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Molecular fingerprint of gilthead seabream physiology in response to pollutant mixtures in the wild

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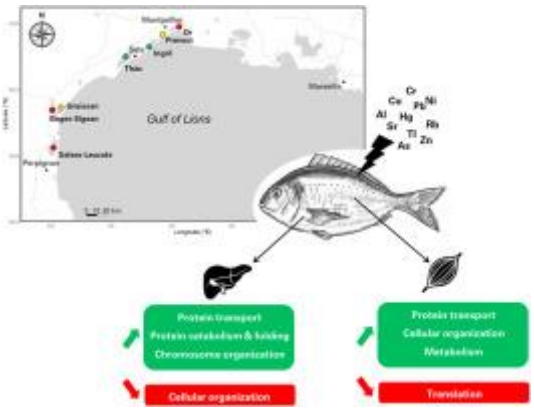
Abstract :

The increase in trace element concentrations in the aquatic environment due to anthropogenic activities, urges the need for their monitoring and potential toxicity, persistence, bioaccumulation, and biomagnification at different trophic levels. Gilthead seabream is a species of commercial importance in the Mediterranean Sea, both for the aquaculture and fisheries sectors, however very little is known about their trace element contamination accumulation and the resulting effect on their health status. In the present study, 135 juveniles were collected from seven coastal lagoons known to be essential nursery areas for this species. We measured seventeen different inorganic contaminants at the individual level in fish muscle (namely Al, As, Be, Bi, Cd, Cr, Cu, Hg, Li, Ni, Pb, Rb, Sb, Sr, Ti, Tl and Zn). Our results revealed the accumulation of multiple trace elements in individuals and distinct contamination signatures between lagoons which might lead to contrasted quality as nurseries for juveniles of numerous ecologically and economically relevant fish species in addition to seabreams. We further evaluated the potential adverse effect of these complex contamination mixtures on the liver (the main organ implicated in the metabolism of xenobiotics) and red muscle (a highly metabolic organ) using a proteomic approach. Alterations in cellular organization pathways and protein transport were detected in both tissues (albeit they were not similarly regulated). Chromosome organization and telomere maintenance in the liver appeared to be affected by contaminant mixture which could increase mortality, age-related disease risk and shorter lifetime expectancy for these juveniles. Red muscle proteome also demonstrated an upregulation of pathways involved in metabolism in response to contamination which raises the issue of potential energy allocation trade-offs between the organisms' main functions such as reproduction and growth. This study provides new insights into the cellular and molecular responses of seabreams to environmental pollution and proposed biomarkers of health effects of trace elements that could serve as a starting point for larger-scale biomonitoring programs.

Graphical abstract



Highlights

► Anthropogenic activities drive aquatic trace element rise, demanding monitoring. ► Little's known about trace element impact on seabream health. ► Seabreams show diverse trace element accumulation in lagoons. ► Contamination affects liver and muscle proteins, impacting health. ► Seabreams face metabolic trade-offs and increased stress due to contamination.

Keywords : Shotgun proteomic, Ecotoxicology, Sparus aurata, Fish health, Lagoon, Cocktail effect

Introduction

Coastal lagoons are critical transition zones between the marine and continental domains that function as nursery areas and feeding grounds for numerous fish species (Franco et al., 2009; Velasco et al., 2018). However, their proximity to various sources of pollution could reduce their quality as nurseries for juveniles of numerous highly prized fish species (Brusle & Cambrony, 1992; Quignard et al., 1984; Tournois et al., 2017), such as the gilthead seabream *Sparus aurata* (Audouin, 1962). In the Gulf of Lions, *S.aurata* performs ontogenetic and trophic migrations between coastal lagoons and the sea (Isnard et al., 2015), related to spawning (Audouin, 1962), settlement and recruitment (Morais et al., 2017). Juveniles that colonise sheltered coastal areas in early winter migrate out to sea the following autumn (Audouin, 1962). However, despite its commercial importance both for the aquaculture and fisheries sectors (Farrugio et al., 1994), very few studies have explored the health effects resulting from contaminant exposure (Isani et al., 2009; Minghetti et al., 2008,2010).

Among natural and anthropogenic pollutants, trace elements are present in minute quantities in all environmental compartments. Some of them, such as Cu, Zn and Fe, are essential for animals, including fish, for maintaining basic cellular metabolic processes, in particular through their functional role as co-factors of different enzymes (Wood et al., 2011). However, an excessive concentration of these elements can lead to detrimental effects on a wide range of biological pathways (Gaetke & Chow, 2003). On the other hand, non-essential trace elements, such as Pb, Hg, Cd, As and Ni, can cause toxicity even at very low concentration (Wood et al., 2011). Metals are well-known inducers of oxidative stress in fish, causing an imbalance between the production of oxidative species including reactive oxygen species (ROS) (e.g. H_2O_2, $HO^{\cdot}$, O-2, $R^{\cdot}$, ROO) and cellular antioxidant activity (Halliwell & Gutteridge, 2015). Increased ROS can damage DNA structures and therefore alter the expression patterns of important proteins, different hormones, enzymes, etc. (Javed et al., 2017; Morcillo et al., 2016).

Monitoring pollution impacts on living organisms through the use of biomarkers associated with trace element toxicity, such as antioxidant enzymes (Pereira et al., 2013; Williams & Yoshida-Honmachi, 2013), heat shock proteins (HSP, Downs et al., 2006) and metallothioneins (Sakuragui et al., 2013; Williams & Yoshida-Honmachi, 2013) is widespread in environmental and ecotoxicology programs. However, as highlighted by some authors (see e.g., Laviale et al., 2010; Stachowski-Haberkorn et al., 2008), most earlier research lacks environmental realism. The main reason is that past experiments were mainly based on laboratory tests typically performed under controlled conditions, from single-contaminant tests. Yet, wildlife species, including many fish populations, reside and reproduce in an environment where they are continuously and increasingly exposed to complex mixtures of anthropogenic chemicals (especially in coastal areas). These cocktails of contaminants, unlike the effects of single compounds, can have additive, synergistic or antagonistic effects resulting from contaminant interactions (DeLorenzo & Fleming, 2008; Relyea, 2009). Biomarkers based on studies of the effects of single elements may therefore prove useless, as they may respond differently to complex mixtures of pollutants (Celander, 2011). Greater attention must therefore be paid to the toxicity of mixtures for the health of wildlife.

The emergence of “omics” tools offers powerful prospects for the analysis of complex, integrated responses to contaminants, and are more sensitive and specific in studying molecular changes in organisms (Benninghoff, 2007; Denslow et al., 2005). Moreover, high throughout shotgun proteomics compared to traditional biochemical methods offers the unique possibility to simultaneously examine, in a single experiment, the thousands of proteins expressed in a specific tissue, which greatly improves our understanding at the molecular level (López-Pedrouso et al., 2020; Sanchez et al., 2011). Furthermore, it presents a unique opportunity to identify few relevant biomarkers, which could be then used to develop more effective monitoring programs for assessing the impact of pollutants on marine species (Apraiz et al., 2006; Benninghoff, 2007).

The present study assessed *in-situ* inorganic pollutant contamination in juveniles of gilthead seabreams in the Gulf of Lion (NW Mediterranean Sea) and the impact of these cocktails of contaminants on individual health using a proteomic approach. Our objectives were: 1) to assess trace element contamination patterns in wild *S.aurata* juveniles across 7 lagoons of the Gulf of Lion, 2) to investigate their impact on the proteome of the liver (the main organ implicated in the metabolism of xenobiotics) and red muscle (a highly metabolic organ), in order to unravel the possible adverse effects of accumulated pollutants on individual's health and to identify for both tissues a few potential biomarkers that could serve as valuable tools for long-term monitoring of gilthead seabream health.

Materials and Methods

Study sites and sampling

In this study conducted in the Gulf of Lions (Northwestern Mediterranean Sea), 135 juveniles of *Sparus aurata* were collected from seven coastal lagoons known to be essential nursery areas for this species (Tournois et al., 2017, ESM1). Specimens were collected in October-November 2021 using traditional passive fishing gear, called "capechade", composed of a pound net and several fyke nets, commonly used by fishermen in lagoons. Among those 135 juvenile individuals, between 17 and 20 fishes were sampled in each lagoon and were measured (total length TL, in mm) and weighed (total mass M, in g). Their body condition was estimated based on the Fulton's index K (Rätz & Lloret, 2003), commonly used as an indicator of general well-being and calculated as follows:

$$K = 10^5 M \,/\, TL^3$$

where M is the total wet mass in g and TL the total length in mm.

Liver and red muscle (taken on the midline of the flank) were removed, snap-frozen in liquid nitrogen and stored at −80 °C for proteomic analysis. White muscle of left fish sides taken from the epaxial mass was removed and stored at -40°C for trace element analysis. Red muscle was cleanly separated from white muscle by scraping the lateral skin stripped from the animal.

Trace-element analysis

All samples were lyophilized using the freeze dryer Lyovac GT4 (Leybold, Cologne, Germany). For Al, As, Be, Bi, Cd, Cr, Cu, Li, Ni, Pb, Rb, Sb, Sr, Ti, Tl and Zn determination, the samples underwent a pre-treatment process where 0.5 grams of each muscle sample were subjected to acid digestion using 4 mL of HNO3 (69%) and 1 mL of H2O2 (33%) in special Teflon reaction tubes within a microwave digestion system (UltraClave-Microwave Milestone®) for 20 minutes at 220 °C, and then diluted to 10 mL with double deionized water (Milli-Q). Samples were analyzed using inductively coupled plasma optical emission spectrometry (ICP-OES, ICAP 6500 Duo, Thermo) to determine the levels of this elements. The detection limit was 0.001 mg/kg. Each sample was read in duplicate and averaged. Based on UNE-EN ISO reference 11885, multi-element calibration standards (SCP Science, in 4% nitric acid) were assembled with different concentrations of inorganic elements. For calibration, see (Romero et al., 2020).

Total mercury (Hg) content was measured using an atomic absorption spectrometer AMA254 Advanced Mercury Analyzer (Leco) without pre-treating or pre-concentrating the samples (wavelength = 253.65 nm, detection limit (DL) = 0.003 $\mu g.g^{-1}$). The recovery rate for reference materials (Mercury ICP Standard 1000 mg L−1 Hg, Merck) was above 95%.

Quantitative proteomic analysis

Proteomic analyses of liver and red muscle were conducted as two separate experiments on a subsample of 31 individuals selected based on their inorganic contamination levels in white muscle, so that one half of juveniles displayed the lowest trace element load (n = 15) and the other half the highest (n = 16) (see Statistical analysis section for further details on individuals 'selection). The process of sample preparation, nanoLC-MS/MS analysis, and mass spectrometry data analysis is described in detail in ESM2. The procedure involved protein extraction and electrophoresis using SDS-PAGE, followed by in-gel digestion with trypsin (Promega, Madison, WI, USA). The resulting peptides were extracted and analyzed using a nanoUPLC-system (nano-Acquity, Waters, Milford, MA, USA) coupled to a quadrupole-Orbitrap hybrid mass spectrometer (Q-Exactive HF-X, Thermo

Scientific, San Jose, CA, USA), controlled by XCalibur software (v4.0.27.9; Thermo Fisher Scientific). The Q-Exactive HF-X was operated using a data-dependent acquisition (DDA) strategy by selecting the Top-20 most intense ions in MS1 for fragmentation in MS2. MS raw data processing was performed in MaxQuant (v2.0.3.1) (Cox et al., 2014), using Andromeda algorithm to search peaklists against a protein database containing the 69,200 protein sequences from the *S. aurata* UniprotKB proteome (TaxID: 8175; Reference proteome ID: UP000472265; July 2022). Only the proteins identified with at least two unique peptides were retained. Protein quantification was performed using unique peptides only via the MaxLFQ option implemented in MaxQuant (Cox et al., 2014). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al., 2019) partner repository with the dataset identifiers PXD037424 (liver) and PXD037412 (red muscle). During the analysis, protein extraction was unsatisfactory for one red muscle sample, which was subsequently removed. QC-related measurements indicated stable performances of the analysis system all along the two experiments, with median coefficients of variation (CV) of 0.41% (liver) and 1.2% (red muscle) for retention times of iRT peptides over all injections. Median CVs of only 8.8% (liver) and 11.1% (red muscle) were obtained for LFQ values of the proteins quantified from the repeated injections of reference samples.

Protein functional annotation

In order to analyse our proteomic data from a functional point of view, we examined the functional annotations of proteins listed by the AMIGO consortium (Gene Ontology, GO; http://geneontology.org/). However, only a handful of *S. aurata* proteins are annotated in GO databases. We therefore took advantage of the crucial evolutionary position of the spotted gar (Lepisosteus oculatus) between teleost fishes and humans, which creates a very valuable bridge between their genomes (Braasch et al., 2016) and of the fact that gar proteins are well annotated in GO databases. We began by searching L. oculatus sequences (UniprotKB, 22,463 protein entries; September 2022) for proteins homologous to S. aurata proteins using BLAST searches (FASTA v36.1.4 program; downloaded from http://fasta.bioch.virginia.edu/fasta_www2/fasta_down.shtml), and only the top BLAST hit for each protein was retained. From L. oculatus protein sequences, the same strategy was employed to search for human homologous proteins (TaxID: 9606; Reference proteome accesed in September 2022). The relevance of the match between homologous proteins was checked manually and only 146 of 3104 matches could not be validated. An automatic extraction of GO annotations was then performed using the MSDA software (Carapito et al., 2014).

Statistical analysis

Element concentrations below LODs (0.001 mg/kg) were set to $LOD/\sqrt{2}$ as suggested as the best substitution method by Verbovsek (2011). Analyses comparing trace elements contamination patterns included only elements that were detected in at least 50% of individuals out of the 135 collected. In order to detect possible relationships between contamination and fish body size, we used a linear model (LM) between each contaminant concentration and total length. Normality and homoscedasticity of the residuals were verified using the Shapiro's and Levene's tests respectively. When residuals distribution did not follow a normal distribution, a Box Cox-based transformation was applied prior to model fitting. If necessary ($P<0.05$), size-corrected contaminant was defined as back-transform residuals of the regression model.

To investigate relationships between contaminants within and between lagoons, we ran Principal Component Analysis (PCA) on trace element concentrations (size-corrected when required) of the 135 individuals. Furthermore, radar plots of the seven sampling sites (lagoons) were drawn based on trace element concentrations.

In the following proteomic approach, we analyzed a subset of 31 individuals who exhibited the most distinct contamination profiles. This selection process involved assigning ranks to seabreams (from all lagoons) based on the level of each contaminant detected. The ranking ranged from 1 (indicating the lowest contamination level) to 135 (representing the highest contamination level). The individuals were then chosen based on the total sum of these ranks. Consequently, we identified two groups, one comprising 15 seabreams with the lowest contamination levels, and the other consisting of 16 seabreams with the highest contamination levels. Then, PCA using the 31 individuals as objects and the 14 contaminants as descriptors was run, using a correlation matrix. PCA axes (PC1, PC2, PC3) were then retained as the three main contaminant mixtures (or cocktails of contaminants) found in seabreams. These three contaminant mixtures were used in further analyses.

Prior to statistical analyses, the proteomic datasets were filtered to keep only proteins that were expressed in at least 70% of individuals in each contamination group leaving 2742 (out of 3104) and 1018 (out of 1218) expressed proteins in the liver and red muscle, respectively. Two and three individuals were removed due to a high number of missing values for liver and muscle proteomic analysis respectively. Missing values were imputed for each contamination group based on random forest method. Previous studies have shown that this method exhibits strong performance and is particularly well-suited for label-free proteomic studies, where the underlying reasons for missing data are not fully understood (Jin et al., 2021; Kokla et al., 2019). The variance stabilization normalization (vsn) method was used to normalize the protein intensities. This method demonstrated good performance in evaluating differential expression statistics. (Välikangas et al., 2018). The aim of performing VSN normalization is to standardize the scale of samples. This is

accomplished by initially removing any variance that may have arisen from systematic experimental factors, followed by the application of a generalized log2 transformation.

Statistical analyses were performed with the statistical open source R software v.4.1.1 (R Core Team 2021) using the packages "FactoMineR" v.2.4 (Lê et al., 2008), "factoextra" v.1.0.7 (Kassambara & Mundt, 2020), "missForest" V.1.5 (Stekhoven & Bühlmann, 2012) and "DEP" V.1.16.0 (Zhang et al., 2018).

Co-expression network and enrichment analysis

Gene co-expression network and identification of hub proteins

Weighted gene co-expression network analysis implemented in the WGCNA R package (Langfelder & Horvath, 2008) was used for both tissues (liver and red muscle) to identify groups, hereafter named "modules", of proteins whose expression significantly correlates with the three retained trace element mixtures. First, a signed adjacency matrix for all pairs of proteins was constructed using the spearman correlation raised to the power (beta) of six and four to approximate a scale-free network for liver and red muscle, respectively. The adjacency matrix was then transformed into a topological overlap dissimilarity matrix and a combination of hierarchical clustering. A dynamic tree-cutting algorithm was used to first define and then merge co-expressed modules of proteins. Proteins outside any modules (indicating low co-expression) were gathered in a "grey module". The module's eigengene (i.e. first axis of a principal component analysis conducted on the expression of all proteins of a given module) summarizes the expression of all proteins in that module. Then, we investigated whether each module's eigengene was correlated with each trace element mixtures (PC1, PC2 and PC3), using spearman correlation. Modules with a significant ($P < 0.05$) spearman correlation of at least 0.4 were retained for further analysis. Positive and negative relationships reflect the up- and down-regulation of the module with increasing contaminant concentration, respectively. To explore the functional and physiological mechanisms associated with each module, we performed functional enrichment analyses.

Functional enrichment analysis and pathway network

For both tissues, enrichment analysis (biological process and cellular component) of the proteins constituting each module was performed using Fisher's exact test using the '*GO_MWU*' package particularly suitable for non-model organisms (https://github.com/z0on/GO_MWU) (Dixon et al., 2015). It allows to determine the functional significance of the identified modules. Enriched GO terms are displayed in a dendrogram plot with distances between terms reflecting the number of shared proteins. Additionally, we assessed the Spearman correlation between each protein and the trace element mixtures (PCA axes) to infer "gene significance" (GS). These GS relationships were compiled within a circle plot for each enriched pathway of the module, focusing on the top 10 enriched biological process terms (GO terms). These plots highlight the up- or down-regulation of the proteins and pathways in response to the three trace element mixtures.

Network visualization

ClueGO was used to illustrate overrepresented Gene Ontology. ClueGO is a Cytoscape plug-in that visualizes the non-redundant biological terms for large numbers of proteins and integrates the GO terms to create a GO/pathway network (Bindea et al., 2009). A network for each tissue was made in order to have a better visualization of the pathways impacted by the three trace element mixtures.

Hubproteins identification

Finally, for each module, GS was correlated with module membership (MM; spearman correlation of protein intensity *versus* the module eigengene) to identify proteins that show the highest degree of connectivity within a module and with the considered trace element mixture (hub proteins). The top 5 proteins with simultaneously the highest module membership and gene significance (rank sum) were selected as hub protein of each module. Due to their central position in the network, hub proteins are expected to play important biological roles within their module and are considered as potential biomarkers.

Functional Comparison between tissues

For each trace element mixture, the similarity in functional response between tissues was compared by plotting their respective GO term delta ranks against each other. A GO term delta rank corresponds to the difference between the mean rank (based on GS values) of annotated proteins it contains *vs* the mean ranks of all the protein not included. Positive and negative delta ranks indicate the GO term tends toward upregulation and downregulation, respectively. The strength of the relationship reflects the similarity between enrichments. It is important to note that these plots do not represent a formal statistical test, as the data points (gene ontology categories) are not independent. Indeed, they often encompass overlapping sets of proteins, nonetheless it allows identifying functional similarity or dissimilarity between functional enrichments.

Results

Contamination profiles

After selection of contaminants detected (>LOD) in at least 50% of individuals (*i.e.* 68 individuals), we retained 14 trace elements in white muscle (Al, As, Cd, Cu, Cr, Hg, Li, Ni, Pb, Rb, Sr, Ti, Tl and Zn, ESM3). Only six exhibited significant but low correlation with fish total length and were size-corrected (i.e., aluminium, arsenic, lithium, strontium, thalium and zinc). While aluminum had positive relation with fish size (R^2 = 0.03, P<0.05), arsenic (R^2 = 0.24, P<0.001), lithium (R^2 = 0.08, P<0.001), strontium (R^2 = 0.07, P<0.01), thallium (R^2 = 0.07, P<0.01) and zinc (R^2=0.05, P<0.01) displayed a negative association.

The first three axes of PCA performed on the 135 individuals as objects and contaminants concentration as descriptors reflected 20.6%, 14.5% and 11% of the total variance, respectively (ESM4). Cu, Cr, Ni, Al, Tl; Rb were all positively loaded on PC1. Rb, Tl and As were significantly and positively loaded to PC2 in opposition to Pb, Al and Ti. Sr and Cr were negatively loaded on PC3 in opposition to Zn, Ti, As and Pb (ESM4). The projection of the seven lagoons within the same PCA highlighted a large overlap of the contamination between lagoons. Considering trace element levels along the PC1 axis, individuals from Prevost and Ingril lagoons displayed low contamination

compared to Salses-Leucate, Or and Bages-Sigean lagoons. Notably Bages-Sigean lagoon revealed a high variance dispersion (inter-individual variability, Figure 1). Moreover, radar plots were used to visually compare the contamination profile across sampling sites (ESM5). The radar plots for the different lagoons varied markedly. Indeed, Ingril and Prevost lagoons displayed generally low concentrations of all elements, except for mercury, which exhibited the highest level in the Prevost lagoon (ESM5e). Seabreams captured in Bages-Sigean and Salses-Leucates presented the highest concentration in 5 elements (Al, Cu, Ti, Sr, Pb; ESM5a and f). High levels of Cr, Rb, Sr and Nickel were found in the Or lagoon (ESM5d). The radar plot of the Thau contamination showed low to medium concentrations for all elements (ESM5g). Finally, Gruissan revealed the greatest concentration in Zn and high levels of As (ESM5b).

The first 3 principal components of the PCA ran on the 31 individuals selected for proteomic analysis, reflected 28%, 17.4% and 10.5% of the total variance respectively (ESM6). PC1 clearly separated the 2 groups of individuals previously selected (high contamination vs low contamination). and reflected higher contamination in Al, Cu, Sr, Pb, Ni, Cr, Tl and Ti. PC2 (contaminant mixture 2) reflected higher contamination to Rb, Tl, As and Zn and lower contamination to Pb while PC3 (contaminant mixture 3) reflected higher contamination to Hg, Zn, Ni and (in a lower proportion) Pb and a lower contamination to Sr. The three PCs independently reflected trace element mixtures/profiles as resumed in Table 1.

Physiological response to inorganic contamination exposure

The three contaminant mixtures based on the PCA with 31 individuals displayed no significant correlation with individual body condition. Therefore, we further investigated the impact of trace element contamination at the proteome level on two central organs in fish (the liver and the red muscle) using a co-expression network analysis.

Liver proteome

After the definition of the protein network, we applied a dynamic clustering method implemented in WGCNA to identify modules of highly correlated proteins. We identified 18 different

315 modules encompassing a total of 2742 proteins. We then examined the relationship between
316 expression modules and the three contaminant mixtures to assess contaminant exposure
317 consequences on individual health. Three co-expressed modules exhibited significant correlation
318 ($P < 0.05$) with either mixtures 1, 2 or 3 (Figure 3a and 4).

319 Mixture 1 and Mixture 3 showed positive relationship with the pink and turquoise modules,
320 respectively, while Mixture 2 was negatively correlated to the magenta module. A positive
321 relationship highlights an overexpression of the proteins in the module with increasing
322 contamination, while a negative relationship reflects an underexpression of the proteins in the
323 module with increasing contamination. In detail, pathway enrichment within the pink module
324 included "vesicle transport" with proteins overexpression with increasing contamination with
325 mixture 1 (Al, Cu, Sr, Pb, Ni, Cr, Tl and Ti, ESM7a and c). Proteins from this module were mainly
326 located in nuclear pores (ESM7b). On the contrary, the negative correlation of the Magenta module
327 with increasing contamination with mixture 2 (Rb, Tl, As, Zn and - Pb) indicated a downregulation of
328 the 52 enriched pathways mainly related to cytoskeleton/cellular organization (ESM8a and c).
329 Accordingly, the GO cellular component analysis highlighted that these proteins were mainly
330 involved in the cytoskeleton (ESM8b). Finally, the positive relationship with the turquoise module
331 revealed an up-regulation of proteins mainly involved in protein catabolism, chromosome and
332 telomere organization and protein folding (ESM9a and c) with increasing levels of Hg, Zn, Ni and Pb
333 and decreasing Sr (mixture 3). As for the cellular component involved, proteins were essentially part
334 of protein complexes involved in protein metabolism (*e.g.* proteasome complex, chaperone complex)
335 and microtubules (ESM9b).

336 To identify potential biomarkers, hub proteins (i.e., the top 5 proteins with the highest GS
337 and MM) were selected for each module (see ESM10). Within the pink module (positively linked to
338 mixture 1), hub proteins were implicated in protein transport and/or localization (ESM10). Notably
339 one of them (Dynamin 1-life protein) is also involved in necrosis pathways. Within the Magenta

module (negatively linked to mixture 2), three hub proteins were involved in metabolism (pyruvate or phospholipid processes) or cellular structure. We noted that one of these proteins (gelsolin, ESM10) is also involved in apoptosis. Finally, hubproteins for the turquoise module (responding to mixture 3) were associated with multiple functions such as DNA damage repair, protein folding, response to ROS and oxygen homeostasis (ESM10).

Muscle proteome

A total of five modules including blue (159 proteins), brown (119 proteins), turquoise (594 proteins), green (48 proteins), yellow (95 proteins) and grey (3 proteins) were obtained from WGCNA analysis of the red muscle proteome. Only two modules (turquoise and green) correlated significantly with contaminant mixtures 1 and 2 (Figure 3b and 5).

Turquoise module correlated positively with mixture 1 ($P<0.01$) and mixture 2 ($P<0.05$) and was enriched in 52 pathways mainly involved in metabolism (carbohydrate metabolism, protein metabolism/translation and energy metabolism), cellular structure and muscle organization (ESM11a, c and d). All GO terms were upregulated with increasing level of contaminants of mixture 1 (including Al, Cu, Sr, Pb, Ni, Cr, Tl and Ti) and mixture 2 (increasing Rb, Tl, As, Zn and decreasing Pb). GO cellular component analysis revealed no significant enrichment (ESM11b). Green module consisted of fewer proteins (48) and was enriched in protein biosynthesis/translation pathways which were all downregulated with increasing level of mixture 2 (ESM12a and c). Here again, no cellular component was significantly enriched (ESM12b).

Hub proteins identified within the turquoise module were all involved in carbohydrate metabolism except the “ryanodine receptor 1” (RYR1, ESM13) being involved in muscle contraction. 4 out of five potential biomarkers of contamination mixture 2 (green module) were ribosomal protein involved in protein translation apart from a subunit of tubulin implicated in cellular structure (ESM13).

Comparison of liver vs red muscle proteome response

Comparison of the delta-rank values obtained from GO enrichment analysis for both tissues in regard to mixture 1 revealed no significant correlation ($P>0.05$, ESM14). However, enrichment in regard to mixture 2 and 3 displayed low but significant correlations (cor = -0.16, $P<0.001$ and cor = -0.12, $P<0.01$ for mixtures 2 and 3, respectively). This highlights that similar processes appear involved in response to these two contaminant mixtures, albeit with opposite responses for the two tissues.

Discussion

The increase in trace element concentrations in the aquatic environment due to anthropogenic activities highlights the importance for a better monitoring of their potential chronic persistence, bioaccumulation, biomagnification and toxicity at different trophic levels (Marengo et al., 2018). We found evidence for the accumulation of multiple elements in gilthead seabreams from seven different lagoons with specific contamination signatures. Physiological responses that involved cellular organization pathways and protein transport were detected in both the liver and red muscle (albeit they were not similarly regulated). We also highlighted processes such as metabolism and translation that were specifically affected in red muscle. From our results, we propose hubproteins as biomarkers of health effects of metal stress that could serve as a starting point for larger-scale biomonitoring programs.

Contamination profile

Fish size is often recognized important in determining the rate of physiological processes that influence uptake, distribution and elimination of inorganic contaminants (Canli & Atli, 2003). Our analysis revealed that 6 out of 14 contaminating elements showed significant correlation with size, highlighting that mean concentration of trace elements in muscle were not systematically linked to fish length. The absence of relationship for essential trace elements likely indicates that fish regulate some element at a concentration required for efficient metabolic activities (Canli & Atli, 2003). When correlation occurred, most relationships with size were negative except for aluminium, which is in agreement with results found by Endo et al. (2008). Previous studies have already shown inter-

specific differences in trace element dynamic (e.g. Gobert et al., 2017; Mille et al., 2018). This may result from a higher metabolic activity in fast-growing young individuals, or through a higher excretion rate and/or dilution of metal burden with growth (Eisler, 2010). Another explanation could be a change in diet with growth, where smaller individuals specialize in certain prey species or foraging areas that are more contaminated. More interestingly, no relationship between mercury concentration and total length were found despite being known to bioaccumulate (Storelli et al., 2007). Similar results were found for one year old juveniles of the Mediterranean sardines which suggests that when focusing at one age-class, the variability in size within necessarily remain low and so further investigating on several age-classes are needed to reveal complex bio-accumulation patterns.

Despite high inter-individual variability within sites suggesting variable accumulation among individuals, our results clearly showed differences in the exposure to trace elements among the seven coastal lagoons. Similar results have been reported by Tournois et al. (2017), who highlighted clear differences among lagoons in the relative level of multiple trace elements in otolith of gilthead seabream juveniles. As fish take up trace elements through the gills, digestive tract, and body surface (Kamunde et al., 2002; Tao et al., 2001) comparison with metal contamination of other matrixes in the close environment of seabreams such as sediment, water and mussel (preys of gilthead seabreams) could help to explain such accumulation differences between lagoons and individuals (Andral & Tomasino, 2010; Grouhel et al., 2018; Munaron et al., 2013). Interestingly, contamination in fish muscle seemed related to prey contamination for Gruissan, Prevost and to a lesser extent Bages-Sigean lagoons (ESM15). The findings align with the well-known phenomenon of biomagnification in aquatic food webs, where trace elements tend to accumulate at higher trophic levels (Fey et al., 2019). However, the contamination in multiple trace elements for Salses-Leucate, Bages-Sigean and Or lagoons could not be explained by contamination in other environmental matrices mostly because some trace elements measured in this study (aluminium, rubidium, thallium, lithium, titanium and strontium) were not monitored in sediment, water nor biota.

Moreover, either weak or no relations were found between muscle trace element concentration and water or sediment contamination (ESM15), even for Ni and Zn that are described as elements with the highest partial correlation with muscle contamination load (Pyle et al., 2005). This observation supports the understanding that in low-contaminated waters, the primary mechanism for metal uptake in fish occurs predominantly through feeding. (Farkas et al., 2003). These patterns underlined that accumulation of trace elements in fish does not directly reflect their simple concentration in the surrounding environment because as it is also affected by environmental factors (pH, organic matter content temperature etc.), species biology and the physico-chemical properties of the contaminants (speciation, solubility, complexation etc., P Cresson et al., 2015). Thus, assessing a site-specific contamination patterns requires a good knowledge of all the environmental and biological factors as well as their potential interactions, which can lead to high inter-individual variance within each site (Schull et al., 2023).

In general terms, trace metal concentrations reported in our study were within the ranges reported in Mediterranean and Black Sea seabream populations from Algeria, Croatia, France, Greece, Italy, Spain and Turkey, except for aluminium and lithium (ESM16). In our study, average Al level was 1.3 to 8 times higher than those reported in the literature (Bouchoucha et al., 2019; Guérin et al., 2011; Marengo et al., 2018). Similarly, Li concentrations were 3 times higher than those described in Guérin et al. (2011) and Marengo et al. (2018) although the former one measured Li levels in only 4 individuals. However, the most striking observation was the lack of data for multiple elements including Rb, Sr, Ti and Tl (but see Bouchoucha et al., 2019; Guérin et al., 2011). This is partially explained by the absence of regulatory limits for those elements in fish muscle (along with Cr and Ni), despite their increasing use in multiple area (industrial, agriculture, high-tech and medication industries) and their toxicity for organisms (Aslam & Yousafzai, 2017; Authman, 2011; Blewett & Leonard, 2017; Exley et al., 1991; Genchi et al., 2021; Kumari et al., 2017). Nonetheless, metal concentrations are expressed, in literature, either per unit of wet (ww) or dry (dw) tissue weight as it was the case in this study. A lack of standardized levels of elemental concentrations

limits the comparability of studies (Jovičić et al., 2015). Cresson et al. (2017) demonstrated that the theoretical wet:dry ratio of 5 traditionally used was not valid for all species, and that conversion values were ranging from 3.6 to 5.5 depending on the species. In our case, we used 4.31 as factor as Minganti et al. (2010) used for *Sparus aurata.*

The European regulation has set contamination thresholds for only three specific inorganic trace elements, namely Pb (lead), Cd (cadmium), and Hg (mercury), while some countries (for instance Turkey and Greece) have encompassed a larger set of contaminants (for a review see Guerra-García et al. 2023). Notably, trace element loads measured in seabreams for this study remained below the threshold values in fish for human consumption provided by most international regulations, with the exception of arsenic (As) which exceeded these limits. These results urge further efforts by European commission and other institutions to establish health limits in fish, especially for As.

Contaminant mixture and their physiological effects

In the light of our results, it appears that juvenile seabream, regardless of the environment in which they are living, are exposed to different mixtures of contaminants, which is in line with previous results concerning the same areas in older fishes (Schull et al., 2023). According to the PCA on metal contamination of the 31 individuals selected for proteomic analysis, three different contamination profiles were identified (Table 1). One of the major interests of using PCs as a continuous proxy for general inorganic contamination level is to work along contamination gradient instead of categorial contamination. However, a limit of this approach is when some elements level increases, others decreases as it the case for PC2 and PC3, preventing specific contaminant effects from being identified.

Liver proteome response

Considering the liver proteome, each mixture elicited a specific physiological response, highlighted by the fact that each PC correlated with only one protein module. Mixture 1 (Al, Cu, Sr, Pb, Ni, Cr, Tl and Ti) impacted vesicle protein transport known to be key for protein localization and

function. Indeed, protein function depend on their subcellular localization, with each cell compartment offering different chemical environments (e.g. pH and redox conditions) and different potential interaction partners or substrates (Lundberg & Borner, 2019). Tight regulation of protein subcellular localization is hence an important layer of control over cell physiology (Bauer et al., 2015) and its up-regulation, appears to be a potential response to metal stress. More precisely, mixture 1 seemed to particularly affect proteins located in nuclear pores. The nuclear pore complex is the major gateway to the nucleus and regulates nucleocytoplasmic transport, which is central to processes including transcriptional regulation and cell cycle control (Paci et al., 2021).This result may represent mechanisms developed by cells to defend against trace element toxicity , either through a direct response to the contaminant or through an indirect process.

Furthermore, mixture 2 (PC2) including contamination in Rb, Tl, As, Zn and -Pb impacted specifically hepatocyte structure and organization with enrichment in pathways, such as regulation of actin filament polymerisation. Actins are abundant cytoskeleton proteins (involved in microfilaments) with an important function in intracellular transport, cell organization and motility processes (Goodson & Hawse, 2002). Another protein particularly affected by mixture 2 was the gelsolin protein involved in cellular structure (downregulated, ESM10). This protein was previously identified in oysters as responsive to inorganic contamination as well (Li et al., 2020) and was linked to inhibition of cellular apoptosis (Koya et al., 2000), which made it a great candidate as a biomarker of metal contamination. Furthermore, dysregulation of cytoskeleton affects many cellular mechanisms, ranging from cell motility to the maintenance of cell shape and polarity. This is in line with the significant downregulation in the pathway involved in establishment and maintenance in cell polarity. Cell polarity plays a particularly important role in hepatocyte function such as the sustainment of two concurrent flow systems (bile and sinusoidal blood components) and in liver regeneration (Treyer & Müsch, 2013). Contaminant effects on cellular integrity were also observed in literature at the tissue level with liver histopathological damage, including hyperemia and leukocyte infiltration (Macêdo et al., 2020). Consequently, the variations of cytoskeletal proteins

494 indicated that this contamination mixture caused cellular damage in the hepatocytes as previously
495 suggested in the literature (Hadi & Alwan, 2012; Mela et al., 2007).

496 On the other hand, mixture 3 including contamination in Hg, Zn, Ni, Pb induced upregulation
497 of pathways involved in chromosome organization, protein catabolism and protein folding.
498 Chromosomal abnormalities and DNA damage are known effects of heavy metal pollutants (Järup,
499 2003; Parveen & Shadab, 2012; Whitfield & Elliott, 2002), which can lead to abnormalities in cell
500 behaviour during mitotic division and reproductive system in fish (Çavaş & Ergene-Gözükara, 2003;
501 Ebrahimi & Taherianfard, 2011). In our case, mixture 3 seemed to specifically impact telomeres
502 structure, which are repeated sequences of non-coding DNA at the ends of chromosomes and play
503 an important role in stabilizing and protecting coding sequences in eukaryote genomes (Blackburn,
504 1991). These results are in accordance with a previous article which highlighted that some inorganic
505 contaminant (e.g. As, Pb, Cr) seem to cause telomere shortening and others telomere elongation
506 (Kahl & da Silva, 2021; H. Li et al., 2012). Short telomere length is strongly associated with increased
507 mortality and age-related disease risk (Zglinicki & Martin-Ruiz, 2005) and shorter life time expectancy
508 compared to individuals with longer telomere length (Pauliny et al., 2006; Salomons et al., 2009; Vera
509 et al., 2012). Future research on fish response to trace element stress should then consider the
510 assessment of telomere length and telomerase activity. Such effect is likely to be caused by oxidative
511 stress (Epel et al., 2004), either by reactive oxygen species (ROS) generation (by redox-active metals)
512 or by reducing antioxidants (redox-inactive metals) (Koivula & Eeva, 2010). This would explain the
513 over-expression of thioredoxin-like protein, which is believed to serve as a cellular antioxidant by
514 reducing protein disulfide bonds produced by various oxidants such as metals (Hu et al., 2005).
515 Simultaneously trace metal stress activated overexpression of DNA damage repair proteins and
516 chaperone proteins to encounter cellular damages (Table S2). Indeed, another potential biomarker in
517 response to mixture 2 is a component of chaperonin-containing T-complex (TriC) that assists the
518 folding of proteins and participate in telomere maintenance. This protein was also dysregulated in
519 the golden clam (*Corbicula fluminea*) in response to water contamination (Bebianno et al., 2016).

Moreover, upregulation of protein catabolism pathway suggests that proteolysis might be activated to remove metal-damaged proteins. Altogether, these regulations could be seen as an early response due to the presence of some trace elements and it underlines the implication of the liver in detoxification processes.

Finally, we highlighted that metal contamination level represents a stressor for gilthead seabream. Our results indicate that wild *S.aurata* may be vulnerable to telomere attrition, alterations in redox status and cytoskeleton disorganization in liver as a result of pollution and during juvenile phase. As seabreams mainly store lipids in the liver (McClelland et al., 1995), alteration of this key organ could reduce their chance of survival for their first winter in open water.

Muscle proteome response

Only mixtures 1 and 2 displayed significant impacts on red muscle proteome. Both mixtures caused an up-regulation of multiple pathways involved in cellular organization and protein localization. This highlights similarities in the process involved, displaying for some of them opposite responses for both tissues. Here again, our results showed that metal stress would reorganize the biological structure of cells which seemed to affect muscle development and contraction. In fact, among the hub proteins affected, ryanodine receptor 1 is an intracellular Ca^{2+} release channel that mediates excitation-contraction coupling in skeletal muscle (Zalk et al., 2015), which also explain the enrichment in the “response to calcium ion” pathway. The disturbance of these pathways suggest that these trace elements are likely to affect muscle function in *S.aurata*, which could result in limitation of seabream juvenile movement.

It has been reported that exposure to some environmental pollutants can cause impairment in energy homeostasis and energy allocation (Epelboin et al., 2015; Gardon et al., 2018; Li et al., 2021). Our enrichment analysis results highlighted that mixtures 1 and 2 triggered a general up-regulation of energy metabolism pathways. Carbohydrate metabolism seemed to be particularly affected, as 4 of the 5 hub proteins selected were involved in this process. Particularly, fructose 1,6-bisphosphatase, a key enzyme in carbohydrate metabolism also reported as impacted by metal

546 contamination in previous work (Y. Li et al., 2020; Sabir et al., 2019). Interestingly, we identified one
547 protein, which was activated by metal stress and was related to all previous affected pathways. Beta-
548 enolase protein is found in skeletal muscle cells where it has known role in glycolysis and
549 gluconeogenesis (Butterfield & Lange, 2009), but also may play a role in muscle development and
550 regeneration (Merkulova et al., 2000). We suggest that beta enolase may be an enzyme of choice for
551 assessing myocyte response to metal stress. The over-expression of proteins involved in
552 gluconeogenesis might reflect an inefficient foraging and/or help to increase the balance of glucose
553 and maintain the energy demand for the organisms under trace metal stress (such as cytoskeleton
554 remodulation). However, more energy allocated in some specific aspects would lead to the shortage
555 of others (energy trade-off, Stearns, 1989), such as growth or reproduction (Wood et al., 2011). An
556 energy trade-off could also explain the general down-regulation of translation in response to mixture
557 2. In eukaryotic cells, ribosome production indeed represents one of the most-energy consuming
558 process, which adapts to changes in intracellular energy status (Murayama et al., 2008). Monitoring
559 daily growth rates and reproductive investment of individuals with differential metal burden would
560 help to identify the potential trade-offs that occur for this species in a challenging contaminated
561 area. Reading daily increments of otolith may also allow rebuilding growth rate a posteriori and
562 should deserve further investigation in the future of seabream monitoring (Kruitwagen et al., 2006).

563 # Conclusion

564 The present study highlighted both the extent of trace element accumulation in juveniles of
565 gilthead seabreams in the Gulf of Lions and its effect on fish physiology. Results revealed differential
566 contamination pattern between lagoons, which might lead to contrasted quality as nurseries for
567 juveniles of numerous highly prized fish species in addition to seabreams (Brusle & Cambrony, 1992;
568 Quignard et al., 1984; Tournois et al., 2017). Moreover, we highlighted that proteomic signatures
569 provide a powerful discriminator of metal contamination in biomonitoring and holds potential the
570 classification of contaminated areas or fish populations. Checking the validity of the potential
571 biomarkers highlighted here by their targeted analysis in larger cohorts and/or other marine species

would improve our ability to better monitor trace element pollution. By analysing the effects of multiple elements simultaneously, our study improves resolution over modular studies of individual contaminants, which may lack biological significance, since contaminants do not exist in isolation (Escher et al., 2020). Because the proteome of the most contaminated fishes was altered while their general condition factor was not, we can suppose that these alterations may be an early indicator of adverse effects that can potentially translate into population relevant outcomes. The results of this study—and particularly the complex changes in protein expression—demonstrate the need for future studies to test for simultaneous effects of multiple concomitant stressors in both controlled condition and in the wild.

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Table captions

Table 1. Main variables that contribute to the first three principal components of the PCA on the 31 individuals selected for proteomic analysis. Each PC represent a contaminant profil/mixture.

Figure captions

Figure 1. Biplot of PC1 vs PC2 (a) and PC1 vs PC3 (b) of the PCA built with level of inorganic contaminants present in at least 50% of the 135 sampled juveniles. Each point represents an individual. The larger circles represent the barycentre of the individuals for a given lagoon.

Figure 2. Biplot of PC1 vs PC2 (a) and PC1 vs PC3 (b) of the PCA built with level of inorganic contaminants present the 31 individuals selected for proteomic analysis. Each point represents an individual. Colours indicate contamination level of individuals (HI: High contamination and LI: Low contamination). The larger circles represent the barycentre of the individuals for a given group.

Figure 3. Correlations between module eigengenes (columns) and trace element mixtures (rows) for liver (a) and red muscle (b) proteome. Values with module names indicates the number of proteins belonging to each module. Values in the cells are Spearman's correlation coefficients. Blank cells indicate non-significant correlation (P>0.05). *: P<0.05, **: P<0.01

Figure 4. Significant GO terms (FDR <0.001) and ontological relationships in liver. Circle size indicates the level of FDR-adjusted statistical significance. Terms enriched in the same module were grouped and presented in the same color. Each leading term, which has the highest significance, is indicated by colored font. Biological processes were grouped in larger pathways (large grey circles).

Figure 5. Significant GO terms (FDR <0.001) and ontological relationships in red muscle. Circle size indicates the level of FDR-adjusted statistical significance. Terms enriched in the same module were grouped and presented in the same color. Each leading term, which has the highest significance, is indicated by colored font. Biological processes were grouped in larger pathways (large grey circles).

Tables

Table 1.

Mixture 1- PC1		Mixture 2 - PC2		Mixture 3 - PC3	
-	+	-	+	-	+
None	Al, Cu, Sr, Pb, Ni, Cr, Tl, Ti	Pb	Rb, Tl, As, Zn	Sr	Hg, Zn, Ni, Pb

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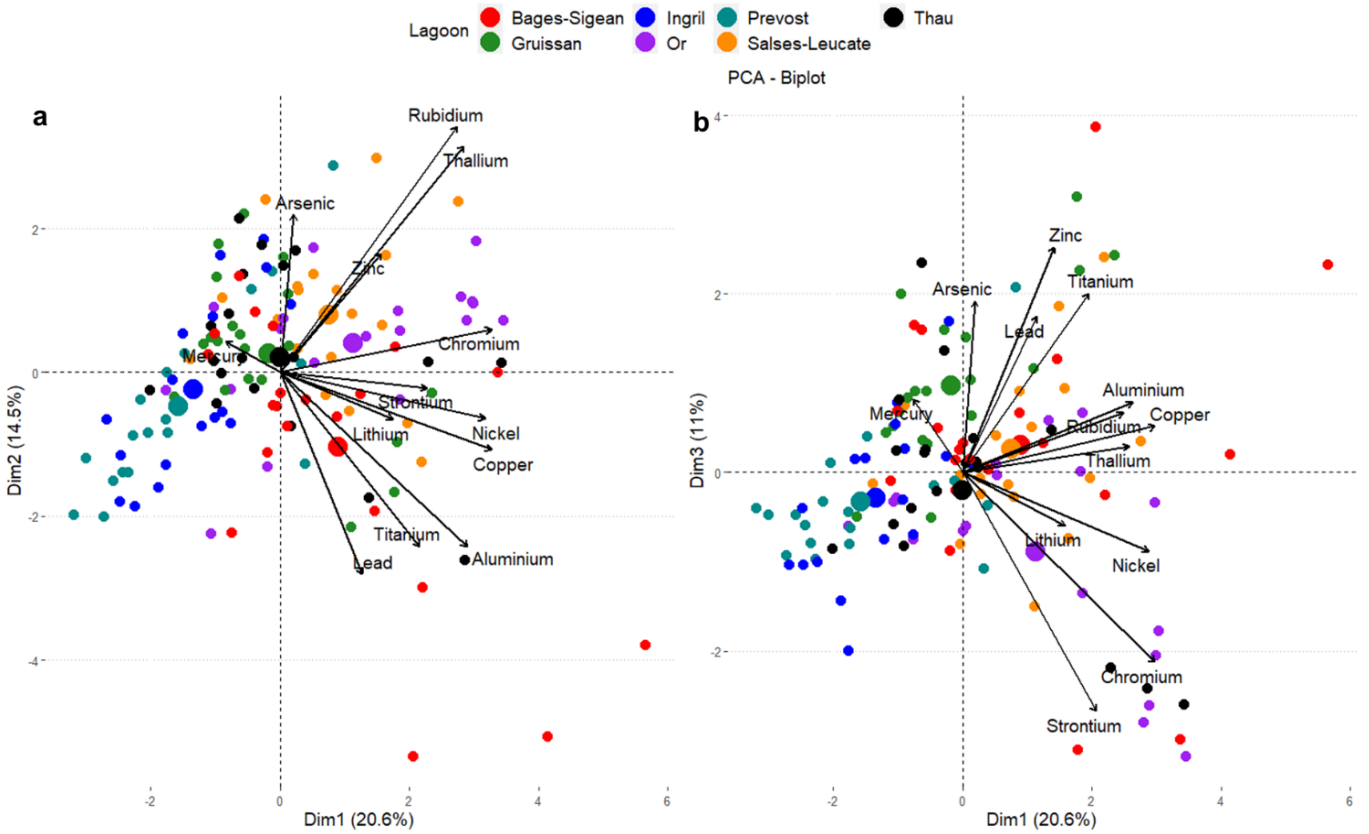
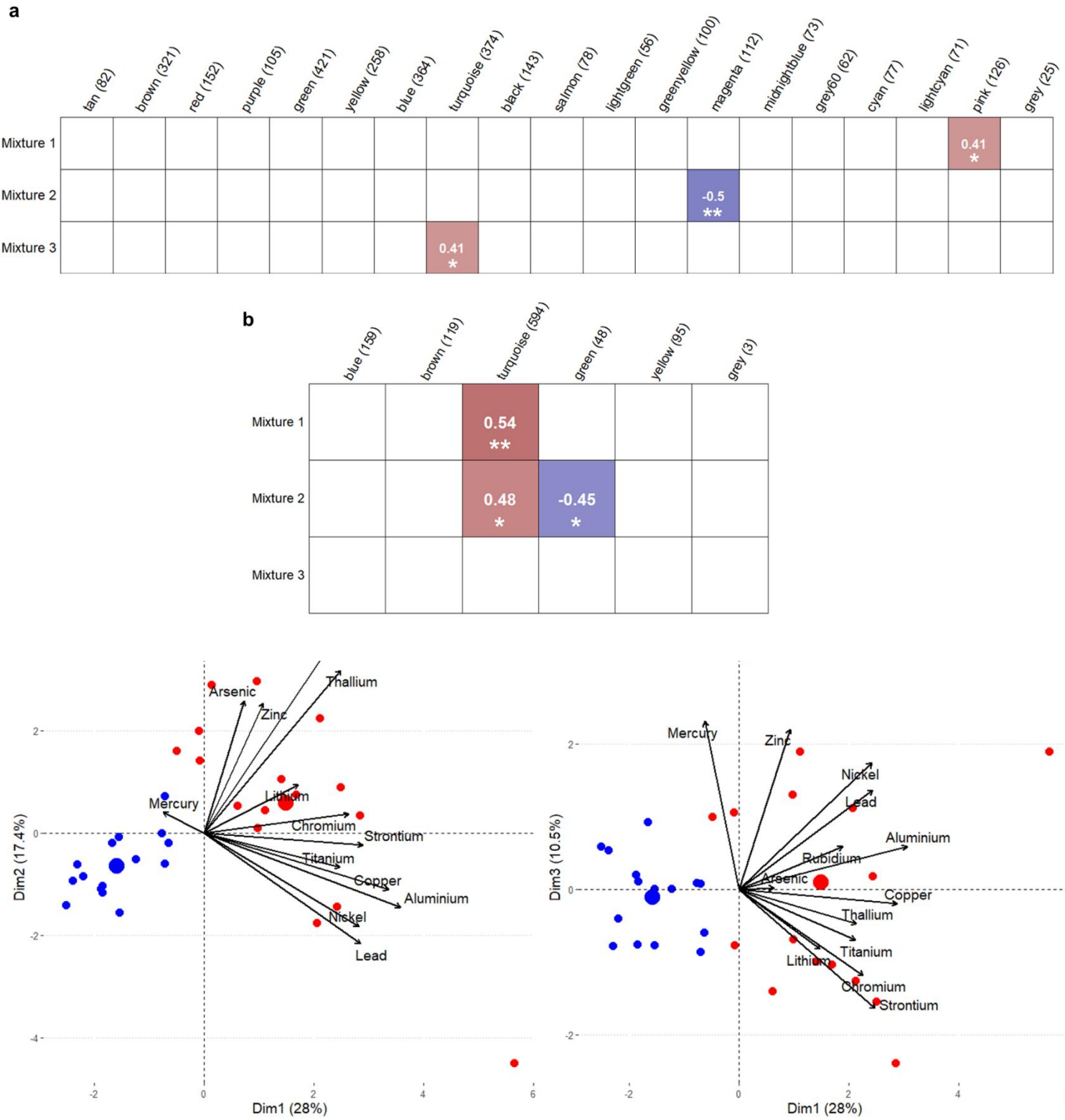
928 ## Figures



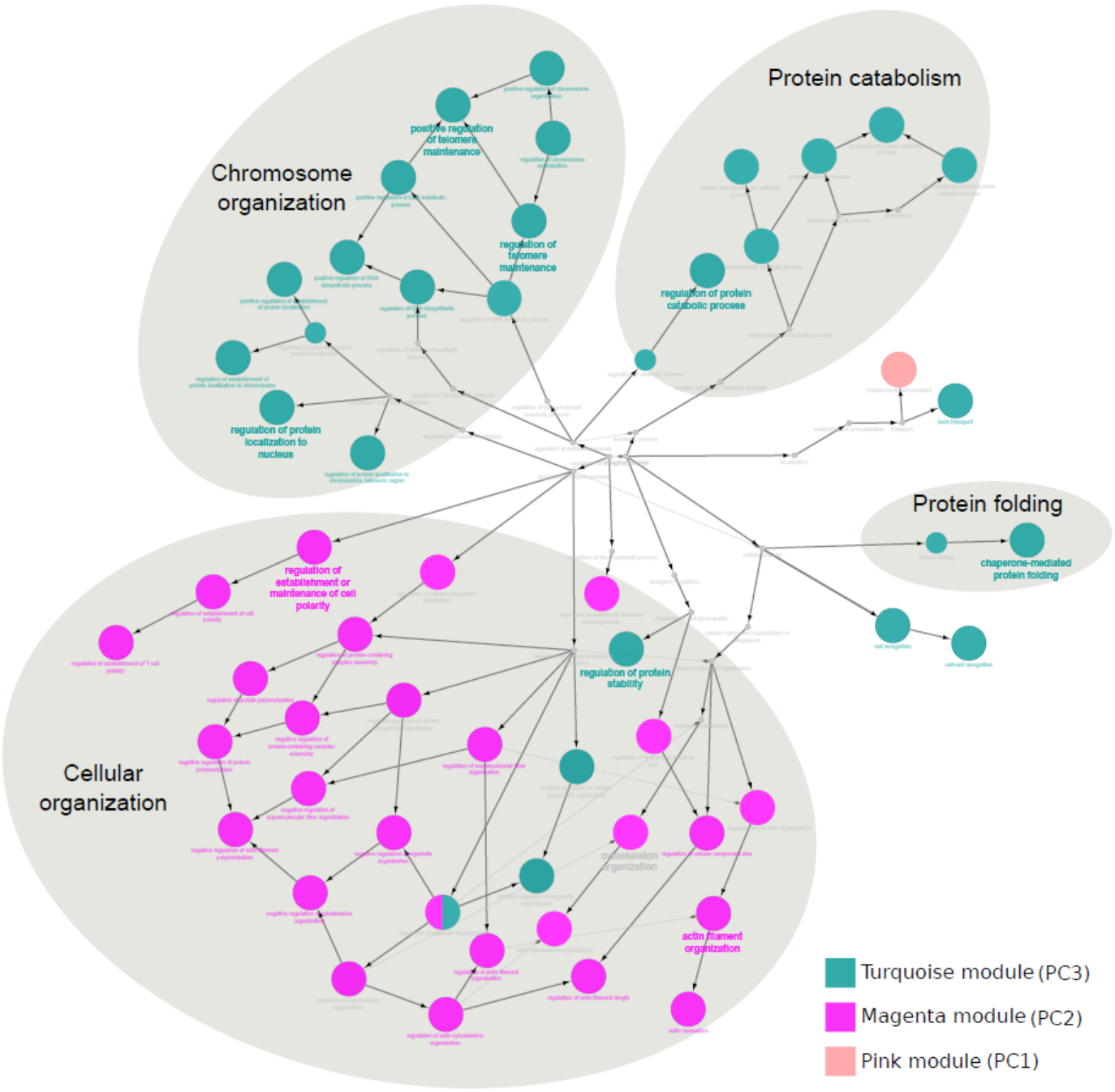
929 **Figure 1.**

930 **Figure 2.**

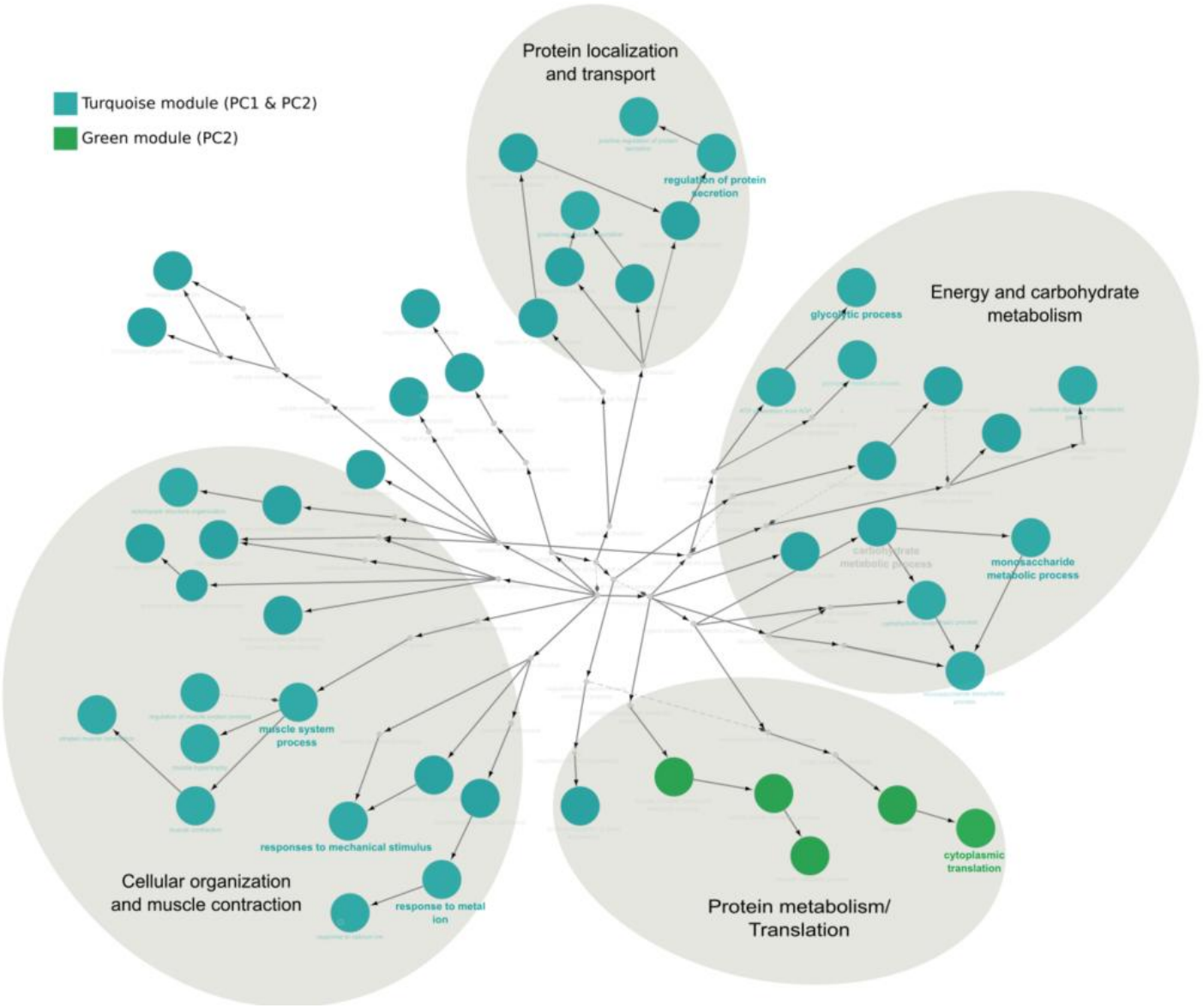


931 **Figure 3.**

932


Chromosome organization
Protein catabolism
Protein folding
Cellular organization
positive regulation of telomere maintenance
regulation of telomere maintenance
regulation of protein catabolic process
regulation of protein localization to nucleus
chaperone-mediated protein folding
regulation of establishment or maintenance of cell polarity
regulation of protein stability
actin filament organization
Turquoise module (PC3)
Magenta module (PC2)
Pink module (PC1)

946 **Figure 4.**


Turquoise module (PC1 & PC2)
Green module (PC2)
Protein localization and transport
regulation of protein secretion
Energy and carbohydrate metabolism
glycolytic process
monosaccharide metabolic process
muscle system process
responses to mechanical stimulus
response to metal ion
Cellular organization and muscle contraction
cytoplasmic translation
Protein metabolism/ Translation

947 **Figure 5.**