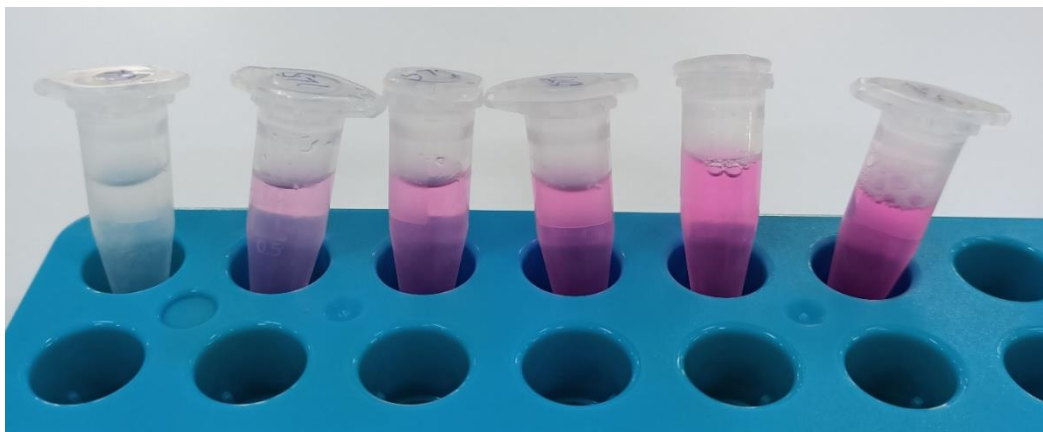


Figure 4.20 Series of MDA Standards in microcentrifuge tubes.

4.5.4.5 Measurements

200 μ L standard or samples at least triplicates were placed into the microplate wells. MDA contents were determined at 532 nm (Figure 4.21).

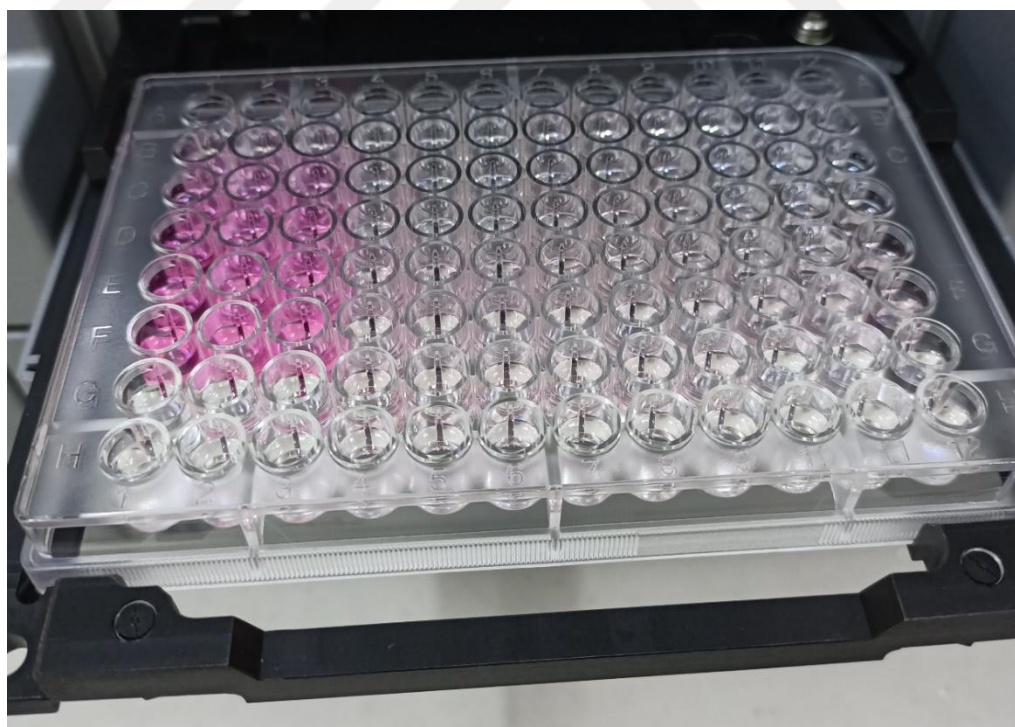


Figure 4.21 Standards (first three columns on the left) and samples of MDA contents in microplate

4.5.4.6 Quality Control

Spectrum scanning was performed for tracing the reaction of MDA on exact wavelength (Figure 4.22).

4.5.4.7 Calculations

OD₅₃₂ values were applied to the TMP standard curve to get concentration of malondialdehyde. MDA content can be calculated by following formula (Eq. 4)

$$MDA = \frac{C}{(mg/20)} = nmol\ MDA / mg\ tissue \quad (4.4)$$

C: MDA amount (nmol)

mg: Used sample amount from the beginning

20: Correction for using 200 μ L of 4 mL reaction mix.

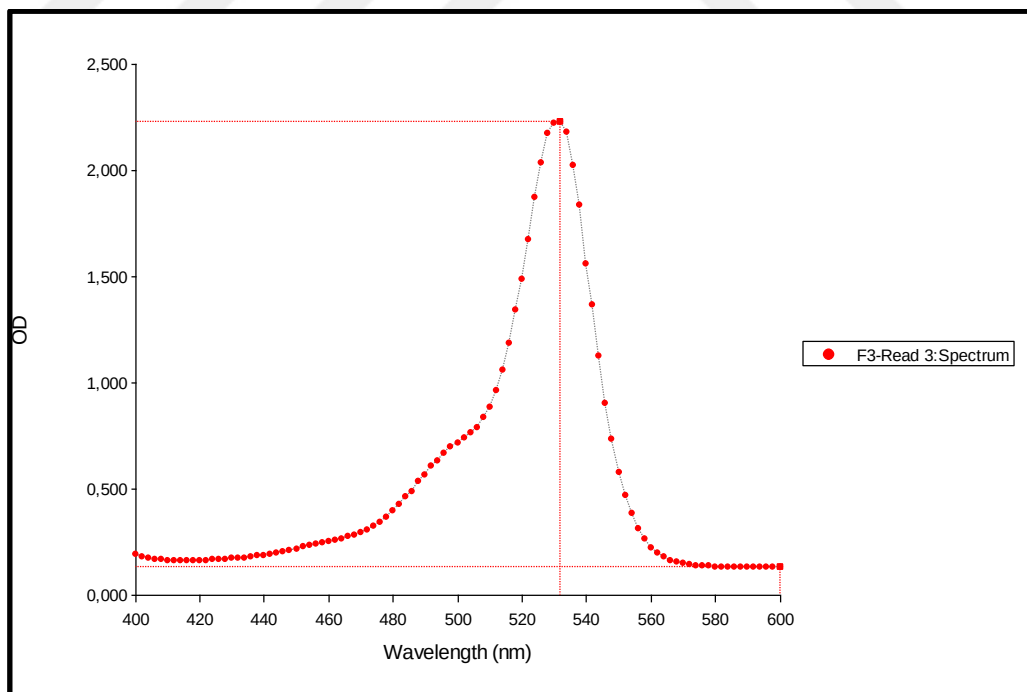


Figure 4.22 Spectrum scanning of TMP. Maximum OD was observed at 532 nm.

4.5.5 Determination of MT Contents

MT method was used prescribed by Viarengo, Ponzano, Dondero, & Fabbri (1997) and adapted onto microplate reader. This method was based on gathering MT phase with serial extractions using organic solvents. After that, MT phase was reacted with Ellman's Reagent (5,5-dithio-bis-(2-nitrobenzoic acid), DTNB in short) and MT contents were determined measuring against reduced glutathione.

4.5.5.1 Reagents

- Homogenization Buffer: This mixture was included 20 mM Tris-HCl (pH 8.6), 0.5M Sucrose, 0.005 mM Leupeptin, 0.5 mM Phenylmethylsulfonyl fluoride (PMSF), and 0.01% β -Mercaptoethanol (β -ME)
- Chloroform: Stock material was used
- Ethanol: Stock material was used. EtOH used in reactions as ice-cold absolute form.
- HCl: Stock material was used
- RNA: Lyophilized powder was used.
- NaCl: 0.25M was prepared (different from Ellman's Reagent Reaction Mix)
- HCl-EDTA mix: 4 mM EDTA was added to 1N HCl.
- Ellman's Reagent Reaction Mix: This mixture was included 2M NaCl and 0.43 mM DTNB buffered with Na-phosphate (pH=8.0).
- Reduced Glutathione (R-GSH): R-GSH was used as standard to determine MT content.

4.5.5.2 Sample Preparations

Dammed tissue samples (1 volume) were homogenized with homogenization buffer (3 volumes). Centrifugations were carried out at 15000 rpm for 1 hour at 4°C. After that, supernatants were collected (Figure 4.23).



Figure 4.23 Tissue preparation for determination of MTs

4.5.5.3 Standards

R-GSH was used as standard. Calibration curve set between 0 – 500 μM . Desired concentrations of R-GSH samples were treated NaCl, HCl-EDTA, and Ellman's Reagent Reaction Mix. Calibration curve and standards can be seen at Figure 4.24-25.

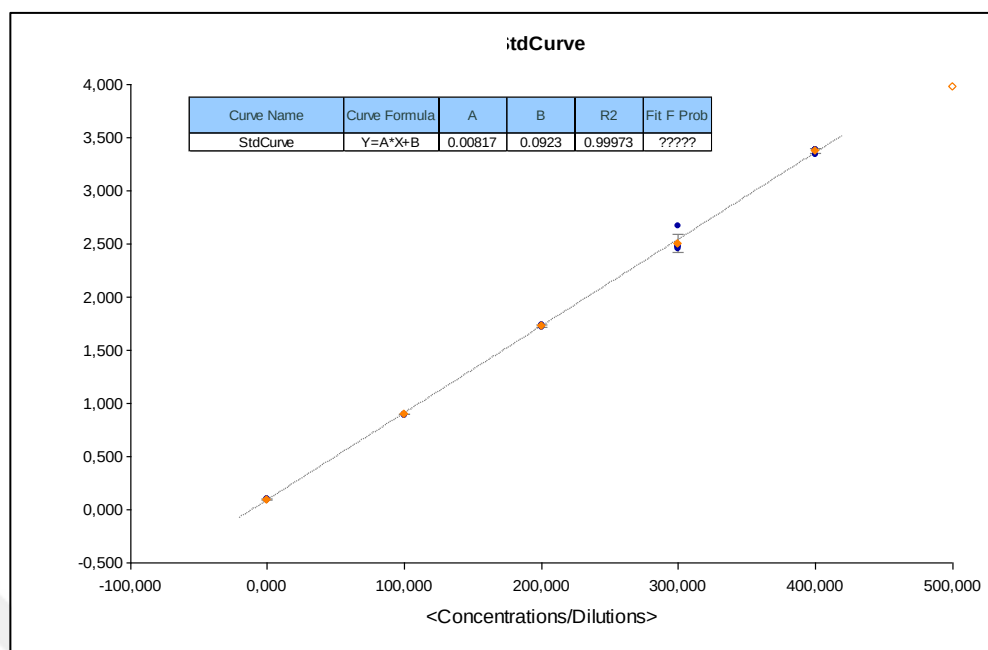


Figure 4.24 Calibration curve of MT determination

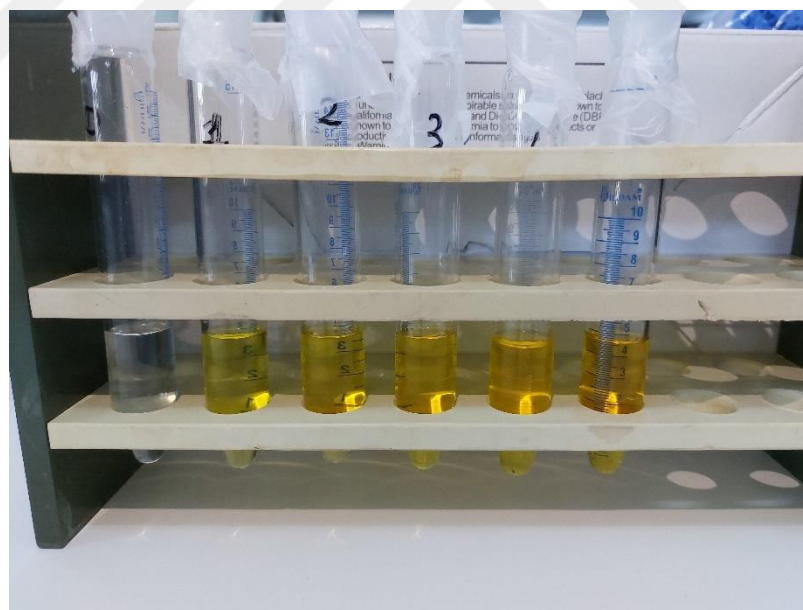


Figure 4.25 Calibration standards of MT determination

4.5.5.4 Development

After homogenization of tissue, Chloroform – ice-cold absolute EtOH mixture were added to gathered supernatants. Centrifugation were carried out (6000 rpm, 15 minutes, and at 4°C). Supernatants were gained again. A mixture (3 volumes) contained stock HCl, 1g RNA, and EtOH was added to supernatants (1 volume) in falcon tubes, and they were placed to the deep freezer (-20°C) for an hour. Suspended mix were centrifuged (6000 g, 15 minutes). Pellets were gathered after that. Then, pellets were treated with a washing mixture contained absolute ice-cold EtOH, chloroform, and homogenization buffer for suspension. The new suspensions were centrifuged (6000 rpm, 15 minutes). Pellets were dried under N₂ stream (Figure 4.26). Dried pellets were resuspended with the adding of NaCl and HCl-EDTA. Finally, Ellman's Reagent Reaction Mix was added to the samples. After final centrifugation (3000 rpm, 10 minutes) were carried out.

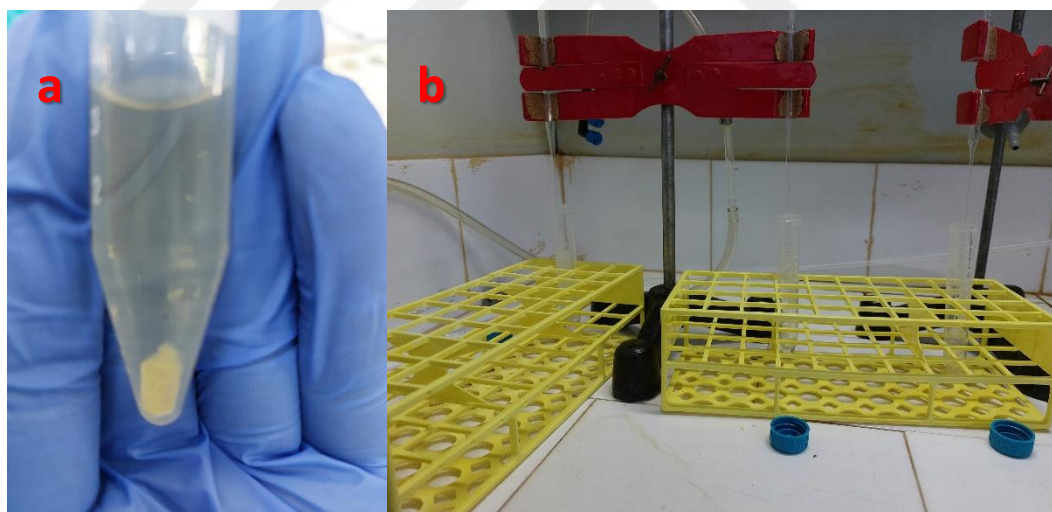


Figure 4.26 a) Forming pellet after centrifugation b) Pellets were treated with N₂ stream

4.5.5.5 Measurement

200 μ L of samples were pipetted to the well plates and MT contents were determined at 412 nm wavelength (Figure 4.27).

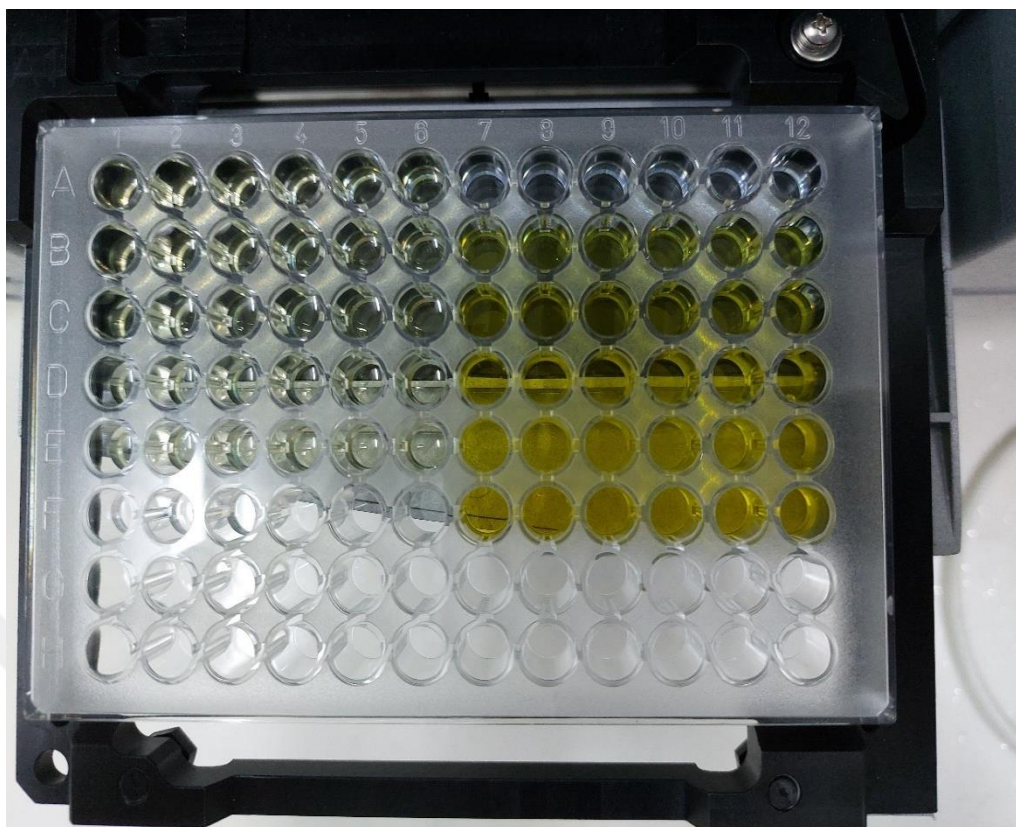


Figure 4.27 Standards of MT (R-GSH) before the determinations on microplate

4.5.5.6 Quality Control

Spectrum scanning was performed for tracing the reaction of MT on exact wavelength (Figure 4.28).

4.5.5.7 Calculations

GSH concentrations were provided by calibration curve. Viarengo et al. (1997) had claimed that 1 μM of GSH contains 0.055 μM MT. With this assumption, conversions from molarity to mass and then MT contents were calculated as “ $\mu\text{g MT /g}$ ”.

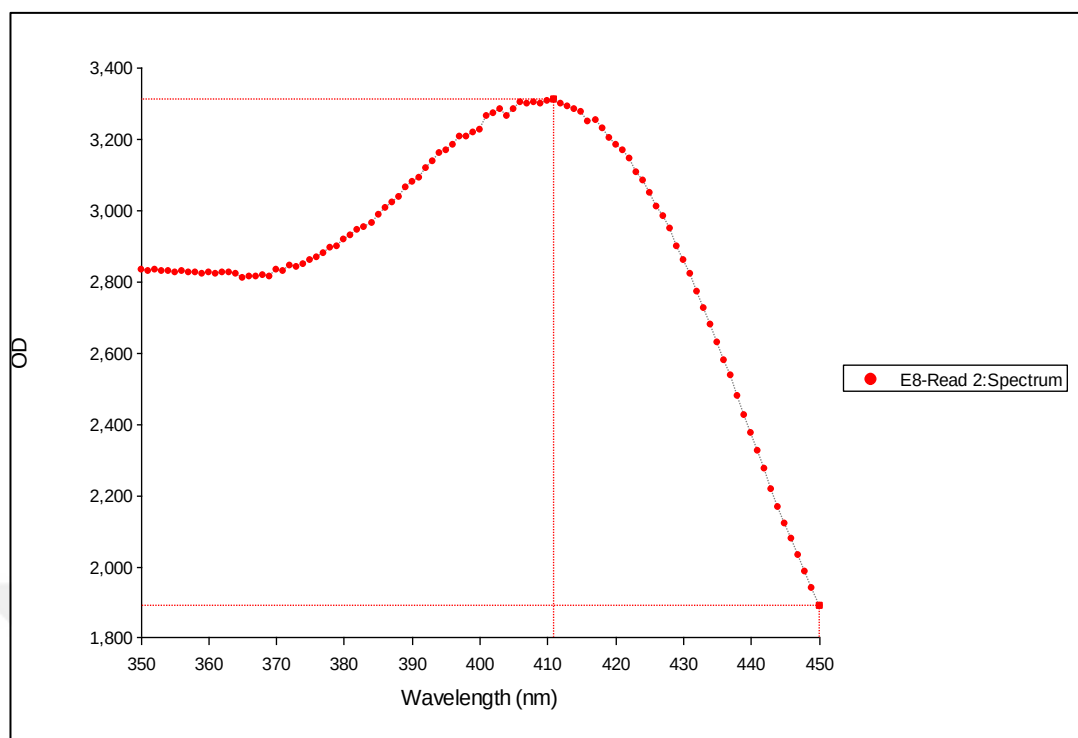


Figure 4.28 Spectrum scanning of R-GSH. Maximum OD was observed at 412 nm.

4.6 Statistical Approaches

Statistical calculations were carried out using JASP statistical software version 0.16.3.0 (JASP Team, 2022) and STATISTICA (version 12). Evaluation of metal and biomarker analyses; by using parametric (ANOVA) and non-parametric (Kruskal-Wallis) statistical tests, the interactions of the analysis results with each other were examined by considering the changes depending on the region, species and tissues ($p < 0.05$). Data were log-transformed where necessary to meet normal distribution and Levene's homogeneity tests. Multiple comparison tests were carried out. Statistics include all fish to distinguish variables. In addition, the interactions of metals and biomarkers were investigated by Pearson Product-Moment Correlation tests ($p < 0.05$). Significant R values were evaluated to investigate relationships. Principal Component Analysis (PCA) were carried out to evaluate the interaction between biomarker responses and metal bioaccumulation. Also, Network Analysis were carried out to exhibit interferences of sub-groups (metals and biomarkers) with each other.

4.7 Metal Pollution Index and Health Risk Assessments

Some indices have been used to evaluate the health risks that can reach people due to metal bioaccumulation and to examine the magnitude of combined metals. “Provisional Tolerable Weekly Intake” (PTWI) and “Target Cancer Risk” (TR) were used for health risk assessment, while “Metal Pollution Index” (MPI) was used for the combined effect of metals.

4.7.1 Metal Pollution Index (MPI)

Based on the obtained trace-metal concentrations, a metal pollution index (MPI) will be determined. This index represents the overall quality of environmental compartments with respect to metals and has shown to be useful in evaluating pollution levels. The metal pollution index (MPI) provides a comparison of the pollution statuses of different locations, i.e. the likely trace metal load (Jović & Stanković, 2014; Lafabrie et al., 2008; Usero et al., 2005). The equation (Eq. 5) was used as described by (Usero et al., 1996).

$$MPI = (C_1 \times C_2 \times C_3 \times \dots C_n)^{1/n} \quad (4.5)$$

4.7.2 Provisional Tolerable Weekly Intake (PTWI)

Provisional Tolerable Weekly Intake (PTWI) values will be calculated for evaluating potential health risks from human consumption. Concentrations of metals on edible tissues will be compared with international authorities' threshold levels.

PTWI values were estimated based on weekly consumption of 70-kg weighted adult person. Wet weight tissue concentrations were calculated via moisture ratio of species (*S. aurata*: 0.2844, *C. labrosus*: 0.2182, *D. vulgaris*: 0.2439) for assessing PTWI values.

Threshold levels were derived from Food and Agriculture Organization [FAO]/World Health Organization [WHO] and Agency for Toxic Substances and Disease Registry [ATSDR]. Limit values were used as: Hg: 0,35 mg/kg (FAO/WHO, 2004), Cd: 0,40 mg/kg (FAO/WHO, 2010), Pb: 1,75 mg/kg (FAO/WHO, 2004), Cr: 2,45 mg/kg (ATSDR, 2022), Cu: 245 mg/kg (FAO/WHO, 2007) (Zn: 490 mg/kg (FAO/WHO, 2007), Mn: 29,4 mg/kg (WHO, 2008), Se: 2.45 mg/kg (ATSDR, 2022), Sn: 980 mg/kg (FAO/WHO, 1989), As: 24.5 mg/kg (FAO/WHO, 1967), and V: 4.90 mg/kg (ATSDR, 2022).

4.7.3 The Lifetime Cancer Risk (TR)

The Lifetime Cancer Risk (TR) will be calculated for potential cancer risk from human consumption. This index is useful for only Cd, Pb and As. TR can be found with the following formula (Eq. 6) described by Bonsignore et al. (2018). No CSF values for other metals were established by USEPA.

$$TR = \frac{EF \times ED \times FIR \times C \times CSF}{WAB \times TA} \times 10^{-3} \quad (4.6)$$

Explanations of abbreviations were given as EF: exposure frequency (365 days per year); ED: average lifetime of Turkey (78.3 years) (Turkish Statistical Institute, 2019); FIR: fish ingestion rate (18.61 g/day per capita) (Republic of Türkiye Ministry of Agriculture and Forestry, 2021); C: concentration; CSF: cancer slope factor (CSF_{Cd}= 6.3 kg-day/mg and CSF_{Pb}= 8.5 × 10⁻³ kg-day/mg and CSF_{As}= 1.5 kg-day/mg) (USEPA, 2010; Bonsignore et al., 2018); average body weight (70 kg); TA: average exposure time (365 days/year × ED). According to the FAO (2014)'s establishment, acceptable risk levels of carcinogens were set at 10⁻⁴ – 10⁻⁶ interval (Keshavarzi et al., 2018).

CHAPTER FIVE

RESULTS

5.1 Metal Results

Eleven metals and metalloids concentrations of collected fish samples at muscle, liver and gills tissues from Foça (Outer Bay) and İnciraltı (Inner Bay) in three different sampling sessions during 2019 – 2021 were determined and given in Table 5.1 – 5.3 and Figure 5.1 – 5.11. All concentrations were represented as mg/kg dry weight.

5.1.1 Metal Concentrations in *Sparus aurata*

5.1.1.1 Muscle Tissue

In muscle tissues; the lowest concentrations of Pb (0.24 mg/kg), Zn (13.8 mg/kg) and Mn (0.25 mg/kg) were detected at November 2020 in Foça. Levels of Hg (0.094 mg/kg), Cd (0.0002 mg/kg), Cr (0.07 mg/kg), and Sn (0.07 mg/kg) were found at November 2020 in İnciraltı. Cu concentrations were determined lowest as 0.38 mg/kg at May 2021 at Foça. The minimum levels of Se (0.79 mg/kg), As (11.7 mg/kg) and, V (0.20 mg/kg) were detected in May 2021 at İnciraltı (Table 5.1).

Highest concentrations of Cd (0.0010 mg/kg), Cr (0.21 mg/kg), Cu (0.91 mg/kg), Zn (17.2 mg/kg), Se (1.26 mg/kg), and V (0.94 mg/kg) were detected at November 2019 in Foça. Mn levels were reached their maximum at November 2020 in İnciraltı as 0.83 mg/kg. The maximum concentrations of Hg (0.71 mg/kg), Cd (0.0010 mg/kg), Pb (0.69 mg/kg), Sn (0.49 mg/kg), and As (58.0 mg/kg) were obtained at May 2021 in Foça (Table 5.1).

Table 5.1 Ranges of metal concentrations (mg/kg dw.) in selected tissues of *S. aurata* from the İzmir Bay.

<i>S. aurata</i>		Hg	Cd	Pb	Cr	Cu	Zn	Mn	Se	Sn	As	V
Nov. 2019												
Foça												
	Muscle	0.67-0.68	0.0008-0.0010	0.28-0.37	0.17-0.21	0.69-0.91	16.6-17.2	0.50-0.81	1.02-1.26	0.19-0.28	36.3-42.5	0.73-0.94
	Liver	0.437-0.438	1.34-1.41	3.61-3.98	0.49-0.58	77.5-79.6	367-368	12.9-13.3	2.99-3.02	0.51-0.52	30.7-34.2	6.29-6.32
	Gills	0.147-0.148	0.0036-0.0039	2.18-2.21	1.18-1.30	2.83-2.99	86.4-91.8	26.1-26.7	4.11-4.68	1.82-1.98	6.51-6.79	8.21-9.24
Nov. 2020												
Foça												
	Muscle	0.55-0.61	0.0004-0.0007	0.24-0.26	0.12-0.15	0.75-0.80	13.8-14.7	0.25-0.39	0.87-1.16	0.09-0.22	19.1-19.3	0.32-0.36
	Liver	0.538-0.540	1.28-1.36	2.40-2.58	0.61-0.63	51.9-54.6	254-272	6.97-7.60	3.66-4.03	0.65-0.69	18.5-17.4	6.13-6.26
	Gills	0.10-0.12	0.0040-0.0042	1.13-1.21	1.23-1.37	1.79-2.11	63.0-98.0	17.0-17.3	2.44-2.89	1.20-1.23	5.46-6.36	5.66-6.58
İnciraltı												
	Muscle	0.094-0.095	0.0002-0.0002	0.45-0.64	0.07-0.12	0.49-0.56	15.2-15.8	0.55-0.83	1.01-1.10	0.07-0.12	17.1-18.9	0.28-0.31
	Liver	0.09-0.10	0.49-0.53	1.44-1.65	0.31-0.45	15.7-16.6	110-118	4.59-5.42	2.31-2.43	0.30-0.32	11.8-12.4	1.52-1.78
	Gills	0.05-0.06	0.0021-0.0023	1.30-1.37	1.17-1.31	2.92-3.09	64.6-65.2	11.9-13.2	2.51-2.68	0.74-0.92	4.81-5.46	2.63-3.14
May 2021												
Foça												
	Muscle	0.66-0.71	0.0008-0.0010	0.28-0.69	0.10-0.14	0.38-0.56	15.1-16.5	0.45-0.58	0.96-1.24	0.26-0.49	46.7-58.0	0.81-0.93
	Liver	0.81-0.84	1.37-1.83	2.32-2.34	0.29-0.30	60.0-61.9	212-219	8.81-9.08	4.30-4.68	0.62-0.63	33.0-39.1	1.03-1.03
	Gills	0.20-0.24	0.006-0.007	0.85-0.90	1.12-1.34	3.18-3.36	78.2-78.6	20.2-21.0	4.30-4.45	1.48-1.69	6.09-6.34	4.92-6.08
İnciraltı												
	Muscle	0.17-0.18	0.0005-0.0005	0.26-0.28	0.08-0.09	0.64-0.69	13.9-16.1	0.58-0.75	0.79-0.96	0.30-0.38	11.7-12.4	0.20-0.26
	Liver	0.21-0.24	1.56-1.76	1.75-1.76	0.37-0.38	19.7-20.6	144-147	6.62-6.77	2.69-2.90	0.47-0.59	16.3-16.9	0.81-0.83
	Gills	0.05-0.08	0.0017-0.0022	0.94-1.13	0.61-0.62	3.86-4.20	78.5-80.6	15.5-19.8	6.11-6.40	1.60-1.66	2.97-3.21	3.53-3.81

5.1.1.2 Liver

For liver tissues; minimum concentrations of Hg (0.093 mg/kg), Cd (0.49 mg/kg), Pb (1.44 mg/kg), Cu (15.7 mg/kg), Zn (110 mg/kg), Mn (4.59 mg/kg), Sn (0.30 mg/kg), Se (2.31 mg/kg) and As (11.8 mg/kg) were found at November 2020 in İnciraltı. While the lowest concentrations for Cr (0.29 mg/kg) were found at May 2021 in Foça, minimum V concentrations (0.81 mg/kg) were determined at May 2021 in İnciraltı (Table 5.1).

Maximum concentrations of Pb (3.98 mg/kg), Cu (79.6 mg/kg), Zn (368 mg/kg), Mn (13.3 mg/kg), and V (6.32 mg/kg) were obtained at November 2019 in Foça. Also, the highest levels of Cr (0.63 mg/kg) and Sn (0.69 mg/kg) were found at November 2020 in Foça. In addition, the maximum levels of Hg (0.84 mg/kg), Cd (1.83 mg/kg), Se (4.68 mg/kg), and As (39.1 mg/kg) were detected for at May 2021 at Foça for livers (Table 5.1).

5.1.1.3 Gills

At gills, minimum concentrations of Cu (1.79 mg/kg), Zn (63.0 mg/kg), and Se (2.44 mg/kg) were detected at November 2020 in Foça. On the other hand, the lowest levels of Hg (0.05 mg/kg), Mn (11.9 mg/kg), Sn (0.74 mg/kg), and V (2.63 mg/kg) were found at November 2020 in İnciraltı. Besides these, the lowest Pb (0.85 mg/kg) and Cr (1.12 mg/kg) concentrations were obtained at May 2021 in Foça. Last but not least, Cd (0.0017 mg/kg) and As (2.97 mg/kg) levels were determined at May 2021 in İnciraltı (Table 5.1).

The highest levels of Pb (2.21 mg/kg), Mn (26.7 mg/kg), Sn (1.98 mg/kg), As (6.79 mg/kg), and V (9.24 mg/kg) were found at November 2019 at Foça, while maximum concentrations of Cr (1.37 mg/kg) and Zn (98.0 mg/kg) were detected at November 2020 in this area. In addition, Hg (0.24 mg/kg) and Cd (0.007 mg/kg) concentrations showed their maximum at May 2021 in Foça. Lastly, the highest concentrations of Cu

(4.20 mg/kg) and Se (6.40 mg/kg) were obtained at May 2021 in İnciraltı for gills of *S. aurata* (Table 5.1).

5.1.2 Metal Concentrations in *Chelon labrosus*

5.1.2.1 Muscle Tissue

The lowest concentrations of Cu (0.55 mg/kg) and Mn (0.18 mg/kg) were found at November 2019 in Foça. On the other hand, minimum levels of Hg (0.01 mg/kg), Pb (0.20 mg/kg), and Se (0.53 mg/kg) were detected at November 2019 in İnciraltı. Besides, minimum levels of Cr (0.07 mg/kg), Zn (11.1 mg/kg), Sn (0.11 mg/kg), As (2.93 mg/kg), and V (0.18 mg/kg) detected at November 2020 in İnciraltı. Cd concentrations were found its lowest point as 0.0003 mg/kg at May 2021 in İnciraltı (Table 5.2).

Maximum levels of Hg (0.27 mg/kg), Cr (0.69 mg/kg), and V (0.75 mg/kg) were found at November 2019 in Foça but, Cd concentrations were found the highest as 0.002 mg/kg at Foça 2019 in İnciraltı. Both Cu (2.40 mg/kg), and Zn (21.9 mg/kg) showed highest levels at November 2020 in Foça. Mn concentrations were reached their maximum values as 0.95 mg/kg at November 2020 in İnciraltı. Pb (0.61 mg/kg) and Se (1.33 mg/kg) were detected the highest concentration at Foça, while Sn (0.48 mg/kg) were obtained at İnciraltı at May 2021 for muscle tissues (Table 5.2).

5.1.2.2 Liver

Minimum values of Hg (0.056 mg/kg) and As (9.11 mg/kg) were detected at November 2019 in Foça. In addition, the lowest concentrations of Cd (2.40 mg/kg), Pb (1.59 mg/kg), Cu (56.1 mg/kg), and Sn (0.29 mg/kg) were obtained at November 2019 in İnciraltı. Results show that, Cr (0.30 mg/kg) and Se (2.46 mg/kg) concentrations were showed their lowest at November 2020 in Foça. Besides, minimum levels of Mn (3.10 mg/kg) and Zn (81.1 mg/kg) were determined at May

2021 in Foça. Lastly, the lowest levels of V (0.81 mg/kg) were found at May 2021 in İnciraltı (Table 5.2).

Most of the highest concentrations of livers were detected at November 2019 in İnciraltı. Hg (0.51 mg/kg), Cr (1.50 mg/kg), Cu (775 mg/kg), Mn (4.36 mg/kg), Zn (180 mg/kg), Sn (0.60 mg/kg), Se (13.7 mg/kg), and As (19.6 mg/kg) were found at this sampling period. Also, V concentrations were obtained the highest as 3.68 mg/kg at November 2020 in Foça, when maximum Cd (5.94 mg/kg) and Pb (4.38 mg/kg) concentrations were determined at May 2021 in Foça for livers (Table 5.2).

5.1.2.3 Gills

Hg concentrations showed their minimum concentrations as 0.015 mg/kg at November 2019 and May 2021 in İnciraltı and at November 2020 in Foça. Apart from these, the lowest concentrations of Cu (2.54 mg/kg) were detected at November 2019 in İnciraltı. Minimum values of Sn (1.13 mg/kg), Se (2.58 mg/kg), and V (1.90 mg/kg) were found at November 2020 in İnciraltı. The lowest concentrations of Mn (21.5 mg/kg) and Zn (50.3 mg/kg) were determined at May 2021 in Foça, however, the lowest concentrations of Cd (0.0016 mg/kg), Pb (0.86 mg/kg), and Cr (0.30 mg/kg) were obtained at May 2021 in İnciraltı (Table 5.2).

The highest values of Hg (0.064 mg/kg) and V (6.37 mg/kg) were found at November 2019 in Foça. The maximum concentrations of Cu (6.79 mg/kg), Se (6.23 mg/kg), and As (2.99 mg/kg) were detected at November 2019 in İnciraltı. On the other hand, highest levels of Cd (0.006 mg/kg), Cr (2.15 mg/kg), Zn (90.8 mg/kg), and Sn (2.04 mg/kg), were obtained at November 2020 in Foça. Mn concentrations were determined maximum as 38.4 mg/kg November 2020 in İnciraltı. Only Pb levels reached their maximum at May 2021 in Foça as 12.1 mg/kg for the gills of *C. labrosus* (Table 5.2).

Table 5.2 Ranges of metal concentrations (mg/kg dw.) in selected tissues of *C. labrosus* from the İzmir Bay.

<i>C. labrosus</i>		Hg	Cd	Pb	Cr	Cu	Zn	Mn	Se	Sn	As	V
Nov. 2019												
Foça	Muscle	0.25-0.27	0.001-0.002	0.25-0.33	0.12-0.69	0.55-0.66	12.4-14.7	0.18-0.20	0.55-0.60	0.33-0.36	14.5-14.8	0.64-0.75
	Liver	0.056-0.058	3.19-3.66	2.17-2.30	1.15-1.44	567-568	162-165	3.20-3.24	4.08-4.10	0.52-0.54	9.11-10.3	2.67-2.91
	Gills	0.055-0.064	0.003-0.004	3.55-4.28	0.50-0.53	2.62-2.84	56.3-61.9	31.0-33.2	4.18-4.67	1.15-1.35	1.60-2.10	5.76-6.37
İnciraltı	Muscle	0.01-0.03	0.001-0.002	0.20-0.34	0.23-0.35	0.95-1.72	11.5-19.0	0.47-0.88	0.53-0.67	0.16-0.33	7.07-8.72	0.23-0.36
	Liver	0.22-0.51	2.40-4.58	1.59-1.89	0.52-1.50	56.1-775	109-180	3.78-4.36	2.60-13.7	0.29-0.60	17.1-19.6	1.12-1.59
	Gills	0.015-0.026	0.0038-0.0040	2.56-2.73	0.49-0.52	2.54-6.79	54.4-59.8	23.4-30.0	4.35-6.23	1.17-1.52	0.89-2.99	2.26-3.84
Nov. 2020												
Foça	Muscle	0.031-0.034	0.0006-0.0007	0.26-0.33	0.19-0.26	1.60-2.40	16.9-21.9	0.50-0.53	1.03-1.26	0.13-0.42	3.41-4.11	0.43-0.56
	Liver	0.14-0.15	3.55-4.00	2.12-2.42	0.30-0.34	144-155	154-174	4.02-4.34	2.46-2.71	0.42-0.46	15.3-17.8	3.54-3.68
	Gills	0.015-0.016	0.005-0.006	7.61-8.40	1.57-2.15	3.36-4.06	88.9-90.8	32.7-35.1	4.37-4.48	1.99-2.04	2.12-2.26	4.33-4.84
İnciraltı	Muscle	0.069-0.073	0.0010-0.0014	0.26-0.31	0.07-0.12	0.89-1.14	11.1-12.2	0.56-0.95	0.67-0.96	0.11-0.25	2.93-3.41	0.18-0.23
	Liver	0.14-0.19	3.26-3.64	2.13-2.37	0.61-0.76	385-419	110-123	3.14-3.45	4.15-4.74	0.54-0.55	13.8-14.6	1.49-1.53
	Gills	0.022-0.023	0.004-0.005	5.97-6.63	1.00-1.24	3.91-3.98	81.2-85.9	36.6-38.4	2.58-2.66	1.13-1.32	1.85-2.13	1.90-1.99
May 2021												
Foça	Muscle	0.12-0.13	0.001-0.002	0.45-0.61	0.08-0.30	0.65-0.74	13.1-13.9	0.58-0.88	1.18-1.33	0.13-0.25	12.6-15.1	0.41-0.50
	Liver	0.18-0.21	5.82-5.94	4.07-4.38	0.57-0.58	206-210	81.1-78.4	3.10-3.79	5.93-6.19	0.51-0.55	15.9-18.1	1.63-1.65
	Gills	0.05-0.06	0.0045-0.0046	12.0-12.1	1.24-1.62	5.36-5.98	50.3-53.0	21.5-24.9	4.587-4.593	1.48-1.67	2.60-2.79	4.871-4.874
İnciraltı	Muscle	0.050-0.053	0.0003-0.0005	0.33-0.40	0.23-0.37	0.69-0.86	12.1-13.2	0.43-0.56	0.54-0.66	0.40-0.48	4.88-5.29	0.20-0.24
	Liver	0.44-0.49	5.30-5.92	2.13-2.18	0.52-0.59	56.4-57.9	152-158	3.90-3.98	3.17-3.56	0.41-0.45	15.1-15.2	0.81-0.83
	Gills	0.015-0.015	0.0016-0.0019	0.86-0.89	0.30-0.31	4.13-4.19	61.6-62.1	30.5-31.0	4.65-5.14	1.30-1.51	0.50-0.56	2.65-2.80

5.1.3 Metal Concentrations in *Diplodus vulgaris*

D. vulgaris sampling was carried out only at November 2019 in Foça. The highest concentrations of Hg (0.60 mg/kg) were detected at muscle tissues. The highest Cu (41.3 mg/kg), Cd (3.82 mg/kg), Zn (165.9 mg/kg), and As (22.0 mg/kg) levels were obtained at liver, while the highest Pb (16.8 mg/kg), Cr (0.75 mg/kg), Mn (25.3 mg/kg), Se (4.96 mg/kg), Sn (1.507 mg/kg), and V (7.97 mg/kg) levels were found at gills (Table 5.3).

Minimum concentrations of Cd (0.0021 mg/kg), Pb (1.00 mg/kg), Cr (0.11 mg/kg), Cu (1.34 mg/kg), Zn (18.4 mg/kg), Mn (1.09 mg/kg), Se (0.89 mg/kg), Sn (0.21 mg/kg), and V (0.14 mg/kg) were detected at muscle tissues. However, minimum concentrations of Hg (0.11 mg/kg) and As (3.11 mg/kg) were detected at gills (Table 5.3).

Table 5.3 Ranges of metal concentrations (mg/kg dw.) in selected tissues of *D. vulgaris* from the İzmir Bay.

<i>D. vulgaris</i>	Hg	Cd	Pb	Cr	Cu	Zn	Mn	Se	Sn	As	V
Nov. 2019											
Foça											
Muscle	0.57-0.60	0.0021-0.0023	1.00-1.16	0.11-0.12	1.34-1.63	18.4-18.7	1.09-1.25	0.89-1.06	0.21-0.54	11.5-12.4	0.14-0.16
Liver	0.20-0.22	3.30-3.82	9.78-10.3	0.43-0.58	40.9-41.3	165.5-165.9	9.05-9.36	3.46-3.74	0.71-0.86	21.4-22.0	2.67-2.91
Gills	0.11-0.12	0.0040-0.0042	16.2-16.8	0.70-0.75	2.44-3.13	84.5-91.7	24.2-25.3	4.54-4.96	1.505-1.507	3.11-4.14	7.00-7.97

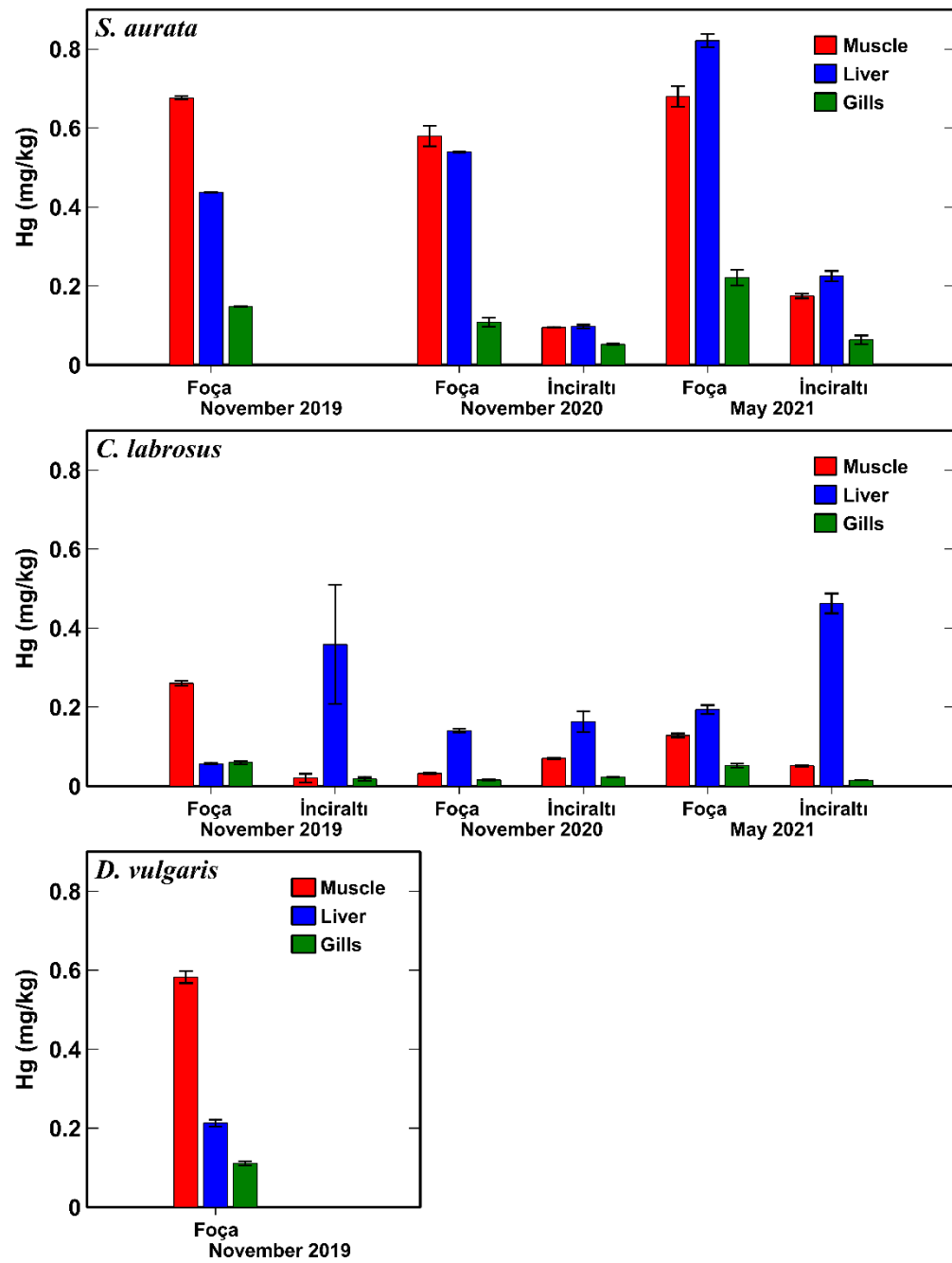


Figure 5.1 Hg concentrations (mg/kg $\pm$ SD) of tissues on fish species.

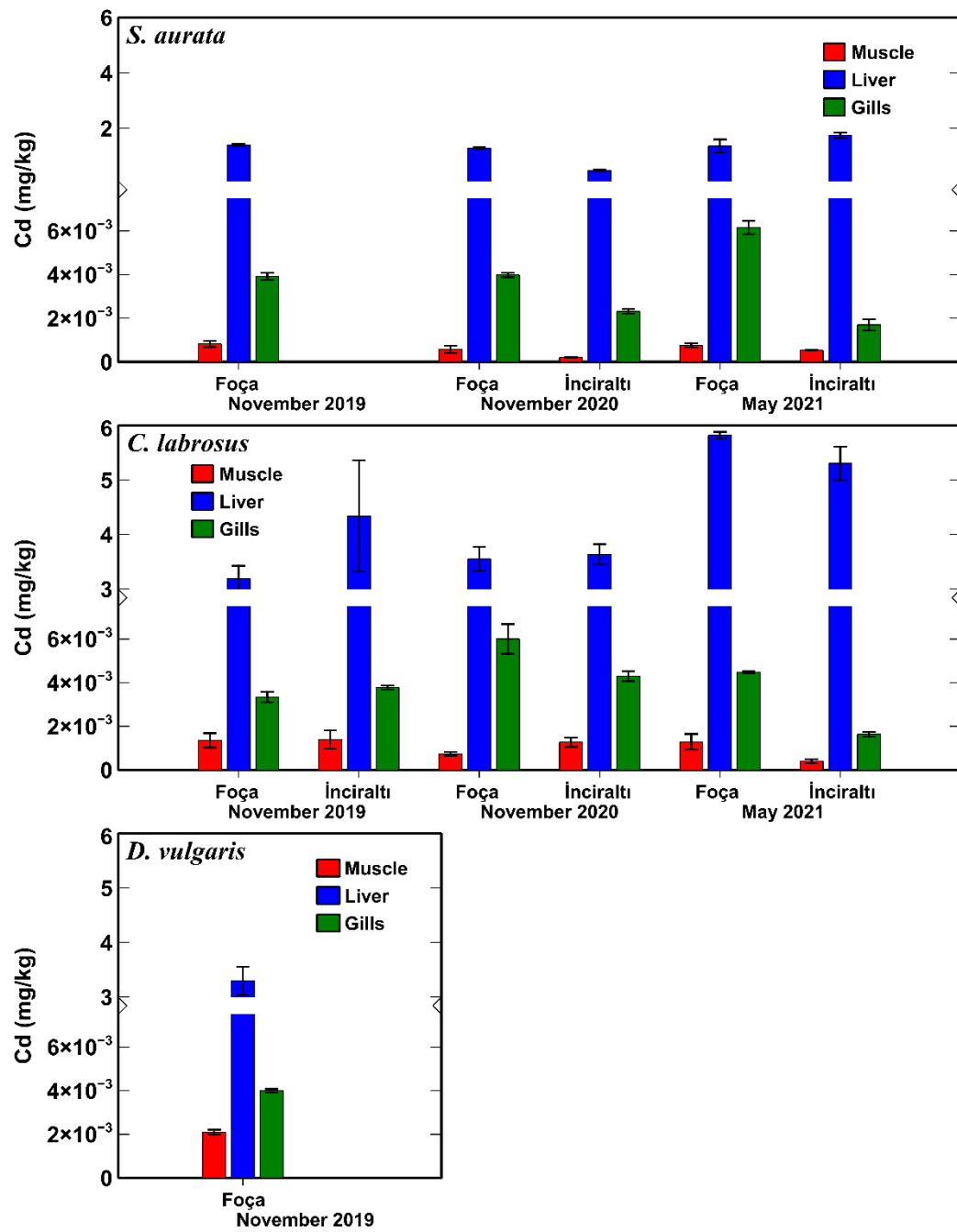


Figure 5.2 Cd concentrations (mg/kg $\pm$ SD) of tissues on fish species.

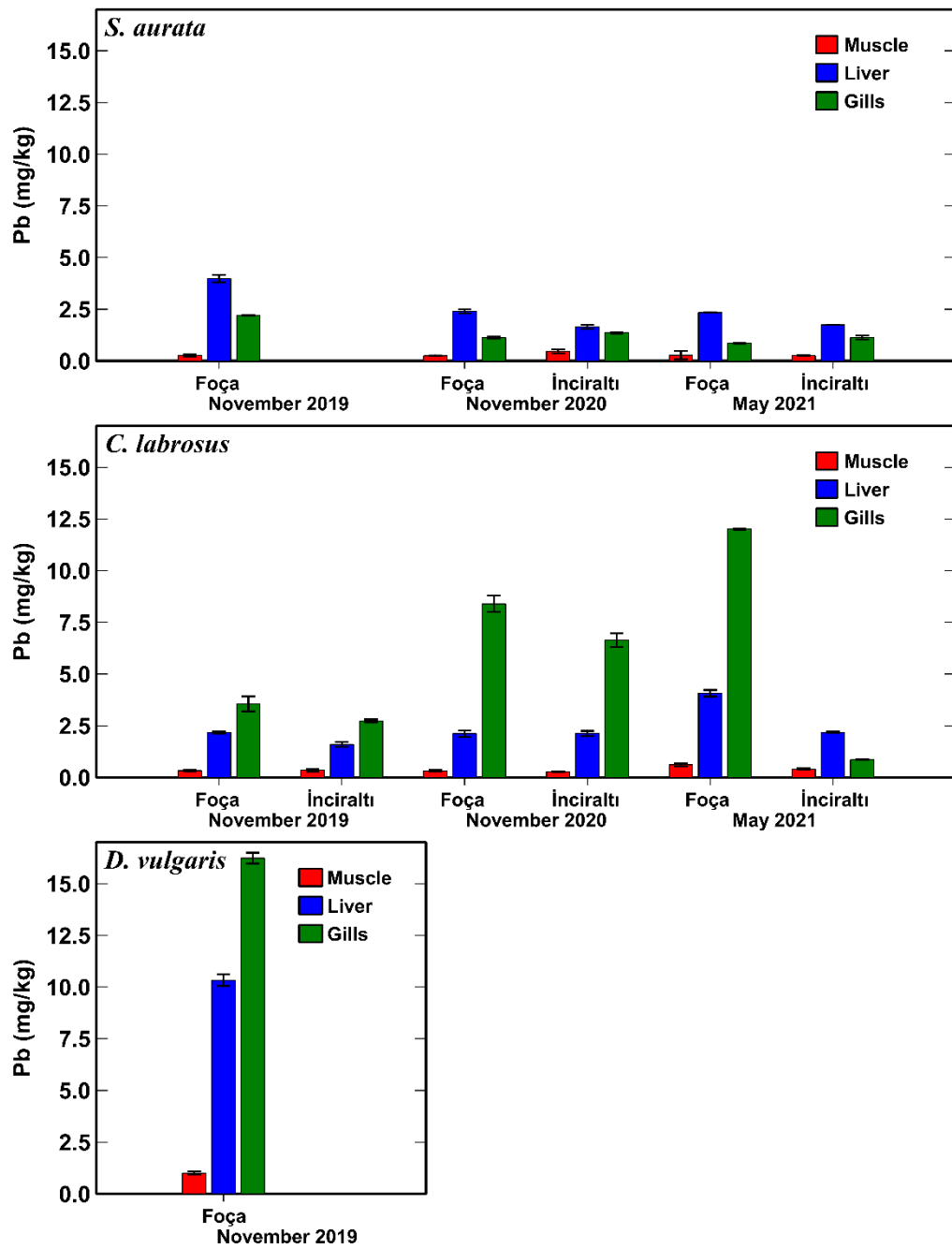


Figure 5.3 Pb concentrations (mg/kg $\pm$ SD) of tissues on fish species.

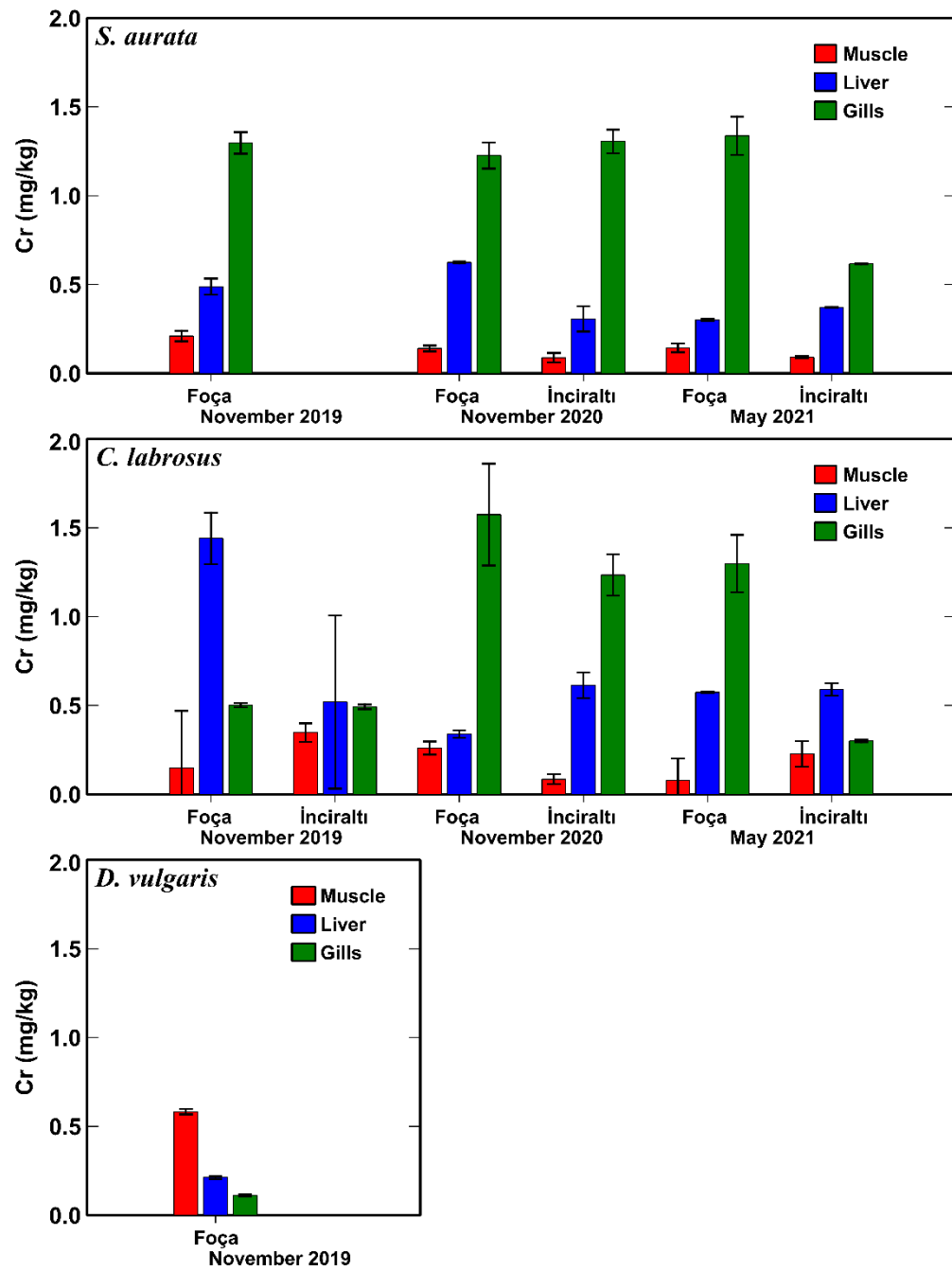


Figure 5.4 Cr concentrations (mg/kg $\pm$ SD) of tissues on fish species.

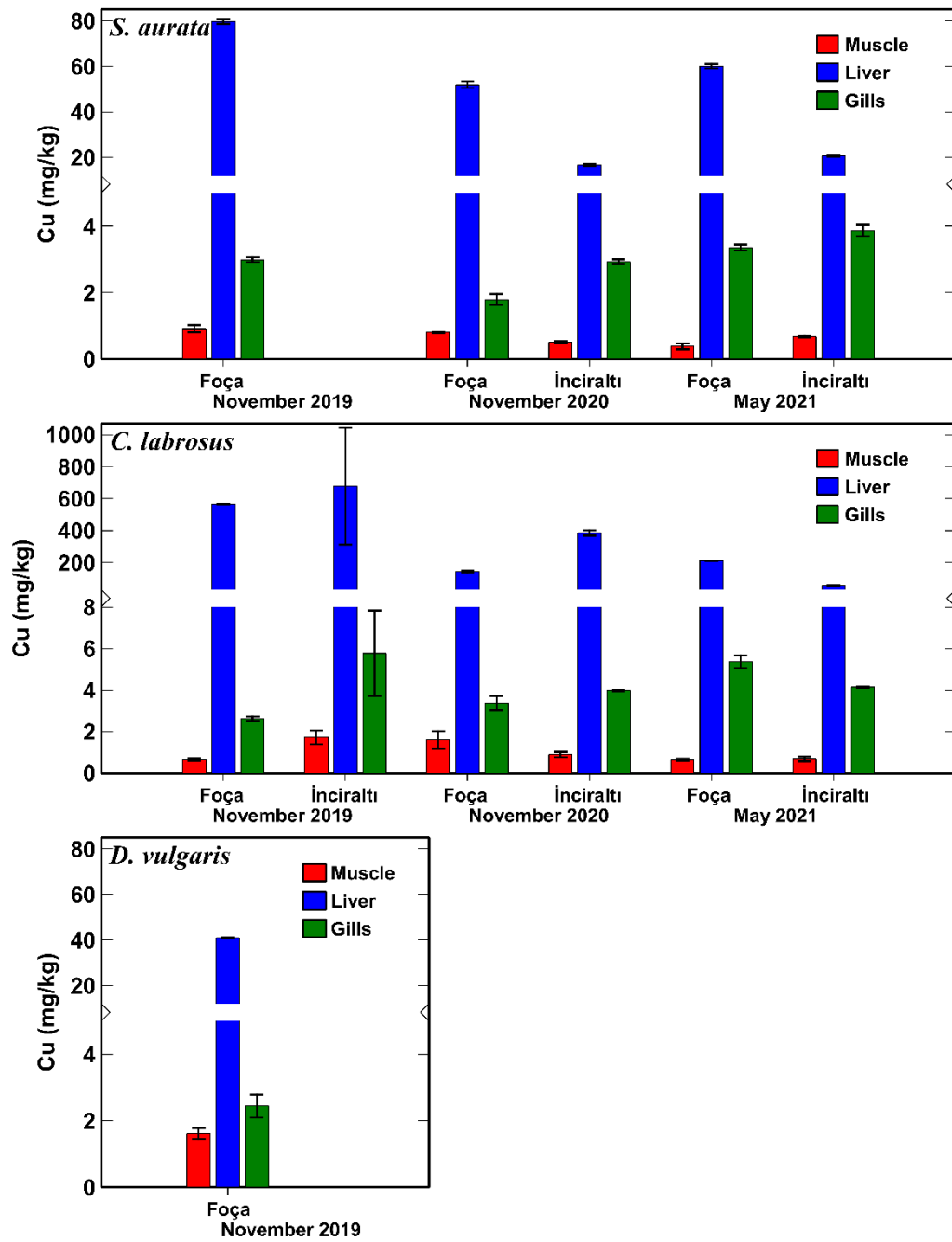


Figure 5.5 Cu concentrations (mg/kg $\pm$ SD) of tissues on fish species.

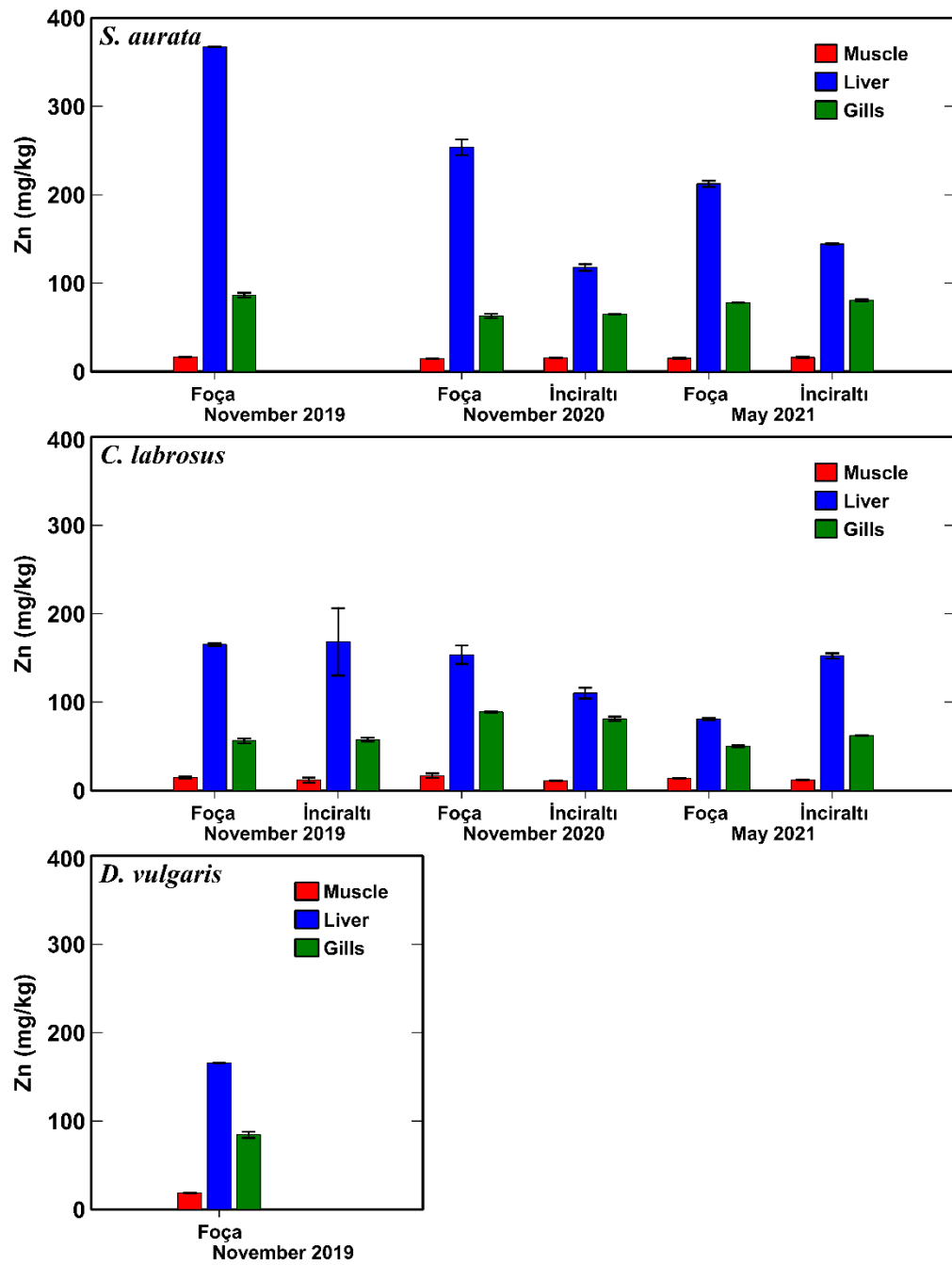


Figure 5.6 Zn concentrations (mg/kg $\pm$ SD) of tissues on fish species.

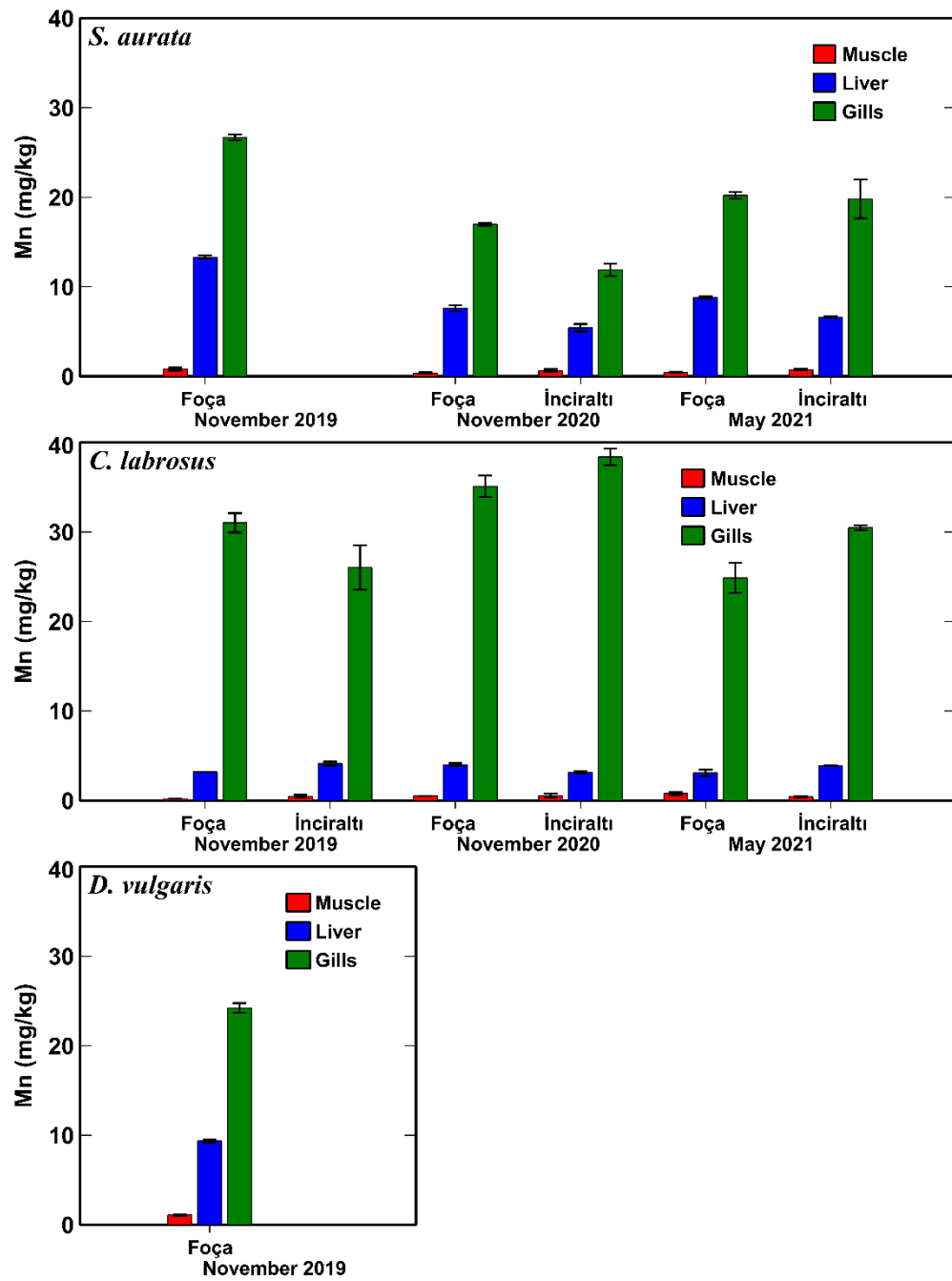


Figure 5.7 Mn concentrations (mg/kg $\pm$ SD) of tissues on fish species.

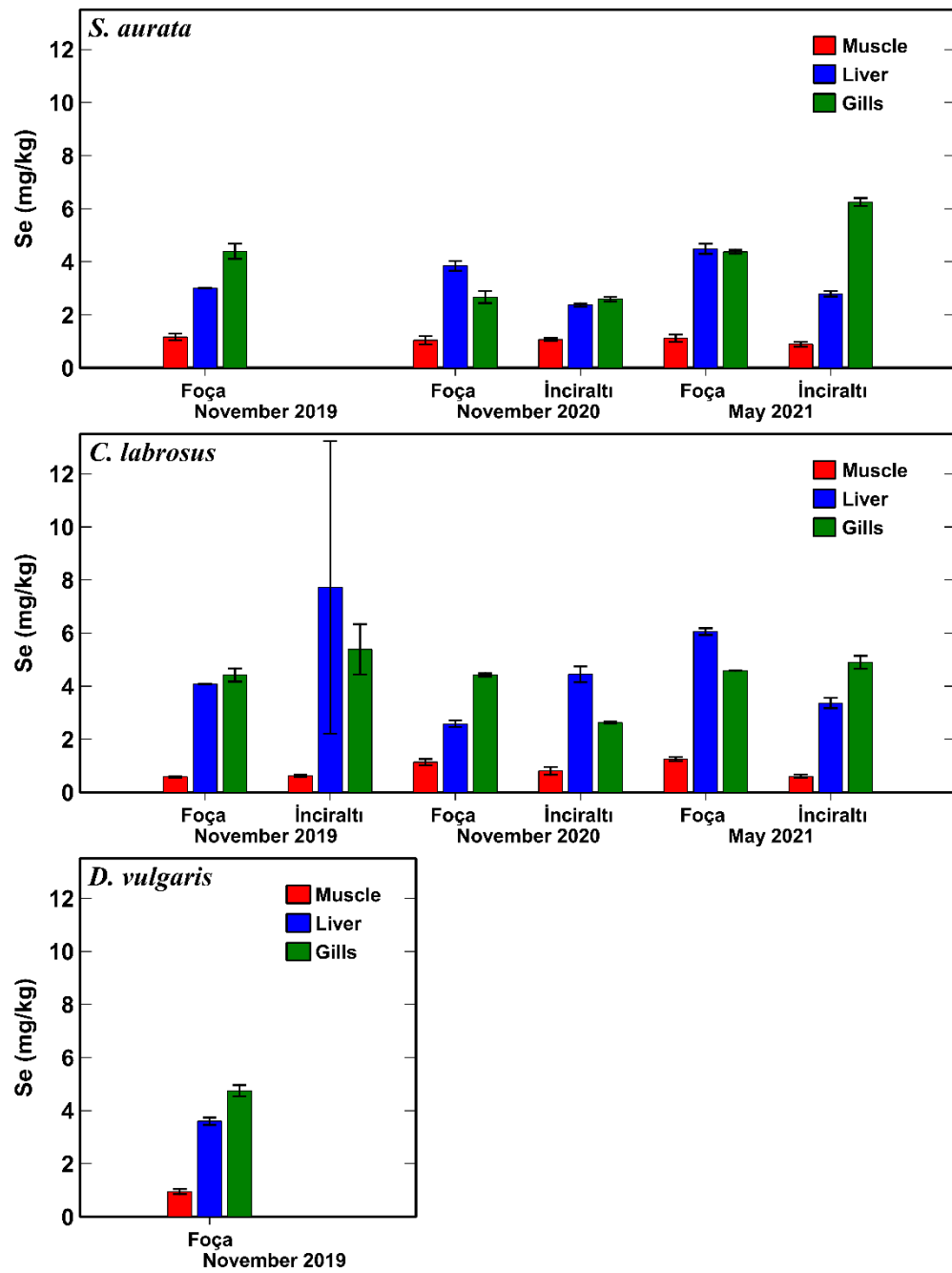


Figure 5.8 Se concentrations (mg/kg $\pm$ SD) of tissues on fish species.

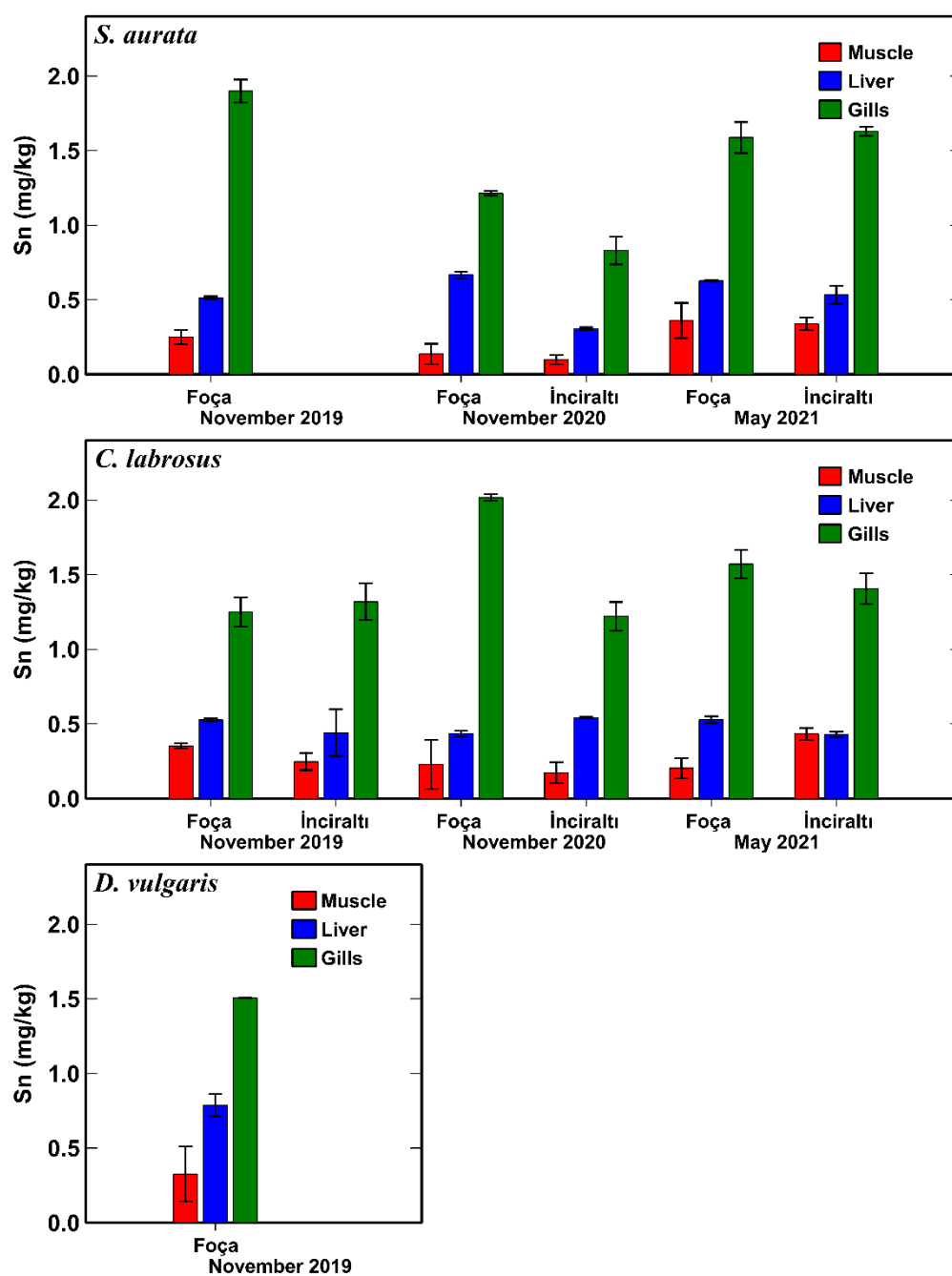


Figure 5.9 Sn concentrations (mg/kg $\pm$ SD) of tissues on fish species.

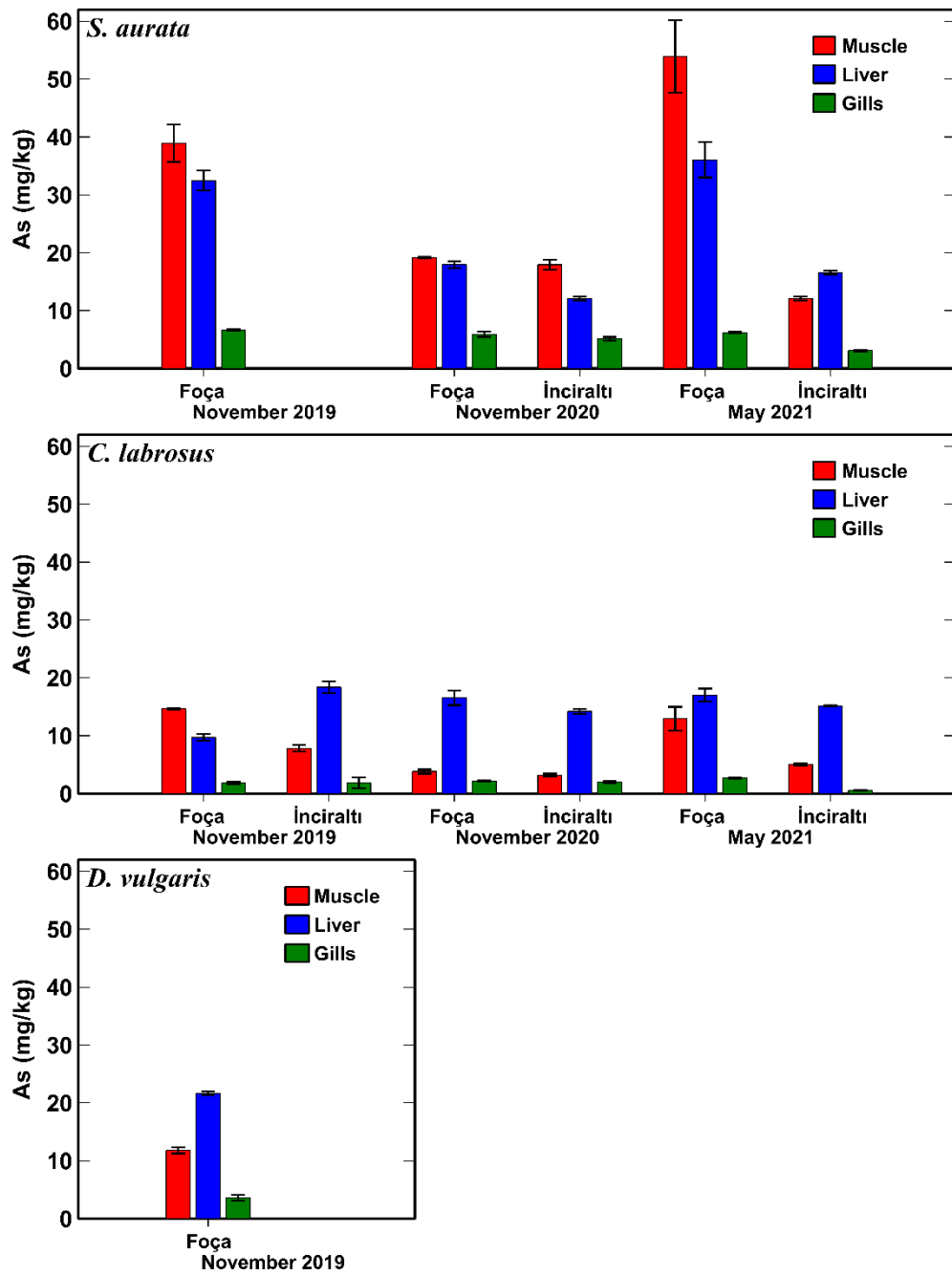


Figure 5.10 As concentrations (mg/kg $\pm$ SD) of tissues on fish species.

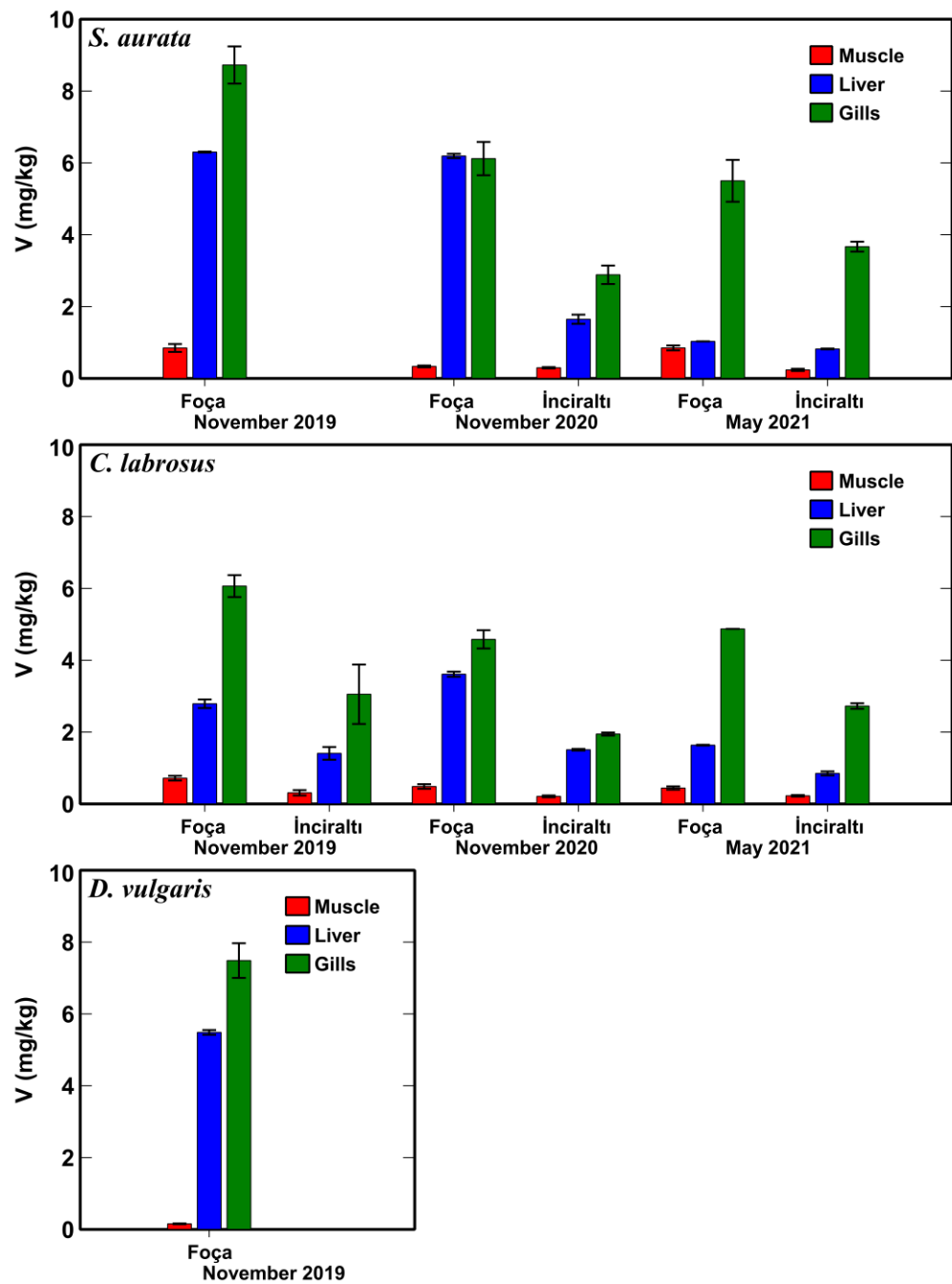


Figure 5.11 V concentrations (mg/kg $\pm$ SD) of tissues on fish species.

5.2 Biomarker Results

Four different biomarkers (CAT, SOD, MDA, and MT) of collected fish samples at muscle, liver and gills tissues from Foça (Outer Bay) and İnciraltı (Inner Bay) in three different sampling periods during 2019 – 2021 were recorded and results were given in Table 5.4 – 5.6 and Figure 5.12 – 5.15.

5.2.1 Biomarker Levels in *Sparus aurata*

5.2.1.1 Muscle Tissue

The lowest CAT activity in muscle tissues of *S. aurata* was determined as 1.52 mU/mg protein at November 2020 in Foça. The minimum SOD activity was recorded as 11.7 mU/mg protein at November 2020 in İnciraltı and at May 2021 in Foça. The lowest MT levels was found at November 2020 in İnciraltı as 80.5 µg MT/g, while the lowest MDA contents was observed at May 2021 in İnciraltı as 8.11 pmol MDA/mg tissue (Table 5.4).

When the highest levels were examined, SOD activity (37.7 mU/mg protein) was found at November 2019 in Foça. The maximum MDA contents (14.0 pmol MDA/mg tissue) was detected at November 2020 in Foça, while the highest CAT (5.54 mU/mg protein) and MT levels (281 µg MT/g) were at May 2021 in İnciraltı (Table 5.4).

5.2.1.2 Liver

The lowest levels for CAT (1.81 mU/mg protein) and MDA (26.9 pmol MDA/mg tissue) were at November 2019 in Foça, and for SOD at November 2020 in İnciraltı as 13.0 mU/mg protein. The minimum MT levels were recorded at November 2020 in Foça as 558 µg MT/g (Table 5.4).

When the maximum biomarker levels are evaluated; MDA contents were seen at November 2020 in Foça as 52.6 pmol MDA/mg tissue. The highest CAT (12.7 mU/mg

protein) and SOD (47.9 mU/mg protein) activities were found at May 2021 in Foça, while the highest MT (909 µg MT/g) was measured at May 2021 in İnciraltı (Table 5.4).

Table 5.4 The minimum and maximum biomarker levels of *S. aurata*.

<i>S. aurata</i>		CAT (mU/mg prot.)	SOD (mU/ mg prot.)	MDA (pmol MDA/ mg tissue)	MT (µg MT/g)
Nov. 2019					
Foça					
	Muscle	1.89-2.88	36.8-37.7	9.11-11.0	-
	Liver	1.81-2.89	22.3-31.3	26.9-31.0	-
	Gills	3.80-7.60	69.4-90.7	21.7-23.2	-
Nov. 2020					
Foça					
	Muscle	1.52-3.90	23.5-25.1	10.1-11.7	198-226
	Liver	2.03-8.60	23.5-25.3	46.3-52.6	558-605
	Gills	2.35-10.2	35.4-37.8	41.6-91.0	141-161
İnciraltı					
	Muscle	1.67-2.68	17.7-21.9	11.1-14.0	138-805
	Liver	3.87-5.77	13.0-17.1	27.4-33.9	776-799
	Gills	3.81-5.31	24.2-36.3	30.9-51.7	485-531
May 2021					
Foça					
	Muscle	2.54-5.17	17.7-25.1	10.4-12.3	188-214
	Liver	4.58-12.7	43.8-47.9	33.8-26.1	718-753
	Gills	6.02-9.54	43.2-54.5	55.4-75.6	286-307
İnciraltı					
	Muscle	3.29-5.54	19.5-32.2	8.11-11.5	245-281
	Liver	4.21-4.49	17.9-24.1	35.5-37.7	852-909
	Gills	4.47-8.59	31.9-43.3	18.1-29.0	392-414

5.2.1.3 Gills

Minimum CAT (2.35 mU/mg protein) and MT (141 µg MT/g) levels were determined at November 2020 in İnciraltı. The lowest SOD activities were recorded at

November 2020 in İnciraltı as 24.2 mU/mg protein. The MDA contents were the lowest at May 2021 in İnciraltı as 18.1 pmol MDA/mg tissue (Table 5.4).

Maximum SOD activities were detected at November 2019 in Foça as 90.7 mU/mg protein. The highest CAT (10.2 mU/mg protein) and MDA (91.0 pmol MDA/mg tissue) levels were found at November 2020 in Foça, while the maximum MT levels were 531 µg MT/g at November 2020 in İnciraltı. (Table 5.4).

5.2.2 Biomarker Levels in *Chelon labrosus*

5.2.2.1 Muscle Tissue

The lowest CAT activities in the muscle tissues of *C. labrosus* were found at November 2019 in İnciraltı as 1.90 mU/mg protein. Foça recorded the lowest SOD activities (17.3 mU/mg protein) and MT contents (107 µg MT/g) at November 2020. The lowest MDA contents were observed at November 2020 in İnciraltı as 7.93 pmol MDA/mg tissue (Table 5.5).

When the maximum levels are examined in muscle tissue; CAT activity (17.3 mU/mg protein) were found at November 2019 in Foça, MDA contents were detected (21.7 pmol MDA/mg tissue) at November 2020 in Foça, MT levels were observed (233 µg MT/g) at May 2020 in Foça and SOD activities (49.3 mU/mg protein) were determined at May 2021 in İnciraltı (Table 5.5).

5.2.2.2. Liver

According to the results in the liver tissues, the minimum MDA contents were found as 20.9 pmol MDA/mg tissue at November 2019 in Foça. The lowest CAT (1.88 mU/mg protein) and SOD (15.1 mU/mg protein) activities were found at November 2020 in Foça. The minimum MTs were observed at May 2021 in İnciraltı as 632 µg MT/g (Table 5.5).

The maximum SOD activities were determined at November 2019 in İnciraltı as 40.3 mU/mg protein. The highest CAT activities (8.20 mU/mg protein) and MT levels (1336 µg MT/g) were detected at November 2020 in İnciraltı. The maximum MDA contents were found at May 2021 in Foça as 71.1 pmol MDA/mg tissue (Table 5.5).

Table 5.5 The minimum and maximum biomarker levels of *C. labrosus*.

<i>C. labrosus</i>		CAT (mU/mg prot.)	SOD (mU/ mg prot.)	MDA (pmol MDA/ mg tissue)	MT (µg MT/g)
Nov. 2019					
Foça					
	Muscle	2.47-17.3	23.2-27.0	10.0-11.3	-
	Liver	4.74-7.51	29.5-36.9	32.0-33.1	-
	Gills	14.9-22.4	112-131	28.7-30.1	-
İnciraltı					
	Muscle	1.90-2.70	17.8-22.7	9.44-11.6	-
	Liver	1.75-4.53	36.6-40.3	20.9-28.2	-
	Gills	2.90-3.70	18.7-24.2	25.4-33.2	-
Nov. 2020					
Foça					
	Muscle	2.42-2.94	17.3-23.4	15.8-21.7	107-127
	Liver	1.88-3.38	15.1-22.5	52.2-56.9	1110-1135
	Gills	10.1-22.9	80.3-98.6	27.0-48.7	193-194
İnciraltı					
	Muscle	2.80-2.41	23.4-44.2	7.93-10.1	165-172
	Liver	5.00-8.20	23.7-31.8	26.9-45.2	1283-1336
	Gills	4.00-5.78	50.5-57.3	21.0-27.0	198-227
May 2021					
Foça					
	Muscle	2.07-8.82	24.8-31.4	9.47-11.6	213-233
	Liver	5.35-7.02	31.6-34.3	60.3-71.1	855-933
	Gills	7.14-12.6	83.9-90.5	28.2-45.5	147-176
İnciraltı					
	Muscle	2.42-6.02	41.6-49.3	10.1-13.2	173-180
	Liver	3.05-7.76	22.3-29.0	30.8-37.0	632-700
	Gills	10.4-21.4	74.8-82.0	29.2-34.7	274-332

5.2.2.3. Gills

When we examined the gill tissues, the minimum CAT (2.90 mU/mg protein) and SOD (18.7 mU/mg protein) activities were found at November 2019 in Foça. The lowest MDA contents were found at November 2020 in İnciraltı as 21.0 pmol MDA/mg tissue, while the lowest MT level was observed at May 2021 in İnciraltı as 147 µg MT/g (Table 5.5).

When the maximum levels in the gills were examined in the muscle tissue; SOD activities were detected (131 mU/mg protein) at November 2019 in Foça. CAT activities (22.9 mU/mg protein) and MDA contents (48.7 pmol MDA/mg tissue) were found at November 2020 in Foça. MT levels (332 µg MT/g) were observed at May 2020 in İnciraltı (Table 5.5).

5.2.3 Biomarker Concentrations of *Diplodus vulgaris*

According to the biomarker results of *D. vulgaris* sampling; minimum CAT (2.23 mU/mg protein) and SOD (17.8 mU/mg protein) were found in gill tissues, while the minimum MDA content was 8.31 pmol MDA/mg tissue in muscle tissue. However, maximum CAT (6.27 mU/mg protein), SOD (51.0 mU/mg protein), and MDA (30.6 pmol MDA/mg tissue) levels were recorded in liver tissues (Table 5.6).

Table 5.6 The minimum and maximum biomarker levels of *D. vulgaris*.

<i>D. vulgaris</i>	CAT (mU/mg prot.)	SOD (mU/ mg prot.)	MDA (pmol MDA/ mg tissue)
Nov. 2019			
Foça (Outer Bay)			
Muscle	2.56-3.12	28.1-30.6	8.31-8.48
Liver	4.70-6.27	48.7-51.0	30.2-30.6
Gills	2.23-3.97	17.8-22.7	9.44-11.6

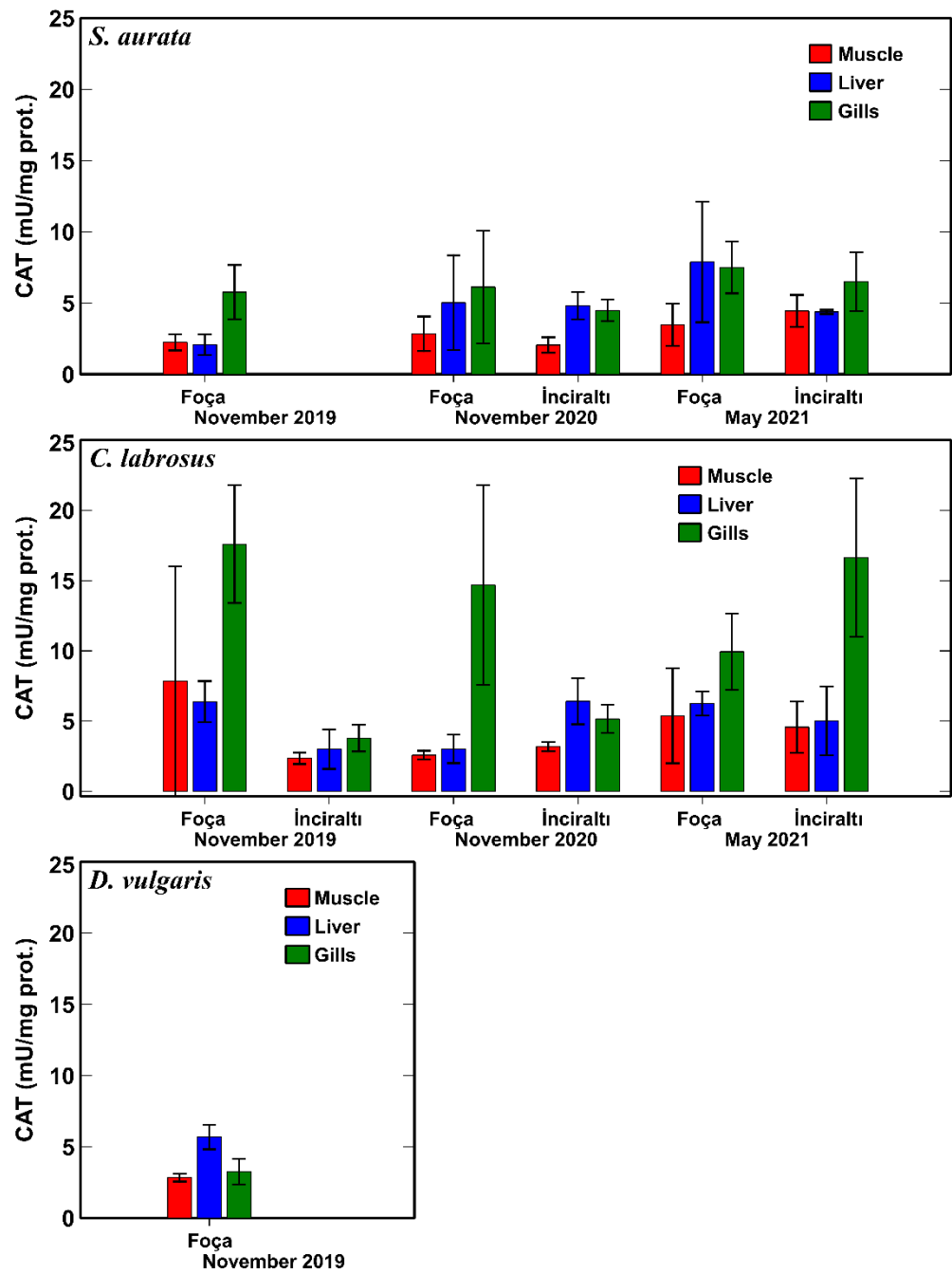


Figure 5.12 CAT activities (mU/mg protein $\pm$ SD) of tissues on fish species.

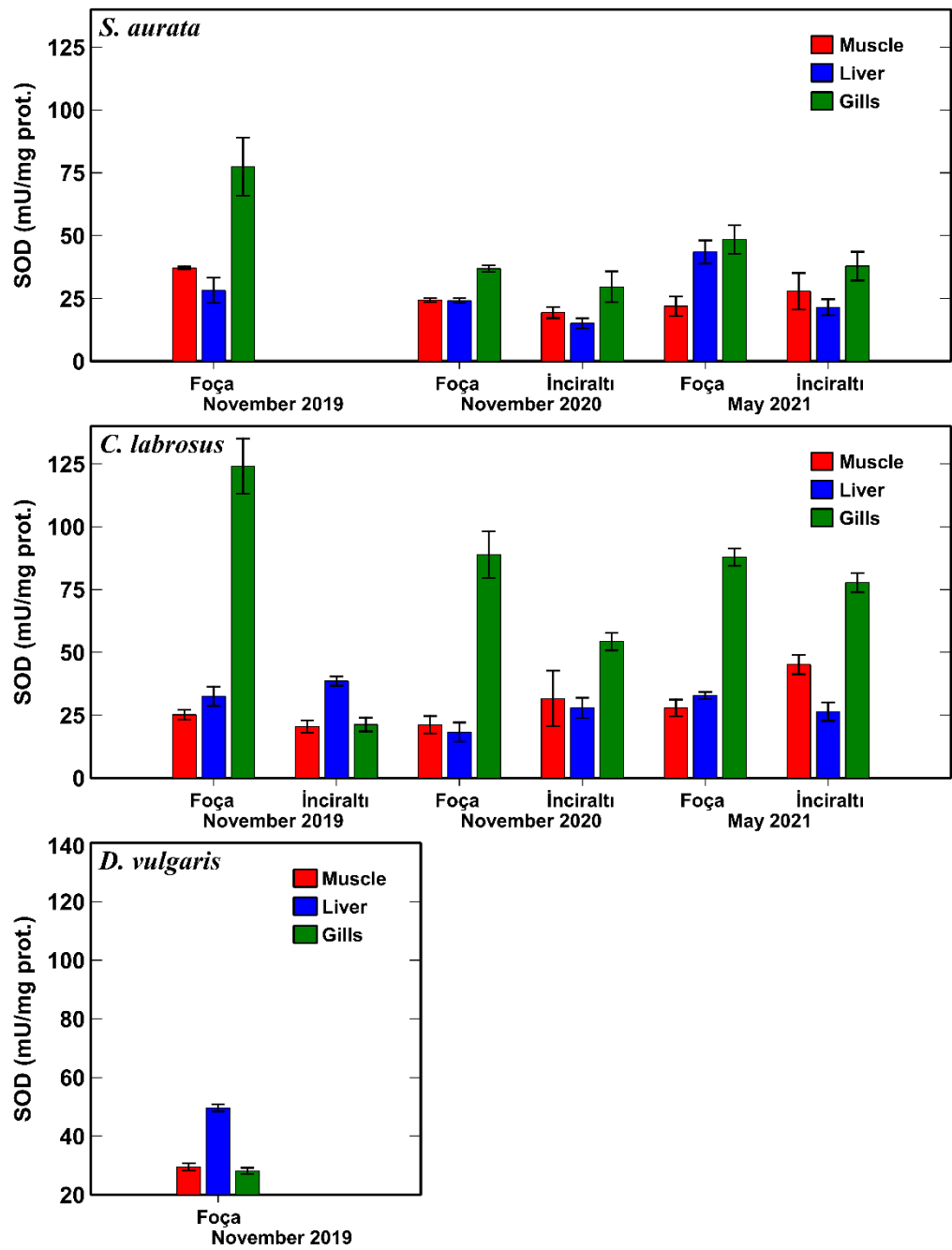


Figure 5.13 SOD activities (mU/mg protein $\pm$ SD) of tissues on fish species.

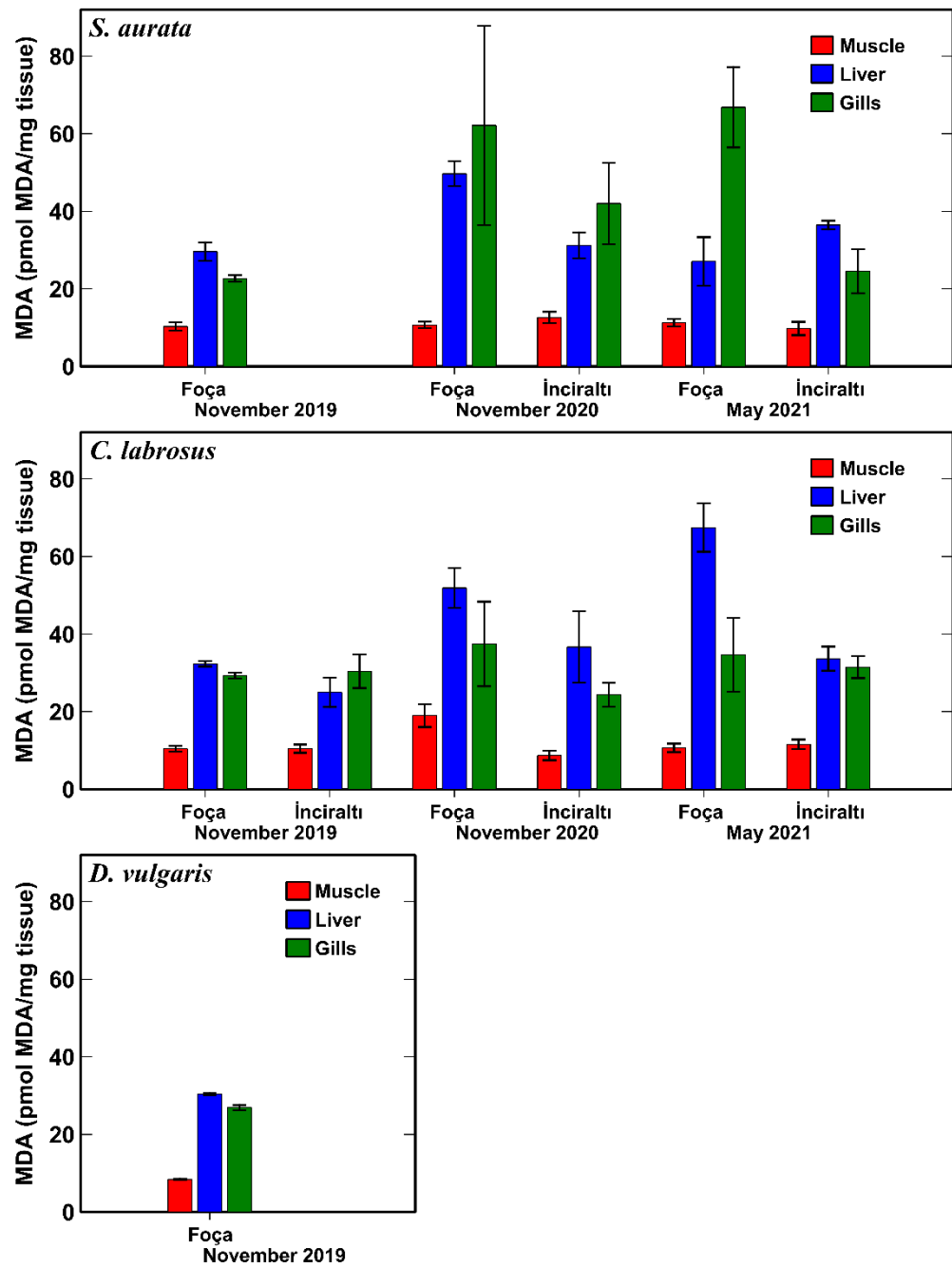


Figure 5.14 MDA contents (pmol MDA/mg protein $\pm$ SD) of tissues on fish species.

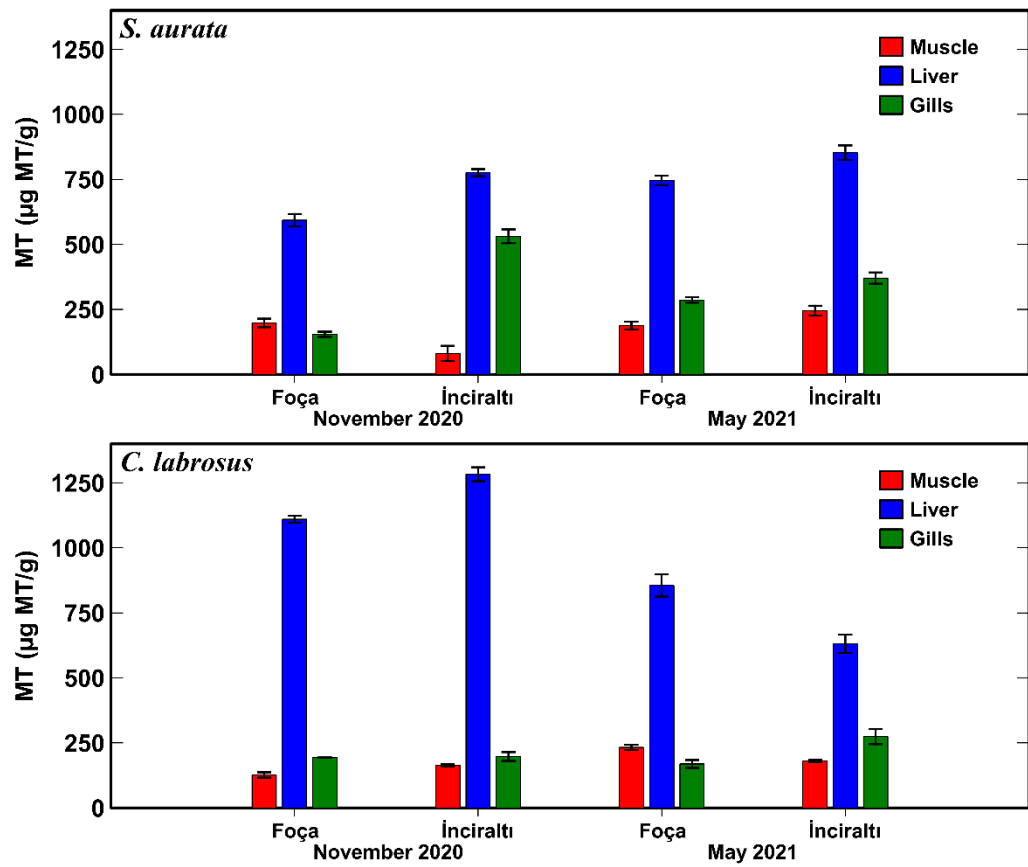


Figure 5.15 MT contents ($\mu\text{g MT/g} \pm \text{SD}$) of tissues on fish species.

CHAPTER SIX

DISCUSSION

6.1 Variations of Metal Concentrations on Fish Species

S. aurata is widely preferred with commercial importance and broadly distribution across the Mediterranean Sea (Rossi, Perrone & Sola, 2006). Hg and As showed higher concentrations on muscle tissues, while Cd, Cu, and Zn concentrations were higher on livers. In addition, Cr, Mn, Se, Sn, and V concentrations were found higher on gills.

Hence data did not applicable for ANOVA, non-parametric Kruskal – Wallis tests were performed to evaluate statistical significances onto *S. aurata* findings ($p < 0.05$). Hg and V concentrations were showed significant differences between sampling periods. Hg, As, and V levels were distinguished significant differences between locations. All metals were showed statistical significances between tissues (Table 6.1).

Table 6.1 p-values ($p < 0.05$) of Kruskal – Wallis test results of metals for *S. aurata* (Significant values were bolded).

<i>S.aurata</i>	Hg	Cd	Pb	Cr	Cu	Zn	Mn	Se	Sn	As	V
Locations	<0.001	0.203	0.372	0.203	0.635	0.342	0.424	0.179	0.354	0.002	0.006
Periods	0.044	0.530	0.289	0.377	0.655	0.253	0.300	0.119	0.310	0.174	0.026
Tissues	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Pearson Product-Moment Correlation tests were applied to metal data to investigate interrelationships of metals ($p < 0.05$). Strong correlations as noted; Hg concentrations were positively correlated with As (0.829), while they were negatively correlated with Cr (-0.478). Cd concentrations were showed positive correlations with Pb (0.741), Cu (0.858), and Zn (0.831). Pb concentrations were positively correlated with Cu (0.864), Zn (0.945), Se (0.496), and V (0.589). Cr concentrations were showed positive correlations with Mn (0.870), Se (0.531), Sn (0.804), and V (0.789), however, they were showed negative correlations with As (-0.604). Cu concentrations were showed

positive correlations with Zn (0.959), which is the highest score. Mn concentrations were positively correlated with Se (0.777), Sn (0.941), and V (0.846), while they were negatively correlated with As (-0.545). Se concentrations were showed positive correlations with Sn (0.789) and V (0.595). Lastly, Sn concentrations were positively correlated with V (0.752), but negatively correlated with As (-0.553) (Table 6.2).

Table 6.2 r-values of Pearson Product-Moment correlation test for *S. aurata* (significant values were bolded).

Metal	Hg	Cd	Pb	Cr	Cu	Zn	Mn	Se	Sn	As	V
Hg	—										
Cd	0.366 *	—									
Pb	0.091	0.741 ***	—								
Cr	-0.478 ***	-0.177	0.281	—							
Cu	0.430 **	0.858 ***	0.864 ***	-0.093	—						
Zn	0.238	0.831 ***	0.945 ***	0.108	0.959 ***	—					
Mn	-0.418 **	-0.046	0.443 **	0.870 ***	0.062	0.260	—				
Se	-0.169	0.266	0.496 ***	0.531 ***	0.314 *	0.440 **	0.777 ***	—			
Sn	-0.409 **	-0.196	0.213	0.804 ***	-0.136	0.052	0.941 ***	0.789 ***	—		
As	0.829 ***	0.263	0.031	-0.604 ***	0.315 *	0.135	-0.545 ***	-0.390 **	-0.553 ***	—	
V	-0.251	0.043	0.589 ***	0.789 ***	0.254	0.451 **	0.846 ***	0.595 ***	0.752 ***	-0.379 *	—

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

C. labrosus, fish species from *Mugilidae*, is commonly consumed by local people and distributed along with Black Sea, Eastern Atlantic, Adriatic, and the Mediterranean Sea (I. Antovic & N. M. Antovic, 2011). Hg, Cd, Cu, Zn, and Se concentrations were found higher on liver tissues, while Pb, Mn, Sn, As, and V concentrations were observed higher on gills. Higher Cr concentrations were showed variations between liver and gills.

Levene's homogeneity test were approved the normal distribution and Three-Way ANOVA tests were performed for evaluating metal data of *C. labrosus*. Hg, Pb, Cr, Mn, Sn, As, and V levels were exhibited significant differences between locations. Hg, Cd, Pb, Cu, Zn, Mn, Sn, As, and V were showed significant differences between sampling periods. In addition, all analyzed metals showed statistical differences between tissues (Table 6.3).

Table 6.3 p-values ($p < 0.05$) of Three-Way ANOVA test results of metals for *C. labrosus* (Significant values were bolded).

<i>C.labrosus</i>	Hg	Cd	Pb	Cr	Cu	Zn	Mn	Se	Sn	As	V
Locations	0.049	0.528	<0.001	<0.001	0.803	0.936	0.007	0.765	<0.001	<0.001	<0.001
Periods	<0.001	<0.001	<0.001	0.070	0.013	<0.001	<0.001	0.158	0.011	<0.001	<0.001
Tissues	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

According to the results of the Pearson Product-Moment Correlation tests ($p < 0.05$), relationships were gathered and strong ones were noted: Hg concentrations were positively correlated with Cd (0.718), Cu (0.510), Zn (0.551), and Se (0.444), and As (0.772), while they were negatively correlated with Mn (-0.407) and Sn (-0.352). Cd concentrations were showed positive correlation with Cu (0.656), Zn (0.783), Se (0.459), and As (0.765). Pb concentrations were positively correlated with Cr (0.734), (0.614), Sn (0.727), and V (0.690). Cr concentrations were positively correlated with Mn (0.421), Sn (0.536), and V (0.491). Cu concentrations were showed positive correlations with Zn (0.715), Se (0.667), and As (0.520). Zn concentrations were positively correlated with Se (0.612), and As (0.490). Mn concentrations were positively correlated with Sn (0.924) (highest value), and V (0.491), while they were showed negative correlations with As (-0.665). Se concentrations were positively correlated with V (0.411). Lastly, Sn concentrations were showed positive correlations with V (0.786), when they were showed negative correlations with As (-0.603) (Table 6.4).

Table 6.4 r-values of Pearson Product-Moment correlation test for *C. labrosus* (significant values were bolded).

Metal	Hg	Cd	Pb	Cr	Cu	Zn	Mn	Se	Sn	As	V
Hg	—										
Cd	0.718 ***	—									
Pb	-0.159	-0.029	—								
Cr	-0.063	0.093	0.734 ***	—							
Cu	0.510 ***	0.656 ***	-0.067	0.152	—						
Zn	0.551 ***	0.783 ***	0.194	0.394 **	0.715 ***	—					
Mn	-0.407 ***	-0.382 **	0.614 ***	0.421 ***	-0.287 *	0.033	—				
Se	0.444 ***	0.459 ***	0.296 *	0.245	0.667 ***	0.612 ***	0.294 *	—			
Sn	-0.352 **	-0.301 *	0.727 ***	0.536 ***	-0.179	0.093	0.924 ***	0.397 **	—		
As	0.772 ***	0.765 ***	-0.280 *	-0.053	0.520 ***	0.490 ***	-0.665 ***	0.176	-0.603 ***	—	
V	-0.249 *	-0.077	0.690 ***	0.491 ***	-0.002	0.303 *	0.724 ***	0.411 ***	0.786 ***	-0.362 **	—

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Diplodus vulgaris is another common species from *Sparidae* family. Despite the limited data, One-Way ANOVA tests were performed after Levene's homogeneity tests were approved normal distribution. Test results were demonstrated that all metals were showed significant differences between tissues (Table 6.5).

Table 6.5 p-values ($p < 0.05$) of One-Way ANOVA test results of metals for *D.vulgaris* (Significant values were bolded).

<i>D.vulgaris</i>	Hg	Cd	Pb	Cr	Cu	Zn	Mn	Se	Sn	As	V
Tissues	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

When metal concentrations of all fish species were evaluated: Higher Hg, Zn, As, and V values were detected in *S. aurata*; higher Cd, Cr, Cu, Mn, Se, and Sn values were found in *C. labrosus*; higher Pb concentrations were found in *D. vulgaris*. Since metal data for all fish species did not meet for ANOVA test, therefore Kruskal – Wallis tests were performed. According to the results; Hg, Pb, and As concentrations were showed significant differences between fish species. Hg, Pb, As, and V concentrations were distinguished significant differences between locations. When all metals were showed significant differences between tissues, none of them were statistically different between sampling periods (Table 6.6). Multiple comparison tests were demonstrated that metal concentrations in muscle tissues were distinguished from other tissues. For locations, metal concentrations in Foça were greater than İnciraltı levels on significantly different cases. *C. labrosus* were distinguished from *S. aurata* and *D. vulgaris* on evaluating species-specific differences (Table 6.6).

Table 6.6 p-values ($p < 0.05$) of Kruskal – Wallis test results of metals included all fish species (Significant values were bolded).

All	Hg	Cd	Pb	Cr	Cu	Zn	Mn	Se	Sn	As	V
Species	<0.001	0.123	0.002	0.566	0.179	0.144	0.640	0.664	0.606	<0.001	0.173
Locations	<0.001	0.304	0.024	0.356	0.749	0.161	0.838	0.464	0.193	0.008	<0.001
Periods	0.150	0.701	0.281	0.400	0.930	0.682	0.930	0.064	0.262	0.682	0.179
Tissues	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Pearson Product-Moment Correlation tests ($p < 0.05$) were performed and strong relationships were noted: Hg concentrations were positively correlated with Zn (0.316) and As (0.809), while they were negatively correlated with Cr (-0.311), Mn (-0.382), and Sn (-0.351). Cd concentrations were showed positive correlations with Cu (0.656), Zn (0.522), and Se (0.434). Pb concentrations were positively correlated with Cr (0.421), Mn (0.507), Se (0.311), Sn (0.509), and V (0.559). Cr concentrations were showed positive correlations with Mn (0.507), Se (0.311), Sn (0.509), and V (0.559), when they were showed negative correlations with As (-0.342). Cu concentrations were positively correlated with Zn (0.381) and Se (0.611). Zn concentrations were showed positive correlation with Se (0.425) and V (0.437). Mn concentrations were showed positive correlations with Se (0.419), Sn (0.900), and V (0.653), when they were showed negative correlations with As (-0.486). Se concentrations were positively correlated with Sn (0.506) and V (0.402). Lastly, Sn concentrations were showed positive correlations with V (0.736), while they were showed negative correlations with As (-0.479) (Table 6.7).

Table 6.7 r-values of Pearson Product-Moment correlation test included all fish species (significant values were bolded).

Metal	Hg	Cd	Pb	Cr	Cu	Zn	Mn	Se	Sn	As	V
Hg	—										
Cd	0.218 *	—									
Pb	-0.174	0.092	—								
Cr	-0.311 ***	0.063	0.421 ***	—							
Cu	0.118	0.656 ***	-0.024	0.137	—						
Zn	0.316 ***	0.522 ***	0.232 *	0.213 *	0.381 ***	—					
Mn	-0.382 ***	-0.284 **	0.507 ***	0.548 ***	-0.211 *	0.104	—				
Se	0.014	0.434 ***	0.311 ***	0.341 ***	0.611 ***	0.425 ***	0.419 ***	—			
Sn	-0.351 ***	-0.232 *	0.509 ***	0.639 ***	-0.140	0.082	0.900 ***	0.506 ***	—		
As	0.809 ***	0.255 **	-0.181	-0.342 ***	0.115	0.294 **	-0.486 ***	-0.134	-0.479 ***	—	
V	-0.164	-0.051	0.559 ***	0.556 ***	-0.054	0.437 ***	0.653 ***	0.402 ***	0.736 ***	-0.217 *	—

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Variations of tissues were showed substantial differences in every metal and metalloid. Besides the muscle tissues, metals are tended to store liver and gills. These tendencies may show the accumulation route and metal loads in fish species. The reason of different amounts of concentration in tissues could be linked that different tissues have different physiological functions which need noticeable affinities (Liu et al., 2015). Cd, Pb (only for *C. labrosus*), Cr, Cu, and Zn were generally showed higher concentrations in liver tissues. It has been widely known that liver tissues include many

hormonal, enzymatic and metabolic processes in the organism such as metallothionein which is inducing metal-binding proteins that provides regulation of unwanted effects of toxic metals (Jakimska et al., 2011). MTs can bind to metals of higher concentrations. Furthermore, liver is the organ where metals coming from different ways through absorption and pollutants involving the detoxification process are stored (Liu et al., 2015). In this study, Pb (for *S. aurata* and *D. vulgaris*), Mn, Se, and Sn were showed considerably higher levels in gills which are initial organ for respiratory. Gills are also having direct interaction with water column. Therefore, gills could uptake metals from water to the tissue's surface. In addition, toxic metals like Pb could related with water rather than food. Another reason of elevated levels in gills could be linked to high density of chloride cells that located gills and these can show chemical affinity to cations like metals (da Costa & Fernandes, 2002).

Metal concentrations in locations were generally balanced, but especially Hg, As, and V showed significant variations for both *S. aurata* and *C. labrosus*. In addition, significant Pb variations were observed for *C. labrosus*. When the metals from which these significant differences are examined, it is seen that the values determined in Foça region are higher than İnciraltı. Katalay et al. (2022) also stated that analyzed metal levels were supported these trends in their recent study carried out in İzmir Bay by using bivalves. Elevated As and Hg concentrations could be natural and anthropogenic oriented on this region, because an abandoned mercury mine is located in Karaburun where the opposite to Foça (Gemici & Oyman, 2003). Also Aksu, Yaşar, & Uslu (1998) found quite higher As levels in sediment from Outer Bay. They had claimed that massive loads of untreated wastes that includes As compounds had been discharged to the Bay from tanning industry. They also had explained that V contamination could be related to oil-combustion by products and vanadium pentoxide which comes to petroleum-fired plants as fly ash and Pb contamination could be related to various facilities includes alloy productions and ceramic industries (Aksu et al., 1998). Another research from China suggested that elevated levels of Hg and As could be related with increasing chlorophyll concentrations originated algae. These consumption might lead to transfer heavy metals like Hg to the fish species via trophic transfer (Chen et al., 2008). İzmir Bay has been host many algal blooms for decades.

Algal traces were found on bivalves (one of main food sources of *Sparidae* sp.) from İzmir Bay (Lok et al., 2010).

Metal levels according to the sampling periods were showed limited variations on *S. aurata* (only Hg and V), almost every metal were showed significances on *C. labrosus* (except Cr and Se). When the trend of change was followed, it was observed that especially toxic metals (Hg and As for *S. aurata*; Hg, Cd, Pb and As for *C. labrosus*) increased from November 2020 to May 2021. Previous studies explained that these seasonal changes might depend on two conditions. First one is increased metabolic activity with increasing temperatures and the second one is increased utilization of synthetic fertilizers in agriculture that includes metal salts and transports along with streams to the open seas (Faggio et al., 2014; Kargin, 1996).

When the difference between species was examined, Hg, As and V showed significant differences. As mentioned before *S. aurata* and *D. vulgaris* are member of *Sparidae*, and *C. labrosus* is member of *Mugilidae* family and they have different life cycle. Liu et al (2015) mentioned that habitat and feeding habits were possess vital importance on metal bioaccumulation of fish species. In addition, increased levels of metal bioaccumulation at livers, gills, and muscle tissues of fish species are under the influenced of efflux rates of elements, lipid contents and ingestion rates, swimming activity, and physiology (Vetsis et al., 2021).

6.2 Variations of Biomarkers on Fish Species

Since there is not enough data for some parameters, some adjustments have been made in the statistical tests of biomarkers. As MTs could not be determined in the November 2019 samples, the MT tests were performed to cover the November 2020 and May 2021 samples. For the same reason, MT values are not available for *D. vulgaris*.

Kruskal – Wallis tests were performed onto biomarker results of *S. aurata* ($p < 0.05$). CAT and SOD activities were showed significant differences between sampling

periods. Only SOD activities were significantly different between locations. Also, all biomarkers were showed significant differences between tissues (Table 6.8).

Table 6.8 p-values ($p < 0.05$) of Kruskal – Wallis test results of biomarkers for *S. aurata* (Significant values were bolded).

<i>S.aurata</i>	CAT	SOD	MDA	MT*
Locations	0.871	0.003	0.763	0.154
Periods	0.017	0.009	0.179	0.229
Tissues	0.001	<0.001	<0.001	<0.001

*: Statistical analysis of MTs was covered November 2020 and May 2021 samplings.

Levene's homogeneity tests were approved and Three-Way ANOVA tests were performed onto *C. labrosus*' biomarker results ($p < 0.05$). According to the results, only MDA contents could not be exhibited significant differences between sampling periods. All other biomarker results were showed significant differences between locations, sampling periods, and tissues (Table 6.9).

Table 6.9 p-values ($p < 0.05$) of Three-Way ANOVA test results of biomarkers for *C. labrosus* (Significant values were bolded).

<i>C.labrosus</i>	CAT	SOD	MDA	MT*
Locations	0.005	<0.001	<0.001	0.002
Periods	0.151	<0.001	<0.001	<0.001
Tissues	<0.001	<0.001	<0.001	<0.001

*: Statistical analysis of MTs was covered November 2020 and May 2021 samplings.

Levene's homogeneity tests were approved and One-Way ANOVA tests were performed for biomarker results of *D. vulgaris* ($p < 0.05$). Results show that, CAT, SOD and MDA findings were showed significant differences (Table 6.10).

Table 6.10 p-values ($p < 0.05$) of One-Way ANOVA test results of biomarkers for *D.vulgaris* (Significant values were bolded).

<i>D.vulgaris</i>	CAT	SOD	MDA
Tissues	0.006	<0.001	<0.001

Kruskal – Wallis tests were applied to biomarker results of all fish species ($p < 0.05$). According to the statistical results, no significant differences were observed between fish species and locations. However, CAT and SOD activities were showed differences between sampling periods. In addition, all biomarkers were showed significant differences between tissues (Table 6.11). According to the multiple comparisons, muscle tissues were distinguished for CAT and MDA, gills were separated for SOD, and livers were differentiated for MT. On sampling periods May 2021 were distinguished previous samplings for CAT and SOD, however, November 2019 samplings were separated for MDA (Table 6.11)

Table 6.11 p-values ($p < 0.05$) of Kruskal – Wallis test results of biomarkers included all fish species (Significant values were bolded).

All	CAT	SOD	MDA	MT*
Species	0.125	0.180	0.378	0.351
Locations	0.551	0.301	0.140	0.402
Periods	0.006	0.011	0.052	0.319
Tissues	<0.001	<0.001	<0.001	<0.001

*: Statistical analysis of MTs was covered November 2020 and May 2021 samplings.

In statistical analyses, all analyzed biomarkers were showed significant differences. Higher concentrations were found on gills for CAT and SOD, however, higher concentrations of MDA and MT were found on liver. Muscle tissues had generally lower biomarker responses. Liver tissues are able to store pollutants like metals and other compounds for detoxification, utilization or excrete, therefore higher biomarker responses observed in liver. Also, in gills, thinnest epithelium cells that pollutants can treat there (Farombi et al., 2007). The reason of observing higher activities of CAT and SOD on gills could be related presence of massive amounts of H_2O_2 concentration from ROS production that insufficiently eliminated (Vlahogianni et al., 2007). Increased oxidative damage parameters are generally observed in liver, therefore elevation of antioxidant enzyme activities might provide increase on MDA contents as a result of oxidative damage in lipids (Solomando et al., 2022). Since many metals and metalloids tend to store in liver, the higher amounts of MTs can be detected in there. MTs take role on regulating trace elements (Cu and Zn), making suitable physiological

conditions by binding (Ag, Pb and Hg), homeostasis of metal ions, detoxification of toxic metals (Cd, Hg, and Pt), apoptosis, and scavenging ROS (Hemmadi, 2016).

When other differences of biomarker responses were evaluated, *S. aurata* showed limited differences (only in SOD for locations and CAT and SOD for sampling periods) activity, while almost all biomarkers (except CAT in sampling periods) showed these differences for *C. labrosus*. Although the studied fish were caught from the same location during the same period, this remarkable variation in biomarkers may be due to the differences in pollutants that the fish are exposed to in their locations. These main differences could be about accumulation of pollutants derived from dietary habits. As mentioned before *S. aurata* and *D. vulgaris* are bottom feeder fishes, but *C. labrosus* can behave both benthic and pelagic feeding like, sedimented detritus, benthic organisms, and suspended particles (Abumourad et al., 2014; Hadj Taieb et al., 2013; Osman & Mahmoud, 2009). These divergent feeding habits might exposed floating or suspended pollutants like oil spill and garbage, and microplastics (Bilbao et al., 2010; Chacón Aranda et al., 2021). Since reproduction cycle of these species generally occurred on late autumn to winter (Abumourad et al., 2014; Hadj Taieb et al., 2013; Osman & Mahmoud, 2009), the increased feeding regime due to increased metabolic activity after spawning and the consequent increase in pollutant intake may explain the elevation through the spring (May 2021) sampling period (Solé et al., 2010).

6.3 Interrelationships Between Metal and Biomarker Findings

The investigation between metal pollution and biomarker responses is getting vital day-by-day. With this relationship, researchers are able to identify the linkage between contaminants and sublethal effects for organisms. In addition, assumptions can be revealed about early detection and/or preventive actions by using these integrated approaches (Sarkar et al., 2006).

In order to obtain presentative results from the statistical analyzes made in this context, the dataset obtained from the November 2020 and May 2021 samplings was used.

Pearson Product-Moment Correlation tests were applied to findings of the metal concentrations and biomarker responses ($p < 0.05$). Test results were obtained as heatmap (Figure 6.1).

Results demonstrated that CAT activities were positively correlated with Pb (0.395), Cr (0.406), Mn (0.586), Se (0.533), Sn (0.640), while they were negatively correlated with As (-0.270). SOD activities were showed positive correlations with Pb (0.687), Cr (0.622), Mn (0.795), Se (0.453), Sn (0.791), and V (0.420), when they were showed negative correlations with Hg (-0.296), Cd (-0.259), and As (-0.425). MDA contents were positively correlated with Cd (0.427), Pb (0.253), Cr (0.539), Cu (0.335), Zn (0.483), Mn (0.304), Se (0.604), Sn (0.418), and V (0.691). MT concentrations were showed positive correlation with Cd (0.752), Cu (0.797), Zn (0.656), and Se (0.401) (Figure 6.1).

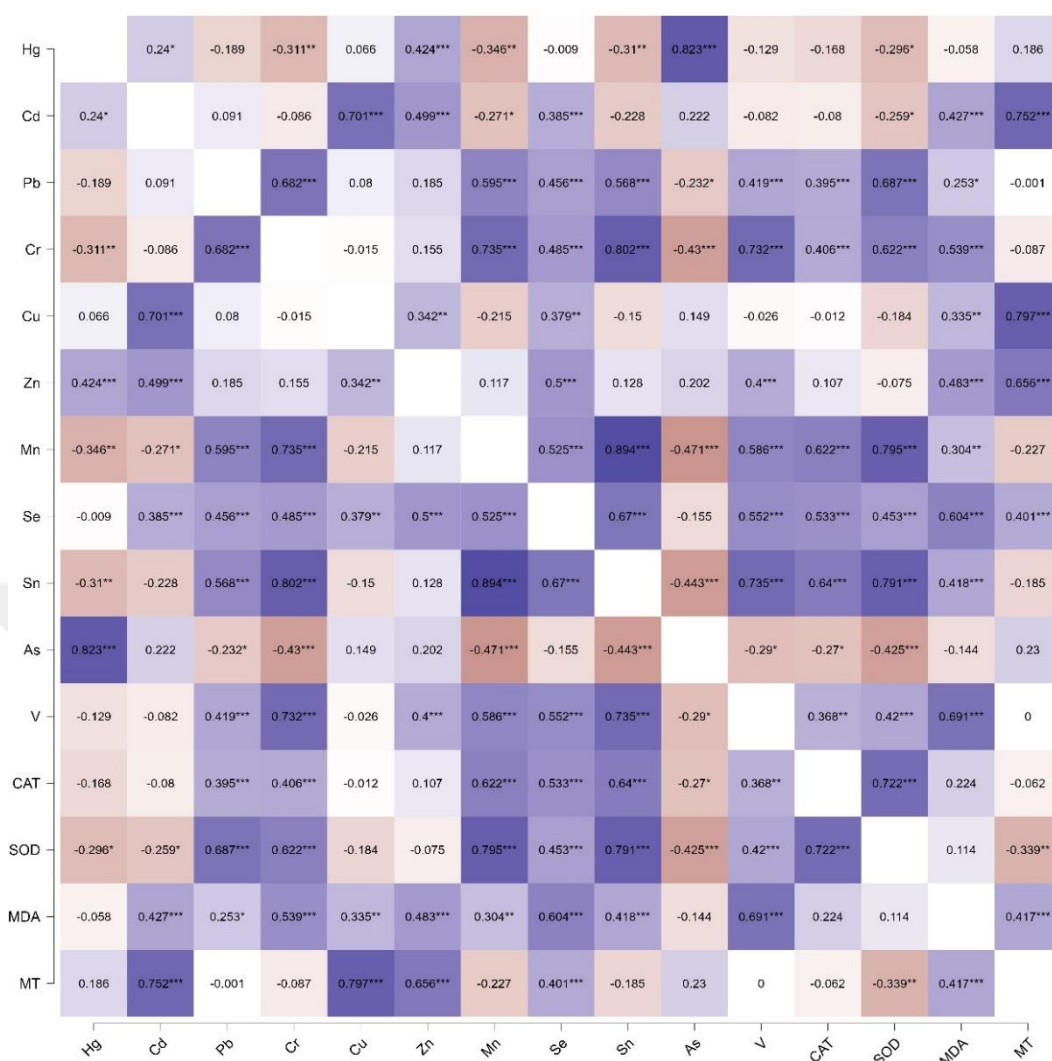


Figure 6.1 Heatmap of Pearson Product-Moment Correlation tests included findings of the metal concentrations and biomarker responses (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Network analysis was applied to dataset to distinguish differences between relationships within sub-groups (metals and biomarkers) (Figure 6.2). Image of network analysis showed that MT was linked with Cu, Zn, and Cd. MDA was linked with Cd and V. CAT was directly linked with Se, while SOD was directly linked with Pb, Mn and Sn. In sub-groups, Hg and As had the strongest relationship with each other, also CAT – SOD linkage was considerably strong. In addition, Mn – Sn, Se – Sn, Cu – Cd, V – Cr, and Pb – Cr were exhibited noteworthy linkages (Figure 6.2).

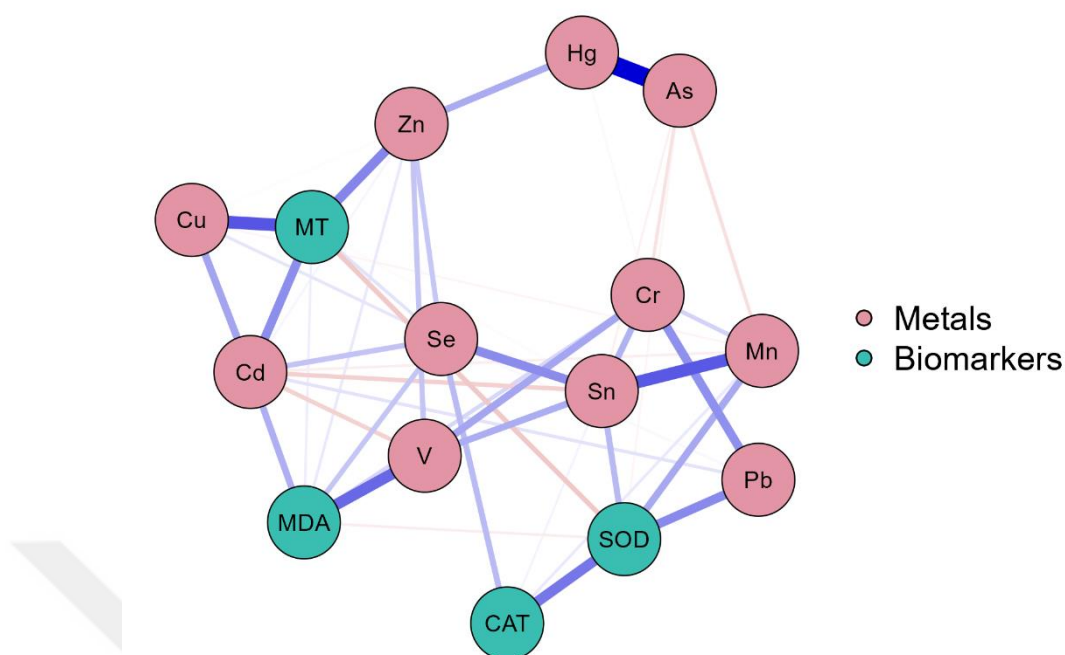


Figure 6.2 Network analysis of two sub-groups (metals and biomarkers)

Principal Component Analysis were applied to the dataset to increase interpretability of variables with the minimizing information loss. Components were selected as Eigenvalues above 1. Test results showed that four main components were loaded (Table 6.12, Table 6.13, and Figure 6.3). PC1 was included Sn, Mn, Cr, SOD, V, Pb, Se, CAT, As, and MDA. PC2 was included MDA, MT, Cd, Cu, Zn and Hg. PC3 was included As and Hg (Table 6.12). Explained variances were found as 39.6%, 25.3%, 9.8%, and 7.3%, respectively. All components were explained 82.1% of variance cumulatively (Table 6.13). Biplot of most loaded principal components was created (Figure 6.4).

Table 6.12 Component loadings of PCA results.

	PC1	PC2	PC3	PC4	Uniqueness
Sn	0.945				0.082
Mn	0.896				0.150
Cr	0.865				0.212
SOD	0.824				0.079
V	0.768			-0.502	0.103
Pb	0.706				0.401
Se	0.693	0.537			0.208
CAT	0.689			0.431	0.319
As	-0.535		0.646		0.154
MDA	0.529	0.585		-0.442	0.171
MT		0.902			0.115
Cd		0.847			0.188
Cu		0.772			0.182
Zn		0.767			0.256
Hg		0.407	0.788		0.064

Table 6.13 Component characteristics of PCA loadings

	Eigenvalue	Proportion var.	Cumulative
Component 1	5.940	0.396	0.396
Component 2	3.802	0.253	0.649
Component 3	1.477	0.098	0.748
Component 4	1.096	0.073	0.821

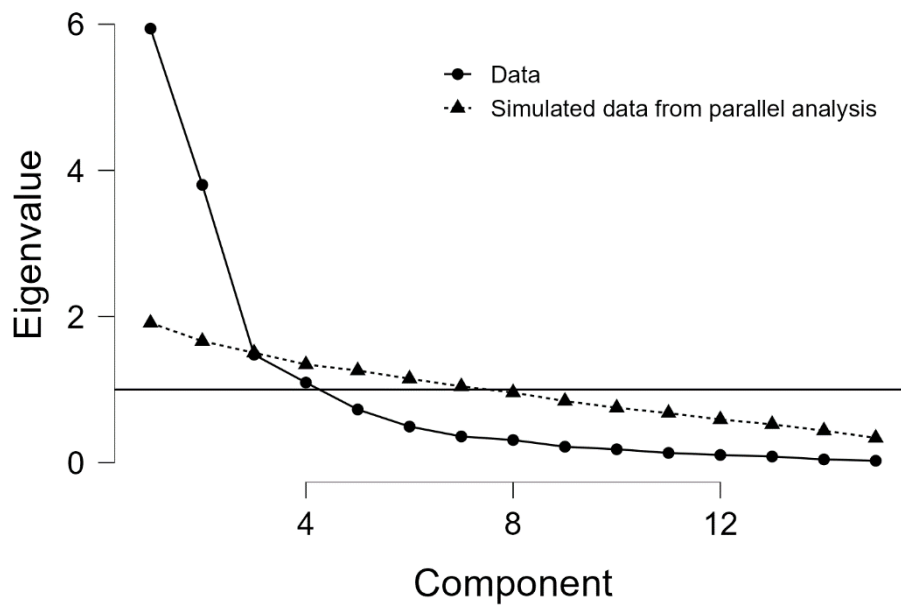


Figure 6.3 Scree plot of Eigenvalues with simulated data from parallel analysis

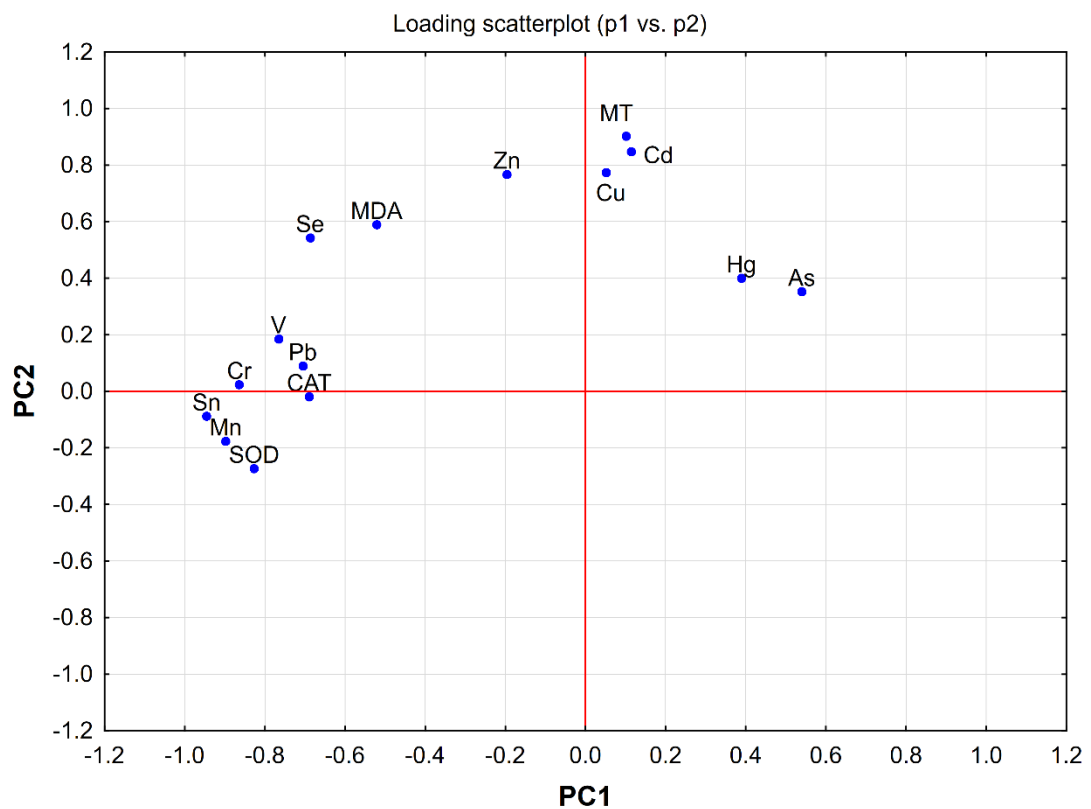


Figure 6.4 Biplot of PCA loadings.

All these tests demonstrated that biomarker responses and metal bioaccumulations were having noteworthy relationships.

Previous studies also explain that levels of MDA content are strongly associated with essential and toxic metals (Beg et al., 2015; Nesto et al., 2007). Depending on the increased ROS concentration in the cell, metabolite products, including MDA, are released with lipid degradation reactions. Therefore, MDA is a useful biomarker for diagnosing oxidative stress (Mishra, 2022).

Some studies about exposure of metals on fish species had explained that exposure some metals including Cd, Cu, and Zn could significant impacts on concentrating MDA an MT (Baker et al., 1998; Ghedira et al., 2010). These results were evident that the higher metal concentrations and MT induction levels support the main role of MT in processes of metal homeostasis and detoxification (Ghedira et al., 2010). Also, MDA might provide indicating oxidative stress, hence it is byproducts of polyunsaturated lipids which degraded by ROS (Dzoyem et al., 2014).

Antioxidant enzymes, CAT and SOD were showed distinguished correlations with metals. Higher activities were generally observed with higher metal loadings. It is widely known that antioxidant enzymes might be varied by metals due to dose amounts, species and exposure route (Ferreira et al., 2008). Mn concentrations were made vital relationship with biomarkers. Mn takes a role as co-factor on antioxidant mechanism. Elevated Mn-SOD activities had been found in gills on a previous study. Researched had claimed that gills are the first defensive line against xenobiotic and microbes (Lu et al., 2015).

Strong linkages were observed on statistical analyses between Hg and As, one of the most toxic components. As mentioned before, effect of both elements could be originated from environment. According to the literature, the results of significant relationships between metals in aquatic organisms; it depends on the metals being of similar origin, as well as the degree of assimilation of the metals, the life span of the

living thing and the residence time of the metals in the body, the accumulation and detoxification capacity (Marsden & Rainbow, 2004).

To summarize, in this study, there was a remarkable linkage between metal bioaccumulation and the biomarker responses of organisms. The fact that the first two components formed because of the PCA test include all metals and biomarkers, and the covering of biomarkers by metals in network analysis also supports this idea. As we know, metals are one of the prime pollutants in environment. Valavanidis, Vlahogianni, Dassenakis, & Scoullou, (2006) were also stated that the use of biomarkers is very important in the monitoring of environmental pollution, as they are an indicator of the damage of ROS products, which reveal as a result of oxidative stress, to lipid, DNA, and proteins, as well as the side effects caused by antioxidant, enzymatic, and non-enzymatic metabolic reactions.

6.4 Interactions among Metals, Biomarkers and Physicochemical Parameters

Pearson Product-Moment Correlation test was applied to November 2020 and May 2021 dataset with physicochemical parameters. Results show that Hg concentrations were correlated with pH (-0.306), salinity (-0.352), and DO (0.397). Pb concentrations were correlated with DO (0.248). Se concentrations were correlated with pH (-0.292) and salinity (0.278). As levels were correlated with pH (-0.337), salinity (-0.408), and DO (0.337). V concentrations were correlated with DO (0.369). Lastly, MDA contents were correlated with DO (0.312).

Inverse correlation between salinity and metal ions could be linked with variability of uptake kinetics on aquatic environments. High salinity waters might cause reducing absorption rates (Veltman et al., 2008). Bienvenue et al., (1984) had claimed that pH levels of environment could affect Hg bioaccumulation with a large surface area (phytoplankton). In their study, diffusion flux of mercury salts had decreased with increased pH. They had thought that liposolubility might be one of the factors (Bienvenue et al., 1984). In one study, metal bioaccumulation and biomarker responses

were positively correlated with dissolved oxygen, associated with feed uptake, metabolic activity and growth (Azmat et al., 2016).

6.5 Environmental Indexes and Health Risk Assessments

In this context; MPI, PTWI, and TR values were calculated to investigate environmental indexes and health risk assessments.

6.5.1 MPI

MPI values were calculated to investigate combined evolutions of metals. For fish species, highest and lowest values were found on *C. labrosus*. For tissues, liver > gills > muscle descending order were observed on each species (Figure 6.5). Kruskal – Wallis test was applied to MPI values. Fish species (p-value = 0.281) and sampling periods (p-value = 0.418) did not show significant differences, however, locations (p-value = 0.007) and tissues (p-value < 0.001) were showed significant differences.

MPI values were followed similar patterns with previous studies (Jia et al., 2017; Kontas et al., 2022). Liver and gills metabolically active organs and provide higher amounts of storage for metals. Higher MPI values could be related metal bio-transfers through the food chain (Jia et al., 2017). MPI results of Foça were higher than İnciraltı results (Figure 6.5). These findings demonstrated that Gediz River could be the main source for the pollutants. Previous studies claims that Gediz River has polluted with agricultural, industrial, and domestic wastewaters (Bilgin & Uluturhan-Suzer, 2017).

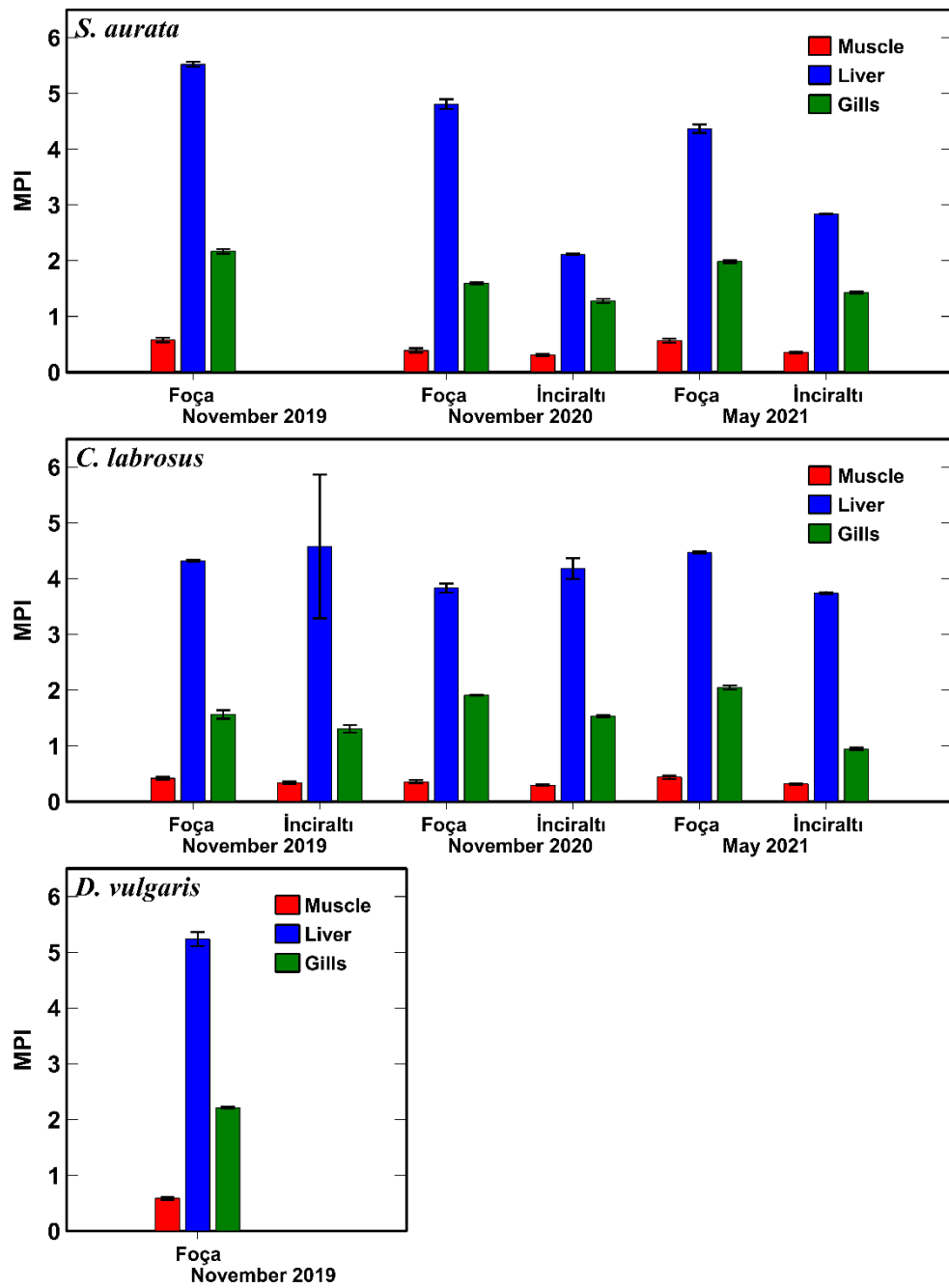


Figure 6.5 MPI values (mg/kg $\pm$ SD) of tissues on fish species.

6.5.2 PTWI

Potential health risks based on human consumption were investigated using PTWI values. Mean concentrations of edible tissues (muscle) and PTWI values were given at Table 6.14. According to the results, no metals were exceeded threshold values. Even though no potential risks were found on analyzed metals, it should be monitored whether the Hg and As values could approach the limit values in further years.

It is known that Hg and As are highly toxic elements. Chronic exposure of Hg may lead cardiovascular, neurologic, immunotoxic, nephrotoxic, and carcinogenic diseases (Genchi, Sinicropi, Carocci, Lauria & Catalano, 2017). In addition chronic As consumption may cause skin lesions, neurological effects, diabetes, high blood pressure, respiratory system problems, and cancers (Rahman, Ng & Naidu, 2009).

Table 6.14 Mean metal concentrations (mg/kg wet weight) of muscle tissues and PTWI values

Species	Location	Sampling Period	Hg	Cd	Pb	Cr	Cu	Zn	Mn	Se	Sn	As	V
<i>S.aurata</i>	Foça	Nov. 2019	0.192	0.0002	0.079	0.060	0.259	4.715	0.230	0.332	0.071	11.07	0.241
	Foça	Nov. 2020	0.165	0.0002	0.071	0.040	0.227	4.184	0.107	0.297	0.039	5.447	0.095
	Foça	May 2021	0.193	0.0002	0.080	0.041	0.107	4.286	0.127	0.317	0.103	15.33	0.242
	İnciraltı	Nov. 2020	0.027	0.0001	0.132	0.025	0.143	4.496	0.186	0.305	0.028	5.098	0.084
	İnciraltı	May 2021	0.050	0.0002	0.073	0.026	0.191	4.570	0.212	0.254	0.096	3.443	0.068
<i>C.labrosus</i>	Foça	Nov. 2019	0.057	0.0003	0.072	0.032	0.143	3.210	0.044	0.128	0.077	3.198	0.156
	Foça	Nov. 2020	0.007	0.0002	0.069	0.057	0.348	3.679	0.112	0.249	0.050	0.832	0.106
	Foça	May 2021	0.028	0.0003	0.133	0.017	0.141	3.026	0.171	0.275	0.044	2.823	0.097
	İnciraltı	Nov. 2019	0.004	0.0003	0.075	0.076	0.375	2.579	0.105	0.135	0.054	1.704	0.068
	İnciraltı	Nov. 2020	0.015	0.0003	0.059	0.019	0.194	2.422	0.122	0.176	0.038	0.696	0.046
<i>D.vulgaris</i>	İnciraltı	May 2021	0.011	0.0001	0.088	0.050	0.149	2.642	0.095	0.131	0.094	1.098	0.049
	Foça	Nov. 2019	0.142	0.001	0.245	0.029	0.393	4.561	0.265	0.231	0.079	2.875	0.038
PTWI			0.35 ^a	0.40 ^b	1.75 ^a	2.45 ^c	245 ^d	490 ^d	29.4 ^e	2.45 ^c	980 ^f	24.5 ^g	4.90 ^c

a: (FAO/WHO, 2004), b: (FAO/WHO, 2010), c: (ATSDR, 2022), d: (FAO/WHO, 2007), e: (WHO, 2008), f: (FAO/WHO, 1989), and g: (FAO/WHO, 1967).

6.5.3 TR

TR values were calculated to assume about lifetime cancer risk of toxic metals through the human consumption. According to the results, TR_{Cd} values were found between 9.73×10^{-8} and 8.54×10^{-7} . TR_{Pb} values were found 1.33×10^{-7} and 5.51×10^{-7} . Lastly, TR_{As} values were found 2.76×10^{-4} and 6.09×10^{-3} . TR findings were explained that TR_{Cd} and TR_{Pb} values were in acceptable limits, but TR_{As} values could pose a threat.

In marine organisms, arsenic is generally found as organic-As, which is a non-toxic form. The percentage of toxic inorganic-As varies between 0.02% and 11% in marine organisms (Falcó, Llobet, Bocio & Domingo, 2006). Considering all this information, it is suggested that As consumption in fish species from İzmir Bay may carry a potential carcinogenic risk. Inorganic-As provides the initiation of ROS production which may cause DNA mutations (Molin, Ulven, Meltzer & Alexander, 2015). IARC recognized inorganic-As as carcinogenic substance for skin, lung, and bladder cancers (Pullella & Kotsopoulos, 2020).

6.6 Comparisons of Metal Levels from The Literature

Metal levels of tissues in this study. were compared with some previous studies on different regions. In muscle tissues, Hg concentrations were similar with Ligurian Sea, Italy. Cd results were close with Canary Islands, but Cd concentrations were higher than this study. Pb concentrations were lower than İskenderun, Tuzla Lagoon, Adana, and Hurmaboğazı Lagoon. Pb results were similar with Canary Islands, Ria de Huelva, and Loukkos River Estuary. Other Pb concentrations were higher than this study. Cr and Cu concentrations in this study were generally lower than İskenderun, Adana, and Mumbai; other Cr and Cu concentrations were similar. Zn concentrations were lower than İskenderun, Tuzla Lagoon, Ria de Huelva, Adana, and Uralo River. Zn concentrations were higher than Canary Islands and Greek coasts of Aegean Sea. Mn concentrations were lower than Mumbai and as similar as other studies. When compared Sn and Se values with *L.parsia*, Sn and Se concentrations were found to be

higher and lower, respectively. As values were higher than Mumbai. Canary Islands had lower V concentrations than this study (Table 6.15).

Literature studies were conducted to compare the metal concentration values on the gills. In this study, Cd concentrations were lower than others. Pb concentrations were lower than Loukkos River Estuary, Morocco and as similar as other studies. Cr results were close with Aegean Sea, but lower than Adana and Mumbai. Cu concentrations in this study were generally lower than Ria De Huelva, Adana; other Cu concentrations were very close. Zn concentrations were higher than Tuzla Lagoon, Greek coasts of Aegean Sea, Loukkos River Estuary, and Mumbai. Mn concentrations were lower than the Uralo River and higher than Mumbai. When compared Sn and Se values with *L.parsia*, Sn and Se concentrations were found to be lower and higher, respectively. As values were similar than Mumbai and lower than Ria De Huelva (Table 6.16).

In liver tissues, Cd results were close with all other studies. Pb concentrations were lower than İskenderun, and higher than Canary Islands and Mumbai. Other Pb concentrations were found to be similar with this study. Mumbai and Adana had higher Cr concentrations. Other studies were very close to this study. Cu concentrations in this study were generally higher than Tuzla Lagoon, Greek coasts of Aegean Sea, Hurmaboğazı Lagoon, and Mumbai. Zn concentrations were lower than Ria de Huelva and higher than Tuzla Lagoon, Greek coasts of Aegean Sea, Hurmaboğazı Lagoon, Loukkos River Estuary, and Mumbai. In this study, all Mn concentrations were found to be higher than others. Se values were similar with Mumbai, but Sn concentrations were lower than Mumbai. When compared As results with others, the concentrations were found to be higher. Canary Islands had a close V concentration than this study. (Table 6.17).

Table 6.15 Metal levels in muscle tissues of fish species from İzmir Bay and different areas.

Location	Species	Hg	Cd	Pb	Cr	Cu	Zn	Mn	Se	Sn	As	V	References
Foça	<i>S.aurata</i>	0.55-0.71	0.0004-0.0010	0.24-0.69	0.10-0.21	0.38-0.91	13.8-17.2	0.25-0.81	0.87-1.26	0.09-0.49	19.1-58.0	0.32-0.94	This study
İnciraltı	<i>S.aurata</i>	0.094-0.18	0.0002-0.0005	0.26-0.64	0.07-0.12	0.49-0.69	13.9-16.1	0.55-0.83	0.79-1.10	0.07-0.38	11.7-18.9	0.20-0.31	This study
Foça	<i>C.labrosus</i>	0.031-0.27	0.0006-0.002	0.25-0.61	0.08-0.69	0.55-2.40	12.4-21.9	0.18-0.88	0.55-1.33	0.13-0.42	3.41-15.1	0.41-0.75	This study
İnciraltı	<i>C.labrosus</i>	0.01-0.073	0.0003-0.002	0.20-0.40	0.07-0.37	0.69-1.72	11.1-19.0	0.43-0.95	0.53-0.96	0.11-0.48	2.93-8.72	0.18-0.36	This study
Foça	<i>D.vulgaris</i>	0.57-0.60	0.0021-0.0023	1.00-1.16	0.11-0.12	1.34-1.63	18.4-18.7	1.09-1.25	0.89-1.06	0.21-0.54	11.5-12.4	0.14-0.16	This study
Ligurian Sea, Italy	<i>S.aurata</i>	0.29-0.72	-	-	-	1.3-2.2	15.0-22.0	0.3-0.6	-	-	18.4-39.6	-	A*
İskenderun, Türkiye	<i>S.aurata</i>	-	3.69-8.58	12.2-28.9	-	3.78-11.7	19.1-34.5	-	-	-	-	-	B
Tuzla Lagoon, Türkiye	<i>S.aurata</i>	-	0.06-0.13	0.63-4.71	-	0.55-0.83	6.08-129	-	-	-	-	-	C
Ria De Huelva, Spain	<i>S.aurata</i>	-	0.01	0.32	-	1.87	44.86	-	-	-	13.58	-	D
İskenderun, Türkiye	<i>S.aurata</i>	-	1.40-1.78	1.99-2.67	1.16-1.44	0.96-1.47	3.13-6.30	0.73-1.70	-	-	-	-	E
Aegean Sea, Greece	<i>S.aurata</i>	-	-	-	0.09-0.19	0.21-0.49	0.60-3.00	-	-	-	-	-	F**
Adana, Türkiye	<i>S.aurata</i>	-	0.26-0.48	4.80-6.28	0.78-1.70	2.41-3.27	19.04-34.28	-	-	-	-	-	G
Adana, Türkiye	<i>M.cephalus</i>	-	0.58-0.74	2.99-7.65	1.26-1.86	2.74-6.08	30.5-44.27	-	-	-	-	-	G
Uralo River, Spain	<i>C. labrosus</i>	-	nd-0.04	-	-	nd-6.0	12.0-86.0	nd	-	-	-	-	H
Canary Islands, Spain	<i>C. labrosus</i>	-	0.0011-0.0029	0.017-0.041	0.04-0.20	0.35-0.91	1.45-5.05	0.06-0.26	-	-	-	0.009-0.027	I***
Hurmaboğazi Lagoon, Türkiye	<i>C. labrosus</i>	-	0.11-0.13	0.96-1.48	0.32-0.44	0.49-0.73	7.63-9.81	0.75-1.53	-	-	-	-	J***
Loukkos river estuary, Morocco	<i>D.vulgaris</i>	-	0.01	0.64	0.39	29.49	34.67	-	-	-	-	-	K
Mumbai, India	<i>L. Parsia</i>	-	0.02	0.16.30	4.02-5.22	1.25-2.61	9.00-17.30	2.40-5.00	0.28-0.56	1.32-3.24	0.28-0.56	-	L***

References and Notations

A: (Minganti, Drava, De Pellegrini & Siccardi, 2010), B: (Kargin, 1996), C: (Dural, Göksu & Özak, 2007), D: (Vicente-Martorell, Galindo-Riaño, García-Vargas & Granado-Castro, 2009), E: (A. Türkmen, M. Türkmen, Tepe & Akyurt, 2005), F: (Castritsi-Catharios, Neofitou & Vorloou, 2015), G: (Canli & Atli, 2003), H: (Legorburu, Canton, Millan & Casado, 1988), I: (Afonso et al., 2017), J: (A. Türkmen, Tepe & M. Türkmen, 2016), K: (M. El Morhit, Fekhaoui, A. El Morhit, Elie & Yahyaoui et al., 2013), and L: (Kumar et al., 2021).

*: Wild samples were picked, **: Farmed individuals, ***: results were presented as wet weight.

Table 6.16 Metal levels in liver tissues of fish species from İzmir Bay and different areas.

Location	Species	Hg	Cd	Pb	Cr	Cu	Zn	Mn	Se	Sn	As	V	References
Foça	<i>S.aurata</i>	0.437-0.84	1.28-1.83	2.32-3.98	0.29-0.63	51.9-79.6	212-368	6.97-13.3	2.99-4.68	0.51-0.69	18.5-39.1	1.03-6.32	This study
İnciraltı	<i>S.aurata</i>	0.09-0.24	0.49-1.76	1.44-1.76	0.31-0.45	15.7-20.6	110-147	4.59-6.77	2.31-2.90	0.30-0.59	11.8-16.9	0.81-1.78	This study
Foça	<i>C.labrosus</i>	0.056-0.21	3.19-5.94	2.12-4.38	0.30-1.44	144-568	81.1-174	3.10-4.34	2.46-6.19	0.42-0.55	9.11-18.1	1.63-3.68	This study
İnciraltı	<i>C.labrosus</i>	0.14-0.51	2.40-5.92	1.59-2.37	0.52-1.50	56.4-775	109-180	3.14-4.36	2.60-13.7	0.29-0.60	13.8-19.6	0.81-1.59	This study
Foça	<i>D.vulgaris</i>	0.20-0.22	3.30-3.82	9.78-10.3	0.43-0.58	40.9-41.3	165.5-165.9	9.05-9.36	3.46-3.74	0.71-0.86	21.4-22.0	2.67-2.91	This study
İskenderun, Türkiye	<i>S.aurata</i>	-	8.25-21.8	33.8-60.0	-	67.3-92.8	188-223	-	-	-	-	-	B
Tuzla Lagoon, Türkiye	<i>S.aurata</i>	-	0.13-0.25	1.85-3.93	-	2.11-3.12	30.0-74.5	-	-	-	-	-	C
Ria De Huelva, Spain	<i>S.aurata</i>	-	4.24	1.58	-	394	230	-	-	-	7.13	-	D
Aegean Sea, Greece	<i>S.aurata</i>	-	-	-	0.08-0.27	0.15-2.36	1.80-30.9	-	-	-	-	-	F**
Adana, Türkiye	<i>S.aurata</i>	-	0.72-1.20	6.74-11.0	1.33-1.99	17.13-49.61	59.06-93.88	-	-	-	-	-	G
Adana, Türkiye	<i>M.cephalus</i>	-	0.73-2.55	6.79-18.39	1.13-8.03	202.8	75.5-145	-	-	-	-	-	G
Uralo River, Spain	<i>C. labrosus</i>	-	0.86-3.4	-	-	250-400	180-260	0.4-1.9	-	-	-	-	H
Canary Islands, Spain	<i>C. labrosus</i>	-	0.11-0.49	0.47-1.33	0-0.54	50.65-396.6	50.92-178.3	0.74-1.30	-	-	-	1.52-7.48	I***
Hurmaboğazi Lagoon, Türkiye	<i>C. labrosus</i>	-	0.13-0.15	0.75-1.55	0.35-0.39	0.76-1.70	8.57-10.47	0.84-1.82	-	-	-	-	J***
Loukkos river estuary, Morocco	<i>D.vulgaris</i>	-	0.01	1.06	0.80	34.13	72.27	-	-	-	-	-	K
Mumbai, India	<i>L. Parsia</i>	-	0.08-0.12	0.67-1.31	6.44-8.40	2.25-2.53	8.51-10.77	2.72-5.60	4.91	7.72-9.66	0.61-1.05	-	L***

References and Notations

B: (Kargin, 1996), C: (Dural, Göksu & Özak, 2007), D: (Vicente-Martorell, Galindo-Riaño, García-Vargas & Granado-Castro, 2009), F: (Castritsi-Catharios, Neofitou & Vorloou, 2015), G: (Canli & Atli, 2003), H: (Legorburu, Canton, Millan & Casado, 1988), I: (Afonso et al., 2017), J: (A. Türkmen, Tepe & M. Türkmen, 2016), K: (M. El Morhit, Fekhaoui, A. El Morhit, Elie & Yahyaoui et al., 2013), and L: (Kumar et al., 2021).

** : Farmed individuals, ***: results were presented as wet weight.

Table 6.17 Metal levels in gills of fish species from İzmir Bay and different areas.

Location	Species	Hg	Cd	Pb	Cr	Cu	Zn	Mn	Se	Sn	As	V	References
Foça	<i>S.aurata</i>	0.10-0.24	0.0006-0.0042	0.85-2.21	1.12-1.37	1.79-3.36	63.0-98.0	17.0-26.7	2.44-4.68	1.20-1.98	5.46-6.79	4.92-9.24	This study
İnciraltı	<i>S.aurata</i>	0.05-0.08	0.0017-0.0023	0.94-1.37	0.61-1.31	2.92-4.20	64.6-80.6	11.9-19.8	2.51-6.40	0.74-1.66	2.97-5.46	2.63-3.81	This study
Foça	<i>C.labrosus</i>	0.015-0.064	0.003-0.006	3.55-12.1	0.50-2.15	2.62-5.98	50.3-90.8	21.5-35.1	4.18-4.59	1.15-2.04	1.60-2.79	4.33-6.37	This study
İnciraltı	<i>C.labrosus</i>	0.015-0.026	0.0016-0.005	0.86-6.63	0.30-1.24	2.54-6.79	54.4-85.9	23.4-38.4	2.58-6.23	1.13-1.52	0.50-2.99	1.90-3.84	This study
Foça	<i>D.vulgaris</i>	0.11-0.12	0.0040-0.0042	16.2-16.8	0.70-0.75	2.44-3.13	84.5-91.7	24.2-25.3	4.54-4.96	1.505-1.507	3.11-4.14	7.00-7.97	This study
İskenderun, Türkiye	<i>S.aurata</i>	-	4.57-10.3	25.2-44.4	-	7.39-17.2	67.7-92.7	-	-	-	-	-	B
Tuzla Lagoon, Türkiye	<i>S.aurata</i>	-	0.77-1.25	1.90-5.82	-	0.76-3.86	11.5-53.8	-	-	-	-	-	C
Ria De Huelva, Spain	<i>S.aurata</i>	-	0.10	3.68	-	9.96	97.52	-	-	-	11.17	-	D
Aegean Sea, Greece	<i>S.aurata</i>	-	-	-	0.10-0.52	0.36-1.03	17.6-29.6	-	-	-	-	-	F**
Adana, Türkiye	<i>S.aurata</i>	-	1.35-2.23	10.44-16.18	2.60-4.02	4.19-5.85	54.0-72.2	-	-	-	-	-	G
Adana, Türkiye	<i>M. cephalus</i>	-	1.64-2.52	5.88-12.02	3.44-6.26	6.14-20.82	56.97-85.45	-	-	-	-	-	G
Uralo River, Spain	<i>C. labrosus</i>	-	0.12-0.23	-	-	6.5-8.2	62-72	41-60	-	-	-	-	H
Loukkos river estuary, Morocco	<i>D.vulgaris</i>	-	60.57	34.06	0.30	0.25	0.05	-	-	-	-	-	K
Mumbai, India	<i>L. Parsia</i>	-	0.03	0.22-0.34	4.09-6.77	1.86-3.06	13.83-22.99	4.80	0.49-0.63	2.01-2.05	0.46-0.48	-	L***

References and Notations

B: (Kargin, 1996), C: (Dural, Göksu & Özak, 2007), D: (Vicente-Martorell, Galindo-Riaño, García-Vargas & Granado-Castro, 2009), F: (Castritsi-Catharios, Neofitou & Vorloou, 2015), G: (Canli & Atli, 2003), H: (Legorburu, Canton, Millan & Casado, 1988), I: (Afonso et al., 2017), K: (M. El Morhit, Fekhaoui, A. El Morhit, Elie & Yahyaoui et al., 2013), and L: (Kumar et al., 2021).

** : Farmed individuals, *** : results were presented as wet weight.

CHAPTER SEVEN

CONCLUSION

In this thesis, biomarker responses on metal pollution were determined by using different tissues (muscle, liver, and gills) of selected fish species (*Sparus aurata*, *Chelon labrosus*, and *Diplodus vulgaris*) from İzmir Bay. Eleven metals (Hg, Cd, Pb, Cr, Cu, Zn, Mn, Se, Sn, As, and V) were determined to assess biomarker responses (CAT, SOD, MDA, and MT). Three different sampling seasons (November 2019, November 2020, and May 2021) on two different locations (Foça and İnciraltı) were carried out.

Higher levels of metal bioaccumulations and biomarker responses were generally found on liver and gills. Statistical analyses were confirmed that the reason of variations on tissues were related to accumulation tendencies of organs. Locational variations were having linkage with natural and anthropogenic activities of Inner and Outer regions of İzmir Bay. Changes between sampling periods could be under influence of increased activity with the increased temperature and post-spawning seasons. Differences of fish species were strongly linked with efflux rates of elements, lipid contents and ingestion rates, swimming activity, and physiology.

According to the health risk assessments, none of analyzed metals were exceeded threshold PTWI limits, however, Hg and As levels could pose a threat on forthcoming years, therefore their levels should be monitored regularly. In addition, TR values were showed that As findings were in carcinogenic risk level.

When metal bioaccumulation in fish species and biomarker responses of organisms are evaluated together, it has been observed that biomarkers can express metal pollution considerably in environmental biomonitoring studies. For this reason, increasing use of biomarkers in terms of biomonitoring the effects of metals and other pollution sources in the marine environment should provide fast, consistent, and cost-effective results.

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