UNIVERSITY OF CRETE

SCHOOL OF MEDICINE

Μελέτη επιμόλυνσης ιχθύων προερχόμενων από ιχθυοκαλλιέργειες και ανοιχτή θάλασσα σε βαρέα μέταλλα και οργανοχλωριωμένους περιβαλλοντικούς μολυντές. Εκτίμηση επιβάρυνσης της διατροφικής αλυσίδας και επιπτώσεις στην ανθρώπινη υγεία.

Study of the contamination of aquaculture and wild fish with Heavy Metals and Organochlorine environmental contaminants. Risk assessment and health implications for humans.

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ABBREVIATIONS

ADI Acceptable daily intake

Cd Cadmium

CF Condition Factor

DL Dioxin like

EC European Commission

EDI Estimated daily intake

EFSA European Food Safety Authority

EU European Union

EWI Estimated Weekly Intake

FAO Food and Agriculture Organization of the United Nations

GC-MS Gas chromatography–mass spectrometry

Hg Mercury

HI Hazard Index

IARC International Agency for Research on Cancer

ICP-MS Inductively Coupled Plasma Mass Spectrometry

LOD Limit of Detection

LOQ Limit of Quantification

MeHg Methyl-mercury

NDL non-dioxin-like

OS overall survival

Pb Lead

PCB Polychlorinated Biphenyls

PTWI Provisional tolerable weekly intake

RfD Reference oral dose

THQ Target hazard Quotient

TWI Tolerable weekly intake

USEPA United States Environmental Protection Agency

WHO World Health Organization

ΣPCB-6 Sum of 6 indicator NDL-PCBs

ΣPCB-7 Sum of 7 indicator PCB

ABSTRACT

The objective of the thesis was to elucidate aspects of contaminant accumulation and toxicity in the fish as well as to monitor the contaminant load namely: Cd, Pb, Hg and PCBs in commonly consumed fish species from two modes of production, aquaculture and fisheries in Greece. Ultimately, the risk for human health through fish consumption was assessed for the Greek population.

For the investigation of metal accumulation and toxicity issues in the fish organism an *in vivo* study, was planned with aim to investigate zebrafish responses to a range of Cd exposure levels, spanning from environmental to toxic. The hypothesis that there is a linear survival response to exposure levels was examined while further histopathological analysis was conducted, in an attempt to correlate Cd effects with mortality. The results of the study showed that zebrafish accumulated Cd in their tissues in a dose dependant, but not time dependant manner. However, zebrafish manifested deviations from the anticipated linear toxic responses. Documented responses regarding mortality rate were non-linear, supporting the increasingly gaining ground hypothesis of non-monotonic and not linear responses to gradient exposures to toxic stimuli. Histopathological findings also deviated from the anticipated dose dependant responses, revealing more severe effects in lower than the most toxic exposures, while adverse effects occurred even at environmental levels. Moreover, there was a low Cd exposure level with surprisingly high mortality rate for zebrafish, which drew the attention and this level could hold a key to the elucidation of the toxicity coping mechanisms, since modifications of these mechanisms beyond this level are implied by our results.

Marine fish, and specifically gilthead seabream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*), are widely farmed and consumed in Greece. Health benefits of fish consumption could be counterbalanced by the intake of contaminants after long term consumption of fish, burdened even in trace levels. In order to assess the contaminant load in the frequently consumed fish species, samples of gilthead seabream and sea bass were collected from aquaculture sites located in the Aegean Sea and the Sea of Crete as well as the fish market of Heraklion, Crete.

The heavy metal load (Cd, Pb and Hg) in the edible part the fish was evaluated and the main factors affecting the heavy metal accumulation in the fish muscle tissue were

investigated. Moreover, risk assessment for the Greek population from fish consumption was conducted based on the determined heavy metal concentrations. Heavy metal levels in fish were determined at levels far below the safe limits for consumption set by authorities, for each metal individually as well as for their sum. Gilthead seabream and seabass demonstrated significant differences in Hg and Cd levels which can be attributed to a number of reasons such as differences in intrinsic factors between species, different metal behaviour, different aquafeed metal load or a combination of all the above. Hg and Pb seem to be more accumulated in closed seas, which could imply that these metals have a similar distribution pattern in the medium of exposure (water), or that they share the same origin of dispersion, possibly waste disposals from human activity. Metal levels, were clearly affected by seasonality and season dependent variations disclosed a species effect as well, since significant differences in metal accumulation amongst seasons were recorded between species. Differences in metal levels between farmed and wild fish were demonstrated which can be the combined result of different feeding behaviour, growth rate and therefore metabolic rate, aside from the effects of waterborne exposure. The risk evaluation we conducted for Greek consumers based on the metal levels we determined in the most frequently consumed fish, both farmed and wild indicates minimal risk for all metals.

Furthermore, the occurrence and burden of the indicator PCBs (ΣPCB-7) in the same fish was determined. Additionally, the association of the ΣPCB-7 accumulation in fish to seasonality, locality, production mode and species was investigated. For the characterization of the hazard of PCB intake through fish consumption for the Greek population, a more elaborate risk assessment method, than those previously used, was developed. Occurrence and levels of the ΣPCB-7 in the muscle tissue of farmed and wild gilthead seabream and seabass reveal that for both species and both modes of production, levels were far below the maximum permissible limits set by the EU (2011). More highly chlorinated congeners such as PCB 138 and PCB 153 were more abundant and more frequently detected in fish tissues, most probably due to higher resistance to degradation and lipophilicity. Wild fish presented higher levels of the ΣPCB-6, while farmed fish accumulated PCB 118 at higher levels. Both ΣPCB-6 and ΣPCB-7 levels are primarily affected by the fish species which is in close relationship to the fact that our results highlighted seasonality, as an important factor affecting PCB accumulation and distribution in fish muscle tissue, as well. Seasonal alterations in PCBs levels seem to be dictated by each species ecology and biological cycle. Distinctions in PCB congeners between open and closed seas were also demonstrated which could be attributed to different types and sources of contamination. The exposure assessment showed that NDL-PCB intake through fish consumption for the Greek adult population is comparable to other European countries. The use of consumption data from the two different sources resulted in slightly divergent exposure results underlying the importance of dietary habits for exposure.

A new food specific HI approach was developed, for which fish contribution to the maximum permitted aggregated dietary exposure was considered, arriving to a lower value for the HI. Risk characterization revealed no risk for Greek consumers.

In summary, two major groups of environmental contaminants, heavy metals and PCBs, which can be hazardous to both fish and humans, were determined in fish tissues and investigation revealed that accumulation and toxicity are affected by both abiotic and biotic factors. Secondly, risk assessment conducted for the Greek population regarding fish consumption revealed no risk. Finally, a new risk characterization method was proposed based on food specific HI approach.

Περίληψη

Το αντικείμενο της παρούσας διδακτορικής διατριβής ήταν να διασαφηνιστούν παράμετροι της συσσώρευσης και της τοξικότητας ρύπων σε ψάρια καθώς και να καταγραφεί η επιβάρυνση τους σε μολυσματικούς παράγοντες και συγκεκριμένα: κάδμιο (Cd), μόλυβδο (Pb), υδράργυρο (Hg) και πολυχλωριωμένα διφαινύλια (PCBs) σε συχνά καταναλισκόμενα είδη, προερχόμενα από ιχθυοκαλλιέργειες και αλιεύματα στην Ελλάδα. Τελικά, ο κίνδυνος για την ανθρώπινη υγεία μέσω της κατανάλωσης ψαριών αξιολογήθηκε για τον ελληνικό πληθυσμό.

Για τη διερεύνηση της συσσώρευσης και της τοξικότητας των μετάλλων στους ιστούς των ψαριών, σχεδιάστηκε μία *in vivo* μελέτη με σκοπό τη διερεύνηση των αποκρίσεων του zebrafish σε ένα εύρος επιπέδων έκθεσης κυμαινόμενων από περιβαλλοντικά σε τοξικά. Εξετάστηκε η υπόθεση ότι υπάρχει μια γραμμική απόκριση επιβίωσης στα επίπεδα έκθεσης, ενώ παράλληλα πραγματοποιήθηκε ιστοπαθολογική ανάλυση προκειμένου να συσχετιστούν οι επιδράσεις του καδμίου με τη θνησιμότητα. Τα αποτελέσματα της μελέτης έδειξαν ότι τα zebrafish συσσωρεύουν κάδμιο στους ιστούς τους κατά τρόπο εξαρτώμενο από τη δόση αλλά όχι από το χρόνο. Εντούτοις, τα zebrafish εκδήλωσαν αποκλίσεις από τις αναμενόμενες τοξικές αποκρίσεις. Οι καταγραφείσες αποκρίσεις σχετικά με το ρυθμό θνησιμότητας ήταν μη γραμμικές, γεγονός που υποστηρίζει την υπόθεση των μη γραμμικών και μη μονοτονικών αποκρίσεων σε τε τοξικά ερεθίσματα, η οποία κερδίζει ολοένα και περισσότερο έδαφος. Τα ιστοπαθολογικά ευρήματα επίσης απέκλιναν από τις αναμενόμενες τοξικές αποκρίσεις, παρουσιάζοντας πιο σοβαρές επιδράσεις σε υψηλά μεν επίπεδα αλλά χαμηλότερα της πλέον τοξικής έκθεσης, ενώ δυσμενείς επιδράσεις καταγράφηκαν ακόμη και σε περιβαλλοντικά επίπεδα. Επιπλέον υπήρξε ένα επίπεδο χαμηλής έκθεσης με εκπληκτικά υψηλό ποσοστό θνησιμότητας για το zebrafish, το οποίο τράβηξε την προσοχή και αυτό το επίπεδο θα μπορούσε να αποτελέσει κλειδί για τη διαλεύκανση των μηχανισμών αντιμετώπισης της τοξικότητας, καθώς τα αποτελέσματά μας υποδεικνύουν τροποποιήσεις αυτών των μηχανισμών πέραν αυτού του επιπέδου.

Τα θαλασσινά ψάρια και ειδικότερα η τσιπούρα (*Sparus aurata*) και το λαβράκι (*Dicentrarchus labrax*), εκτρέφονται και καταναλώνονται ευρέως στην Ελλάδα. Τα οφέλη για την υγεία από την κατανάλωση ψαριών θα μπορούσαν να αντισταθμιστούν από την πρόσληψη περιβαλλοντικών ρύπων μετά από μακροχρόνια κατανάλωση ψαριών, επιμολυσμένα ακόμη και σε ελάχιστα επίπεδα. Προκειμένου να εκτιμηθεί η επιμόλυνση στα συχνά καταναλισκόμενα είδη, συλλέχθηκαν δείγματα τσιπούρας και λαυρακιού από σταθμούς ιχθυοτροφείων που βρίσκονται στο Αιγαίο και το Κρητικό Πέλαγος, καθώς και από την ιχθυαγορά του Ηρακλείου Κρήτης.

Προσδιορίστηκε η επιβάρυνση σε βαρέα μέταλλα (Cd, Pb και Hg) στο βρώσιμο τμήμα των ψαριών και διερευνήθηκαν οι κύριοι παράγοντες που επηρεάζουν τη συσσώρευση βαρέων μετάλλων στον μυϊκό ιστό των ψαριών. Επιπλέον, πραγματοποιήθηκε η εκτίμηση κινδύνου για τον Ελληνικό πληθυσμό από την κατανάλωση ψαριών με βάση τις προσδιορισμένες συγκεντρώσεις βαρέων μετάλλων. Οι συγκεντρώσεις των βαρέων μετάλλων στα ψάρια μετρήθηκαν σε επίπεδα πολύ χαμηλότερα από τα ασφαλή όρια κατανάλωσης που καθορίζονται από τις αρχές, για κάθε μέταλλο ξεχωριστά καθώς και για το άθροισμά τους. Σημειώθηκαν σημαντικές διαφορές στα επίπεδα Hg και Cd ανάμεσα στην τσιπούρα και το λαυράκι, γεγονός που μπορεί να οφείλεται σε διάφορα αίτια, όπως διαφορές σε ενδογενείς παράγοντες μεταξύ των ειδών, διαφορετική συμπεριφορά μετάλλων, διαφορετικά επίπεδα βαρέων μετάλλων στις ιχθυοτροφές ή σε συνδυασμό όλων των παραπάνω. Ο υδράργυρος (Hg) και μόλυβδος (Pb) φαίνεται να συσσωρεύονται στους ιστούς σε μεγαλύτερα επίπεδα στα ψάρια που ζουν σε κλειστές θάλασσες, γεγονός που θα μπορούσε να σημαίνει ότι τα μέταλλα αυτά έχουν παρόμοιο μοντέλο κατανομής στο μέσο της έκθεσης (νερό) ή ότι έχουν την ίδια προέλευση διασποράς, πιθανώς από απορρίψεις λυμάτων εξαιτίας ανθρώπινων δραστηριοτήτων. Για τα επίπεδα των μετάλλων που προσδιορίστηκαν παρατηρήθηκε σαφής επιρροή από την εποχικότητα και οι εποχιακές μεταβολές κατέδειξαν επίσης μια επίδραση είδους, καθώς καταγράφηκαν σημαντικές διαφορές στη συσσώρευση μετάλλων μεταξύ των ειδών ανάμεσα στις διάφορες εποχές. Διαπιστώθηκαν επίσης διαφορές στα επίπεδα μετάλλων μεταξύ εκτρεφόμενων και άγριων ψαριών που μπορεί να οφείλονται σε ένα συνδυαστικό αποτέλεσμα διαφορετικής διατροφικής συμπεριφοράς, ρυθμού ανάπτυξης και επομένως μεταβολικού ρυθμού καθώς και επιπτώσεων της υδάτινης έκθεσης. Η αξιολόγηση κινδύνου που πραγματοποιήσαμε για τους Έλληνες καταναλωτές με βάση τα επίπεδα μετάλλων που προσδιορίσαμε στα πιο συχνά καταναλισκόμενα ψάρια, τόσο εκτρεφόμενα όσο και άγρια, υποδεικνύει ελάχιστο κίνδυνο για όλα τα μέταλλα.

Επιπλέον, προσδιορίστηκε η συχνότητα εμφάνισης και η επιβάρυνση από τους δείκτες PCBs (ΣPCB-7) στα ίδια ψάρια. Επιπρόσθετα, διερευνήθηκε η συσχέτιση της συσσώρευσης των ΣPCB-7 στα ψάρια με την εποχικότητα, την τοποθεσία, τον τρόπο παραγωγής και το είδος ψαριού. Για τον χαρακτηρισμό του κινδύνου πρόσληψης PCB μέσω της κατανάλωσης ψαριών για τον ελληνικό πληθυσμό, αναπτύχθηκε μια πιο λεπτομερής μέθοδος εκτίμησης κινδύνου από αυτές που χρησιμοποιήθηκαν προηγουμένως. Η εμφάνιση και τα επίπεδα των ΣPCB-7 στον μυϊκό ιστό των εκτρεφόμενων και άγριων ψαριών και των δύο ειδών, δείχνουν ότι τόσο για τα δύο διαφορετικά είδη όσο και για τους δύο τρόπους παραγωγής, τα επίπεδα ήταν πολύ χαμηλότερα από τα μέγιστα επιτρεπτά όρια που όρισε η ΕΕ (2011). Περισσότερο χλωριωμένα PCB, όπως το PCB 138 και το PCB 153, ήταν πιο άφθονα και πιο συχνά ανιχνευόμενα στους ιστούς των ψαριών, πιθανότατα λόγω της

μεγαλύτερης αντίστασης αποικοδόμησης και της λιποφιλικότητας τους. Τα άγρια ψάρια παρουσίασαν υψηλότερα επίπεδα ΣPCB-6, ενώ τα εκτρεφόμενα ψάρια συσσώρευσαν το PCB 118 σε υψηλότερα επίπεδα. Τα επίπεδα ΣPCB-6 και ΣPCB-7 επηρεάζονται κυρίως από το είδος γεγονός που είναι συνυφασμένο με το ότι τα αποτελέσματά μας ανέδειξαν την εποχικότητα ως σημαντικό παράγοντα που επηρεάζει τη συσσώρευση και την κατανομή των PCB στον ιστό των ιχθύων των ψαριών. Οι εποχιακές διαφοροποιήσεις των επιπέδων PCB φαίνεται να υπαγορεύονται από την οικολογία και το βιολογικό κύκλο του κάθε είδους. Παρουσιάστηκαν επίσης διαφορές στην κατανομή των διάφορων PCB μεταξύ ανοιχτών και κλειστών θαλασσών, οι οποίες θα μπορούσαν να αποδοθούν σε διαφορετικούς τύπους και πηγές μόλυνσης. Η εκτίμηση της έκθεσης έδειξε ότι η πρόσληψη NDL-PCB μέσω της κατανάλωσης ψαριών για τον ενήλικο πληθυσμό της Ελλάδας είναι συγκρίσιμη με άλλες ευρωπαϊκές χώρες. Η χρήση των δεδομένων κατανάλωσης από δύο διαφορετικές πηγές οδήγησε σε ελαφρώς αποκλίνοντα αποτελέσματα έκθεσης, υπογραμμίζοντας τη σημασία των διατροφικών συνηθειών στην εκτίμηση της έκθεσης. Αναπτύχθηκε μια νέα προσέγγιση δείκτη κινδύνου (HI) για συγκεκριμένο τρόφιμο, για την οποία ελήφθη υπόψη η συμβολή των ψαριών στην μέγιστη επιτρεπόμενη συνολική διατροφική έκθεση, καταλήγοντας σε χαμηλότερη τιμή για το HI. Ο χαρακτηρισμός κινδύνου για τους Έλληνες καταναλωτές δεν αποκάλυψε κανένα κίνδυνο.

Συνοπτικά, δύο μεγάλες ομάδες περιβαλλοντικών ρύπων, τα βαρέα μέταλλα και τα PCB, που μπορεί να είναι επικίνδυνες τόσο για τα ψάρια όσο και για τους ανθρώπους, προσδιορίστηκαν σε ιστούς ψαριών και η διερεύνηση αποκάλυψε ότι η συσσώρευση και η τοξικότητα επηρεάζονται τόσο από αβιοτικούς όσο και από βιοτικούς παράγοντες. Δεύτερον, η αξιολόγηση κινδύνου που διενεργήθηκε για τον ελληνικό πληθυσμό σχετικά με την κατανάλωση ψαριών δεν αποκάλυψε κανένα κίνδυνο. Τέλος, προτάθηκε μια νέα μέθοδος χαρακτηρισμού κινδύνου με βάση μια προσέγγιση HI για συγκεκριμένο τρόφιμο.

CHAPTER 1

General Introduction

Marine ecosystems are ordinarily stable and exhibit very effective self-restoration mechanisms. However, advances in marine sciences have enabled us to document a rapidly declining trend of marine environment quality, as a result of overexploitation of marine resources and other human activities which directly harm marine habitats, such as production and release of contaminants. These activities, apart from exceeding the ecosystems ability for recovery, can additionally compromise human health through consumption of marine species burdened with contaminants. Polychlorinated Biphenyls (PCBs) and heavy metals are included amongst the numerous environmental contaminants that pollute the marine environments. These groups are identified as two of the most hazardous and toxic to both marine life and humans.

PCBs and heavy metal sources and occurrence in the marine environment

PCBs are synthetic compounds classified as Persistent Organic Pollutants (POPs) which consist of 209 congeners and have been produced and distributed commercially as mixtures. Their chemical formula $C_{12}H_{10-x}Cl_x$ renders them high stability (the higher the chlorination level, the higher is the compounds stability), electrical insulating properties, high heat capacity and low flammability. These properties were exploited for industrial purposes and since their manufacture, PCBs have been widely used as dielectric fluids in transformers and capacitors, plasticizers and fireproofing agents among others, for well over 40 years until their toxicity and persistence were determined in the 1960s. Their ban followed several years later by the United States federal law in 1978 and by the Stockholm Convention on Persistent Organic Pollutants in 2001. However, by the time of their ban, it's been estimated that around 1.3 million tons of PCBs had been produced globally, for the most part produced and used in the Northern Hemisphere, while 10% of that production is estimated to have been released into the environment (Breivik *et al.*, 2002a). PCBs enter the marine environment through coastal discharges from rivers, urban and industrial outfalls as well as atmospheric deposition (Breivik *et al.*, 2002b; Serrano, Blanes and López, 2008; Álvarez-Muñoz *et al.*, 2016). Major reservoirs of the environment that can store and redistribute PCBs are the oceans (Álvarez-Muñoz *et al.*, 2016; Lohmann and Dachs, 2019).

PCBs are divided into two categories according to their mode of action and toxicological properties: dioxin like (DL) PCBs and non-dioxin-like PCBs (NDL). Seven

indicator congeners (PCBs 28, 52, 101, 118, 138, 153, and 180) have been suggested as monitoring congeners, which can be used to characterize the presence of PCB contamination. Six of these seven are NDL- PCBs (PCB 28, PCB 52, PCB 101, PCB 138, PCB 153 and PCB 180), and one is a DL-PCB (PCB 118).These seven PCBs, often called "indicator PCBs" were identified by international bodies such as the European commission (European Commission, 1999) and the International Commission for the Exploration of the Seas (ICES). The indicator PCBs have been since, routinely monitored by researchers. PCBs are also included in the Water Framework Directive (European Commission, 2002) for their monitoring. However, due to their persistence and lipophilicity PCBs are not expected to remain in water compartments, since PCBs tend to be bioaccumulated in the fat tissue of exposed marine biota as well as humans (WHO, 2016; Schrenk and Chopra, 2017).

PCB accumulation in fish as a result of diet or waterborne exposure, varies with lipid composition of fish (Vassilopoulou and Georgakopoulos-Gregoriades, 1993; Baptista, Pato, Pereira, *et al.*, 2013; Baptista, Pato, Tavares, *et al.*, 2013). Tissues with higher lipid content accumulate PCBs to a greater extend (Bodin *et al.*, 2014). Other factors influencing PCB accumulation in fish include growth (length, weight, age) and the reproductive cycle, while seasonal variations in PCB levels are mainly determined by the reproduction process (Vassilopoulou and Georgakopoulos-Gregoriades, 1993). Higher level of chlorination in PCB congeners usually translates in higher resistance in metabolic degradation and leads to more frequent detection of highly chlorinated PCBs such as PCB 130, 153 and 180 in marine species (Squadrone *et al.*, 2014). Moreover, biomagnification of PCBs has been demonstrated in large food webs by various authors (Bodin *et al.*, 2014).

Pollution Hotspots Areas in the Hellenic seas which are characterized by increased contaminant loads are usually enclosed gulfs adjacent to urban centres like the inner Saronic Gulf, Elefsis Bay, Thessaloniki Gulf, Gulf of Patras, Pagassitikos Gulf, Larymna Bay, Antikyra Bay where coastal industries are located (Simboura *et al.*, 2019). The presence of highly chlorinated PCB congeners has been identified in sediments of Hellenic coastal areas, in alarming levels, originating from industrial and shipping activities together with the effluents from sewage outfalls (Galanopoulou, Vgenopoulos and Conispoliatis, 2005; Karapanagioti *et al.*, 2011; Kapsimalis *et al.*, 2014). Moreover, PCBs monitoring is included in the International Pellet Watch programme that runs under the assertion, that by using plastic pellets stranded on beaches as passive sampling media (since they are hydrophobic organic materials and thus, they are a favourable medium for persistent organic pollutants to absorb to) it is possible to identify seawater pollution. In Greece, through the analysis of plastic pellets, it has been revealed that pollution in Saronic Gulf is comparable to other heavily industrialized places of the world. However, PCBs values detected in marine fish are

far below those considered dangerous for human consumers (Papadopoulos *et al.*, 2004; Costopoulou, Vassiliadou and Leondiadis, 2016a). On the other hand, monitoring of temporal trends for a period of 20 years (1988–2008) revealed that PCBs levels in fish from Hellenic waters showed no significant differences during this period (Hatzianestis, 2016).

Heavy metals and more specifically cadmium (Cd), lead (Pb) and mercury (Hg) are considered major contaminants primarily because they are non-essential metals and can be toxic to organisms even in traces and secondarily due to the fact that they are non-biodegradable and once they are bioaccumulated they can be biomagnified through the food chain.

Marine ecosystems receive metal loads through erosion processes and subsequent transport by streams and rivers, atmospheric and onshore waste origins, but the main source of metal pollution in the aquatic ecosystems is their discharge as industrial waste. Cd in particular, is distributed from natural sources mainly through volcanic aerosols, while the major anthropogenic sources include its release as component of phosphate fertilizers, battery fluids, pigments and stabilizers (McGeer, Niyogi and Scott Smith, 2011). Pb is widely used in industrial applications and primary uses include mining, battery production, leaded gasoline and lead-based paints (Mager, 2011). Although Hg can be released in the environment through volcanic eruptions, emissions to the atmosphere from fossil fuel combustion or waste incineration as a result of human activity, raise background levels by two to four times in the global environment (Kidd and Batchelar, 2011).

Heavy metal fate, speciation and behaviour in the marine environment is controlled by the physicochemical characteristics of the marine environment, while the metals' bioavailability and potential toxicity to organisms is determined by their chemical form and speciation (Alonso Castillo *et al.*, 2013; Álvarez-Muñoz *et al.*, 2016). Metals usually settle in sediments, which act as adsorptive sinks and can potentially act as a secondary source of the pollutants through diffusion (El-Sorogy and Attiah, 2015; Ranjbar Jafarabadi *et al.*, 2017). Cd and Pb occur at low concentrations in aquatic systems and the concentration of free Cd^{2+} and Pb^{2+} ions is generally associated with toxicity (Mager, 2011; McGeer, Niyogi and Scott Smith, 2011). Methylmercury (MeHg) is of particular concern amongst Hg species, on account of being the most bioavailable form and because of its potent neurotoxicity (Kidd and Batchelar, 2011). Fish assimilate metals via the gastrointestinal track and the gill track and accumulation is influenced by both abiotic (metal speciation, habitat, salinity temperature and pH values etc.) and biotic factors (species, sex, physiologic condition etc.). Lead is reportedly accumulated through waterborne exposure causing disruption of Na^{+}, Cl^{-} and Ca^{2+} regulation and while Cd is principally accumulated through diet, Cd^{2+} ions can disturb the Ca^{2+} uptake at

the gills leading to hypocalcaemia as a result of waterborne exposure. MeHg is strongly biomagnified in fish and the highest levels are detected in top predators (Storelli and Marcotrigiano, 2005; Castro-González and Méndez-Armenta, 2008; Cretì, Trinchella and Scudiero, 2010; Conti *et al.*, 2012; Renieri *et al.*, 2014).

Seawater trace metal levels in Hellenic coastal areas are mainly lower than the permissible levels established by the European Directives, yet they exhibit levels tenfold higher than open waters such as the Aegean Sea (Simboura *et al.*, 2019). However, sediments of coastal areas adjacent to industrial regions (oil platforms, fertilizer factories, smelting plants, aluminium production plants, wastewater treatment plants, regions influenced by mining activities) as well as areas of intensive aquaculture and river estuaries, are enriched in trace metals (Botsou *et al.*, 2011; Farmaki *et al.*, 2014; Pappa *et al.*, 2016). Marine fish collected from aquacultures as well as fisheries in Greece, exhibit low levels of heavy metal bioaccumulation, although literature is scarce (Kalogeropoulos *et al.*, 2012; Kalantzi *et al.*, 2016; Simboura *et al.*, 2019).

Fish consumption and potential implications

Although on a global scale, fisheries still represent a major part of fish production, there is a rapidly increasing trend in aquaculture production, mainly due to the overfishing of stocks at a biologically unsustainable level, which led to a unprecedented peak in 2016 when aquaculture production (54,5%) exceeded fisheries (45,5%) (FAO, 2018). In Greece this was translated in 62% of the domestic fish production originating from aquacultures, while the remaining 38% from fisheries. With regard to Greek fisheries, the fishing ground that generates most of the products is N. Aegean through trawling and seine fishing efforts, while aquaculture sites are distributed in various coastal regions with the majority of them being located in the Peloponnese, the Central Greece and the Dodecanese (Simboura *et al.*, 2019). The Hellenic population consumes fishery and aquaculture products (FAP) regularly, on the basis of the Mediterranean diet and also on account of the fact that Greece is a major producer and exporter country of FAP in the European Union (EU) (Eumofa, 2017). In the Mediterranean countries, two of the most commonly consumed marine fish species are gilthead seabream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*). According to the Federation of Greek Maricultures, these two species represent the 97% of marine fish cultivated in Greece and Greek production of cultivated seabass and gilthead seabream represents the 61% of the EU production and almost the 30% of the international production of these species.

Fish are acknowledged as excellent sources of essential nutrients and micronutrients and fish consumption has been linked to important health benefits. The high nutritional value

of fish is attributed to their rich content in high quality protein, vitamins A, D and B, long-chain omega-3 fatty acids and low cholesterol values. This kind of fish meat composition, has been shown to offer protection against cardiovascular diseases, nutrient deficiencies, visual and cognitive human development (Domingo *et al.*, 2007; Castro-González and Méndez-Armenta, 2008; FAO, 2018). However, health benefits of fish consumption could be counterbalanced by the concurrent intake of contaminants as a result of frequent, long term consumption of fish, burdened with contaminants even in trace levels. Numerous studies in the Mediterranean countries have demonstrated that human dietary exposure to pollutants such as PCBs and heavy metals is to a great extend a result of consumption of fish burdened with these pollutants (Domingo *et al.*, 2007; Martí-Cid *et al.*, 2008; WHO - World Health Organization, 2011; Pastorelli *et al.*, 2012; Renieri *et al.*, 2014b; Rodríguez-Hernández *et al.*, 2016, 2017; Squadrone *et al.*, 2016; WHO, 2016; Costopoulou, Vassiliadou and Leondiadis, 2016b; Giandomenico *et al.*, 2016; Pazi *et al.*, 2017; Bonsignore *et al.*, 2018; Copat *et al.*, 2018; Filippini *et al.*, 2018). Health risks arise from exposure to such toxic stimuli and although safe consumption levels have been established, various parameters must be taken into consideration, in order to sufficiently balance the benefits and risks of regular fish consumption. These parameters include specific fish species consumed, frequency of consumption, meal size, as well as the overall diet habits which could regulate critical physiological parameters such as intracellular lipid composition, oxidative stress and detoxification mechanisms (Domingo, 2016).

Dietary heavy metal intake follows the gastrointestinal track and subsequent adverse health effects, are diverse. Cd is distributed particularly to the kidney and liver where it accumulates and exhibits a biological half-life of 17–30 years and primary health effects associated with Cd oral exposure include renal damage and overall nephrotoxicity. It can additionally exert bone toxicity through oxidative stress as well as reproductive and thyroid disruptive effects through endocrine disruption. Effect on the Central Nervous System (CNS) and neurological disorders has also been reported such as hyperactivity and learning disabilities in children. It has also been classified as a human carcinogen, group I, by the International Agency for Research on Cancer (IARC). Molecularly Cd has been shown to interfere with DNA repair mechanisms as well as reactive oxygen species (ROS) generation (Castro-González and Méndez-Armenta, 2008; Renieri *et al.*, 2014b; Copat *et al.*, 2015; Li *et al.*, 2017; Buha *et al.*, 2018).

Lead once ingested, is primarily distributed and accumulated in the bones and teeth but is also accumulated in liver, kidney and brain. Pb half-life ranges from about a month in blood, 1–1.5 months in soft tissue, and to about 25–30 years in bone. Lead's primary target is the developing nervous system although Pb toxicity can result in various health problems,

such as reduced cognitive development in children while in adults may lead to brain disorders, cardiovascular problems and renal failure. Moreover, on the molecular level it directly interrupts enzyme activity and inhibits trace minerals absorption. Pb exhibits genotoxicity as well, by inhibition of DNA synthesis and repair, oxidative stress, interaction with DNA-binding proteins and tumour-suppressor proteins. Inorganic Pb compounds are classified as probably carcinogenic to humans (group 2A) by IARC(Castro-González and Méndez-Armenta, 2008; Renieri *et al.*, 2014a; Copat *et al.*, 2015)

Mercury toxicity may cause permanent damage to the CNS, such as behavioral disorders and deficiencies in the immune system and development. Exposure in prenatal life may affect the developing CNS, since all forms of mercury pass through the placenta and the foetal brain is more susceptible to Hg harmful effects. Mercury has an extremely long half-life ranging from 15 to 30 years in the CNS. The most toxic form of mercury from environmental exposure, which may affect humans, is MeHg due to its ability to cross the blood-brain barrier and its potential to induce delayed neurotoxicity years after cease of exposure or as a result of low-level long-term exposure. Furthermore, the consumption of marine fish is recognized as the main source of MeHg in the general population (Castro-González and Méndez-Armenta, 2008; Spada *et al.*, 2012; Renieri *et al.*, 2014; Castaño *et al.*, 2015; Copat *et al.*, 2015).

PCBs are distributed to all body compartments and particularly to highly perfused areas, such as liver and muscle and bioaccumulate in the adipose tissue lipophilic compounds. Higher chlorinated PCBs have longer half-lives reaching up to 11.5 years and are more accumulated. Metabolism of PCBs in humans follows the oxidation by cytochrome P450 pathway and NDL-PCBs specifically are metabolized by CYP2B or by CYP2C and CYP3A. The CYP enzymes which are involved in the biotransformation and elimination of PCBs are induced subsequently to PCBs exposure. The most prevalent congener detected in humans is PCB 153 due to its slow rate of biotransformation. Several prospective and cross-sectional studies have shown that NDL-PCBs cause toxic effects primarily to the liver and the thyroid while neurotoxicity has also been suggested (WHO, 2016). In addition, several PCB metabolites have been shown to cause endocrine or toxic effects through mechanistic actions on thyroid hormone homeostasis, neuronal development and functioning (WHO, 2016). NDL-PCBs have also been reported to interfere with steroidogenic pathways and sex hormone synthesis *in vitro* (Xu *et al.*, 2006).

Taking all the above into consideration, the issue of balancing health benefits and risks from fish consumption becomes imperative. With a view to ensure public health protection, international authorities have set maximum permissible limits for contaminants

such as heavy metals and PCBs in fish meat and regularly evaluate the presence of contaminants in foodstuff as well as the dietary exposure to the aforementioned contaminants (USEPA, 2001, 2014; The Commission of the European Communities, 2006; Alexander *et al.*, 2009; European Commission, 2011; EFSA, 2012a, 2012c, 2012b; EFSA CONTAM Panel, 2012; Ceccatelli *et al.*, 2014a, 2014b; WHO, 2016; Ranjbar Jafarabadi *et al.*, 2017). Moreover, many researchers have published monitoring data on contaminant occurrence in fish meat as well as risk estimations of human dietary exposure to heavy metals and PCBs (Renieri *et al.*, 2014; Domingo, 2016). However, risk assessment is quite challenging since dietary habits and contaminant levels in fish vary not only amongst different countries but amongst specific sub regions as well. In addition to site-specific variations, it is important to reckon in the frequency of certain species consumed, besides the contaminant and nutrient profile of each species. Focusing on a global attempt to maximize the health benefits and minimize the contingent health risks of fish consumption, international authorities, health institutions and researchers should join forces under the scope of forming and updating databases as wide and reciprocally accessible as possible, which would enable revising the public policy under which fish consumption is monitored.

Aim and objectives of the thesis

The aim of the thesis is to elucidate aspects of contaminant accumulation and toxicity in the fish as well as to monitor the Cd, Pb, Hg and PCBs load in commonly consumed fish species from two modes of production, aquaculture and fisheries in Greece. Ultimately the risk for human health through fish consumption is assessed for the Greek population.

Specific objectives of this study include:

1. The investigation of metal accumulation and toxicity issues in the fish organism through an *in vivo* study. Cd burden in zebrafish exposed to concentrations ranging from environmental to toxic and its association with fish mortality was studied. The hypothesis that there is a linear survival response to exposure levels was examined and the response features to Cd exposure were investigated as well (Chapter 2).
2. The assessment of the metal load and specifically Cd, Pb and Hg in fish tissue and heavy metal accumulation with regard to mode of production, species, location and seasonality. Estimation the potential health risk for consumers for this group of contaminants was conducted (Chapter 3).
3. The determination of the indicator PCBs in fish tissue and PCB accumulation with regard to mode of production, species, location and seasonality. Estimation the

potential health risk for consumers for this group of contaminants was conducted (Chapter 4).

The initiation of this PhD study was approved by the University of Crete, School of Medicine, on 12 April 2013(Ref. No. 86/21-2-2013). In vivo experiments were conducted in the Animal House Facility at the Department of Biology, University of Crete, which is certified by the Veterinary Unit of the Region of Crete for the rearing (EC91-BIObr-09) and use of laboratory animals for scientific purposes (EL91-BIOexp-10).

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CHAPTER 2

Nonlinear responses to waterborne cadmium exposure in zebrafish. An in vivo study

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ABSTRACT

Cadmium (Cd) has proved to be associated with numerous toxic effects in aquatic organisms via waterborne exposure. With a view to investigate Cd toxicity along a broad spectrum of exposures reaching from environmental to toxic, we employed adult zebrafish (Danio rerio) for an in vivo study. A number of 10 fish per tank were placed in 40 L tanks and were exposed for 30 days to 0.0, 5.0, 25, 50, 75, 100 and 1000 μg Cd per liter. There were 2 tanks for each Cd exposure (duplicate experiment). Mortality was recorded daily, dead fish were collected and tissue samples were obtained for histologic observation, whereas remaining tissues were stored for Cd burden determination. Surviving fish were collected at the end of the experiment. Median overall survival (OS) in days was found to be 9.0, 11.0, 8.0 and 7.0 for 25 μg/L, 50 μg/L, 75 μg/L and 100 μg/L respectively, with all of them showing mortality greater than 50%. Remarkably, fish exposed to the highest Cd concentration (1000 μg/L) survived the longest exhibiting a mean OS of 29.2 days. Cd determination in fish tissue was conducted with an in house ICP-MS method and levels ranged from 3.1 to 29.1 ng/mg. Log Cd tissue levels were significantly correlated with the log Cd exposure levels ($r = 0.535$, $p<0.001$). The highest Cd burden was determined for fish exposed to 1000 μg Cd /L (mean = 12.2 ng/mg). Histopathology supported these results. Our findings disclose a deviation in toxic responses through the range of Cd concentrations, leading to nonlinear responses. These differentiated responses, could be linked to hormesis phenomena.

1. Introduction

Cadmium (Cd) toxicity in aquatic organisms mainly via waterborne exposure, is rising to a major topic, being associated with parameters both of environmental pollution and human health risks. Cd is a non-essential heavy metal widely present in aquatic environments as a result of industrial and mining activities (Hsu et al., 2013) which tends to bioaccumulate in fish tissues (Arini et al., 2015). It can be eventually hazardous to humans with fish consumption being one of the important sources of Cd exposure. (Copat et al., 2013, Kalantzi et al., 2013, Renieri et al., 2014).

Even trace amounts of Cd can be toxic for fish, and its toxicity is dependent on the concentration of Cd^{2+}, which is the most bioavailable form (McGeer et al., 2011). Cd toxicity is affected by the timing of exposure and more specifically life stage, in addition to other factors such as internal biodynamics, temperature, salinity and genetics. The primary route of aqueous Cd exposure in fish is branchial and secondary the olfactory epithelium. Mechanisms of Cd toxicity involve disruption of ion regulation, oxidative damage, endocrine disruption, genotoxicity, olfactory and renal impairments, histopathological effects and adverse effects on behaviour, survival, reproductive parameters and growth (Alsop and Wood, 2011; McGeer et al., 2011; Arini et al., 2015; Sfakianakis et al., 2015; Buha et al., 2013; Acosta et al., 2016).

Zebrafish (*Danio rerio*) has served as a vertebrate model for Cd toxicity studies, however, most literature is focused on its early life stages (embryo and larvae) and few research studies have utilized adult fish. Primary acute toxic effect of Cd in zebrafish appears to be ion loss, especially Ca^{2+} and Na^{+}(Aslop and Wood, 2011) both for larvae and adults. It is stated that Cd^{2+} antagonizes Ca^{2+} for gill binding sites leading to various adverse effects and eventual death (McGeer et al., 2011). Alsop and Wood (2011) also suggest that ion loss is due to the endocrine stress response which highlights the role of Cd as an endocrine disrupting (ED) chemical. Studies with different fish species, support that Cd behaves as an antiestrogenic chemical through several signalling pathways (Denslow, and Sepúlveda, 2007), reduces thyroid hormone levels and disrupts growth hormone expression (Sfakianakis et al., 2015). Moreover, oxidative stress caused by Cd in zebrafish is reported, via alterations in activities of catalase (CAT) and superoxide dismutase (SOD) (Wang and Gallagher, 2013), liver oxidative damage to proteins (Vergauwen et al., 2013a) and alteration of antioxidant capacity in zebrafish brain (Richetti et al., 2011). Exposure of adult zebrafish to Cd oxide nanoparticles also showed liver tissue damage and oxidative stress induction (Balmuri et al., 2017). There is also evidence of over-expressions of genes involved in protection against oxidative stress, following Cd exposure (Arini et al., 2015). With respect to olfactory effects, cell death, structural alterations, potential disruption of foraging, predator avoidance, and

altered behavior are noted (Wang and Gallagher, 2013; Matz and Krone, 2007). Cd-induced deformities are also described in not only zebrafish but other species as well (Sfakianakis et al., 2015).

Tissue accumulation also plays an important part in Cd toxicity, with gills, kidney, liver, and brain exhibiting the highest Cd levels after waterborne exposure (Cambier et al., 2010), although levels appear to be modified after decontamination (Arini et al., 2015). Accumulation is profoundly affected by acclimation responses as well, such as temperature (Vergauwen et al., 2013, Vergauwen et al., 2013), or genetic ones (Arini et al., 2015, Gonzalez et al., 2006, Cambier et al., 2010) which subsequently affect short- and long-term physiological effects.

Each study conducted on zebrafish investigates different Cd effects hence, different LC50s are reported, rendering it difficult to compare and assimilate the results. In order to better elucidate the mechanisms of Cd toxicity, fish responses should be investigated in a broader spectrum of exposure levels, as it is becoming more and more evident that there is a modulation of toxic responses depending on the concentration of exposure as well as tissue accumulation.

In this study, we employed adult zebrafish as means of investigating Cd ecotoxicity through an in vivo study. The aim was to investigate the Cd burden in zebrafish exposed to concentrations ranging from environmental to toxic and its association with fish mortality. We hypothesize that there is a linear survival response to exposure levels and attempt to investigate the response features.

2. Materials and methods

2.1. Chemicals – Reagents

Cadmium chloride hydrate (CdCl2H2O) 99.995% trace metals basis was purchased from Sigma Aldrich. Multielement standard solution for ICP was purchased from Target Analysis (CPA Chem). Tune A (Multielement standard solution) and Tune F (Cross calibration solution) were purchased from Target Analysis (CPA Chem). Nitric acid (HNO3) trace SELECT, for trace analysis ≥ 69%, hydrogen peroxide solution (H2O2) for ultratrace analysis ≥ 30% and hydrochloric acid (HCL) ≥ 37%, trace SELECT, for trace analysis, were purchased from Sigma Aldrich. Type 1 (18.2 MΩ cm at 25 °C) ultrapure water was used (produced by a Direct-Q® Water Purification System). Dorm 4 (Fish protein CRMs, National Research Council of Canada; Joint Research Centre of European Commission) was used for

quality control measures. All glassware and polyethylene vials were kept in 10% HNO3 solution overnight and rinsed thrice with ultrapure water prior to use.

2.2. Experimental set-up and Cd exposures

2.2.1. Zebrafish maintenance

Wild type adult zebrafish (Danio rerio: 0.2–0.8 g), about 12 months old, were used for the study (ZF WT2 F5, Wageningen Agricultural University, The Netherlands). At the beginning of the experiment, fish were placed in 40 L tanks at the average temperature (28 ± 0.5 °C) and photoperiod (artificial 14 h light: 10 h dark) conditions, while oxygen saturation was constantly > 99% (through the use of air pump). Fish were fed twice a day (ad libitum) with industrial dry food in flakes (Sera Vipan, Germany), whereas the freshwater medium of the tanks was not renewed during the experiment due to its short final duration.

2.2.2. In vivo experimental set-up

A series of seven tanks with increasing Cd levels (including blank) were used for exploring the toxic response and the estimation of dose-death response curves. The experiment outline consisted of duplicates of aquaria for each Cd exposure level. Ten adult zebrafish were placed in each aquarium containing 36 L freshwater, after applying Cd (CdCl2) exposures. Subsequently, fish were kept for 30 days in the aquaria or until fish death. Water temperature was monitored daily.

2.2.3. Cd exposures

A wide range of Cd exposure concentrations was selected: 0.0(control), 5.0, 25, 50, 75, 100 and 1000 μg Cd per liter. Cd was administered as $CdCl_2H2O$, on account of being the most bioavailable species. Levels were selected to cover the range from environmental to polluted and finally toxic. (Cambier et al., 2010; Alsop and Wood, 2011;Vergauwen et al., 2013, Vergauwen et al., 2013; Wang and Gallagher, 2013; Wang et al., 2015; Arini et al., 2015; Copat et al., 2013).

2.3. Sample collection, preparation, and digestion

Deaths for each Cd exposure level for both aquaria duplicates were recorded daily. Dead fish were collected from the aquaria and before sample storage, tissue samples of muscle, liver, gills and intestinal tube were obtained in formaldehyde solution 10% for histologic observation, using a pre-cleaned and stainless steel lancet. Remaining tissues were subsequently collected in polyethylene vials and stored at −20 °C until use. Fish which survived until the end of the experiment were sacrificed in liquid nitrogen and stored in

polyethylene vials at −20 °C until use. The whole fish body was homogenized with liquid nitrogen, and 200 mg wet weight (w.w.) of each sample was weighed and placed in acid-cleaned borosilicate glass vials. 6 ml HNO3 ≥ 69% 1:1 UP H2O was added to each vial and left overnight for pre-digestion. For total dissolution, the predigest with the addition of 0.5 ml H2O2 and 1 ml HCl were placed in Teflon digestion vessels, sealed and placed in a high-pressure microwave digestion system. A speedwave MWS- 3+, BERGHOF microwave digestion system with built in, non- contact temperature and pressure measurement was used for the digestion of the samples in PFA Teflon DAP-60+ pressure vessels. Digested samples were stored in borosilicate glass vials at 4 °C until further analysis. Each sample was diluted with ultrapure water up to HNO3 2% final concentration prior to Inductively Coupled Plasma – Mass Spectrometer (ICP-MS) analysis. All method blanks and spiked samples were prepared using the same protocol. Spiking was conducted before the addition of acids and immediately after homogenization.

2.4. Analysis

2.4.1. Instrumentation and Cd analysis

A Thermo Fischer Scientific, XSERIES 2 ICP-MS equipped with an autosampler was used for the determination of Cd at the Laboratory of Toxicology, Medical School, University of Crete. Plasma lab software was used for sample analysis. The instrument was tuned (Tune A) and performance checks (Tune F) were run daily. The adjustment was performed by optimizing the nebulizer gas flow to achieve a high indium (In115) sensitivity while maintaining a low oxide and double charged ions sensitivity (156CeO + /140Ce + < 2% and 138Ba++/138Ba < 3%) and by lenses adjustment for achieving equal sensitivity for both the low mass (Li) and high mass (U) ions. Concentrations were calculated using blank subtraction and a linear fit standardization. A six-point calibration curve was used for Cd analysis. Standard solutions were prepared by diluting certified standard stock solutions with ultrapure water and 2% high purity HNO_3. Indium was used as an internal standard and was added to each sample and calibration standard solutions. Every ten samples a medium concentration standard was added. Cd concentrations were expressed in wet weight. Additional measurements of a series of samples were conducted at A.N. Bach Institute of Biochemistry, Research Centre of Biotechnology of the Russian Academy of Sciences, Moscow, Russia for validation of the method. The additional ICP-MS measurements were carried out with a quadrupole ICP-MS instrument Aurora M90 (Bruker Corp., USA), equipped with an autosampler and a MicroMist low flow nebulizer.

2.4.2. Quality assurance

Sample digestion was performed for blank as well as for calibration standards. Quality control measures included the use of a blank and internationally certified reference materials (CRMs, National Research Council of Canada; Joint Research Centre of European Commission) with every ten samples digested. Mean recovery of Cd of DORM-3 (fish protein) was 96.01 ± 10% (n = 10). Limit of detection (LoD) of the procedure was determined as three times the standard deviation of the procedural blanks (n = 20) by three and was 0.09. Each sample was analysed in triplicate.

2.5. Histopathology

The fish samples were selected from each of the study groups as well as from the control group. We performed Hematoxylin/Eosin staining in 3 μm slices of fish tissue fixed in 40% formaldehyde solution and embedded in paraffin wax. We observed the tissues under light microscope (Nikon Eclipse E400) in 40-400X magnification.

2.6. Statistics

Data was handled separately for each Cd exposure concentration as well as for groups of consolidated Cd exposure concentrations, since certain Cd concentrations were appraised to apply to similar toxicity levels. Groups of Cd exposures (μg/L) were set as follows: group 1 consists of Cd levels 0.0–5.0, representing control and environmental (background) level respectively. Group 2 incorporates concentrations: 25.0–50.0 because there are particular toxicity mechanisms involved in these levels, group 3: 75.0–100.0, as more toxic and group 4 solely Cd level:1000.0.

Death events are expressed as counts and proportions, while the survival time (OS) is expressed in the form of mean and 95%CI (in days). Median OS which corresponds to median Lethal Time (LT50) in days was estimated when possible for each Cd exposure concentration as well as for the four concentration groups. Kaplan –Meier death-response curves are presented for initial Cd exposure concentrations and for groups of Cd exposures.

Micrograms per liter (μg/L) and the log-transformed concentrations (log Cd) were used to describe the Cd exposure and the levels of Cd as measured in fish tissues. Box and Whisker plots were used for graphical representation of data. IBM SPSS was used for statistical analysis.

3. Results

3.1. Mortality- Cd accumulation

The lowest median OS (in days) was observed for group 3: 8.0 (7.1–8.9) while for group 2 was found to be 11.0 (5.3–16.7) (Table 1) with both groups exhibiting mortalities greater than 50%. Fish exposed to the highest Cd concentration of 1000 μg/L, survived longer exerting a mean OS equal to 29.2 [95% CI: 27.8–30.7], close to the corresponding value for group 1 (29.6).

Table 1. Mean overall survival time and LT_{50} (days) of zebrafish for individual and grouped exposure concentrations

Cd exposure conc. (μg/L)	Mean Overall Survival			Median Overall Survival		
	OS	95% CI		LT_{50}	95% CI	
		95%LL	95%UL		95%LL	95%UL
0-5	29,6	29,0	30,1	NE	NE	NE
25	15,7	11,2	20,1	9,0	2,8	15,2
50	14,7	10,6	18,9	11,0	,0	24,1
75	8,3	5,7	10,9	8,0	7,2	8,8
100	11,6	8,0	15,2	7,0	5,7	8,3
1000	29,2	27,8	30,7	.	.	.
Overall	17,6	15,6	19,5	14,0	4,2	23,8

Cd exposure conc. (μg/L)		Mean Overall Survival			Median Overall Survival		
		OS	95% CI		LT_{50}	95% CI	
			95%LL	95%UL		95%LL	95%UL
Group 1	0 - 5	29,6	29,0	30,1	*NE	NE	NE
Group 2	25 - 50	15,3	12,2	18,4	11,0	5,3	16,7
Group 3	75 - 100	10,2	7,8	12,7	8,0	7,1	8,9
Group 4	1000	29,2	27,8	30,7	NE	NE	NE
	Overall	17,6	15,6	19,5	14,0	4,2	23,8

**NE: Not Estimated*

Survival curves of zebrafish for each Cd treatment (Fig. 1a) as well as for the four exposure levels (Fig. 1b) are presented in Fig. 1. Survival times for fish exposed to each Cd treatment were significantly different (χ^2 = 47.89, df=5, p<0.001) as were for the four groups of Cd exposure levels (χ^2= 58.25, df=3, p<0.001). Fish exposed to group 3 Cd levels demonstrate the greatest mortality rate, followed by fish exposed to group 2. Group 4 has a similar mortality rate to group 1 suggesting much less toxic effects of Cd to fish exposed to the highest Cd treatment. Mortality for the fourth group occurs only after long term exposure, particularly in the last ten days, while for group 2 and group 3 as soon as the third day of the experiment. When considering the median OS (LT_{50}) for each Cd treatment separately, a

lower value (LT_{50} = 9) is detected for fish exposed to 25 µg/L than fish exposed to 50 µg/L (LT_{50} = 11) hinting a non-anticipated toxic response.

Figure 1a **Figure1b**

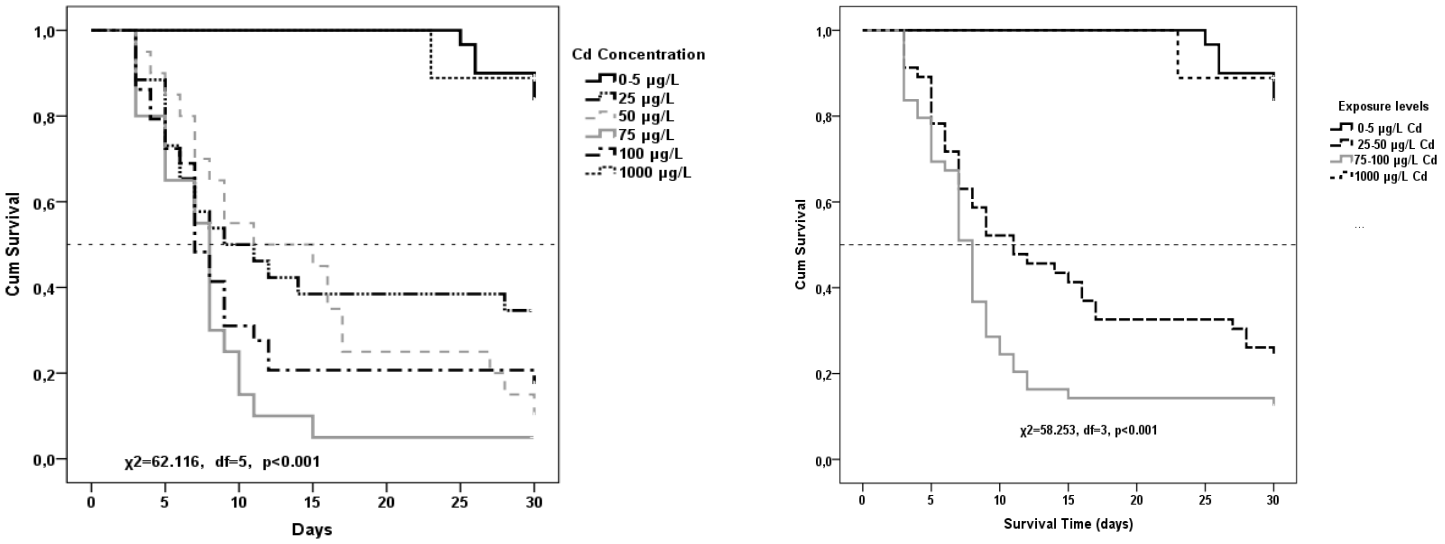

Fig.1 Kaplan – Meier survival curves of *Danio rerio* at each Cd treatment (a) and at different Cd exposure levels (b) throughout the experimental period (30d).

The Cd burden in fish tissue samples exposed to the highest Cd concentration determined with ICP-MS was found to range from 3.1 to 29.1 ng/mg (Table 2). Cd exposure levels were significantly correlated with the log Cd level in fish tissues [r = 0.535, p < 0.001] as presented in Fig. 2. The association of the individual Cd exposures with the Cd burden in tissues of fish is shown in Fig. 2a. Similar correlation is evident when associating the log Cd burden in zebrafish tissue with the groups of Cd exposure levels, as presented in Fig. 2b [r = 0.535, p < 0.001].

Table 2. Cd (ng/mg) wet weight determined in zebrafish tissue with ICP-MS for each Cd treatment and exposure levels.

Cd treatments (µg/L)	Mean	SD	Median	Minimum	Maximum
0	0,1	0,1	0,1	0,0	0,3
5	0,3	0,2	0,2	0,1	0,7
25	0,5	0,3	0,5	0,2	1,4
50	1,0	0,8	0,9	0,2	3,0
75	1,3	0,5	1,2	0,8	3,1
100	2,1	1,4	1,8	1,2	8,8
1.000	12,2	9,0	11,6	3,1	29,1
Total	2,0	3,9	1,1	0,0	29,1
Groups (Exposure levels)	**Mean**	**SD**	**Median**	**Minimum**	**Maximum**
0-5 µg/L	0,2	0,2	0,1	0,0	0,7
25-50 µg/L	0,7	0,6	0,5	0,2	3,0
75-100 µg/L Cd	1,8	1,2	1,5	0,8	8,8
1000 µg/L Cd	12,2	9,0	11,6	3,1	29,1

Figure 2a

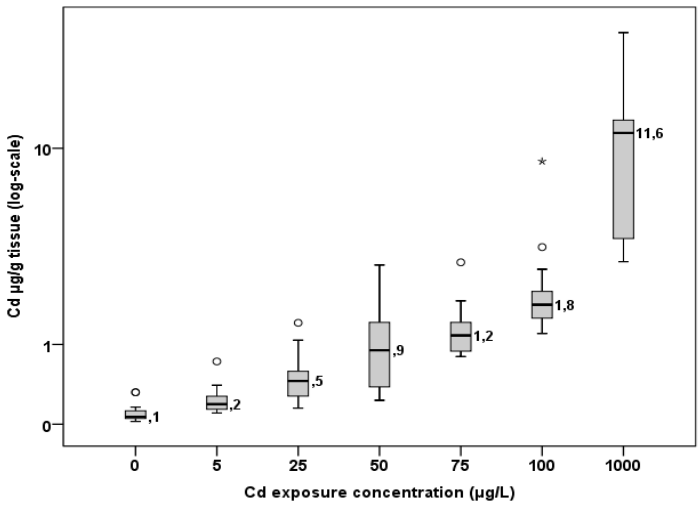

Figure 2b

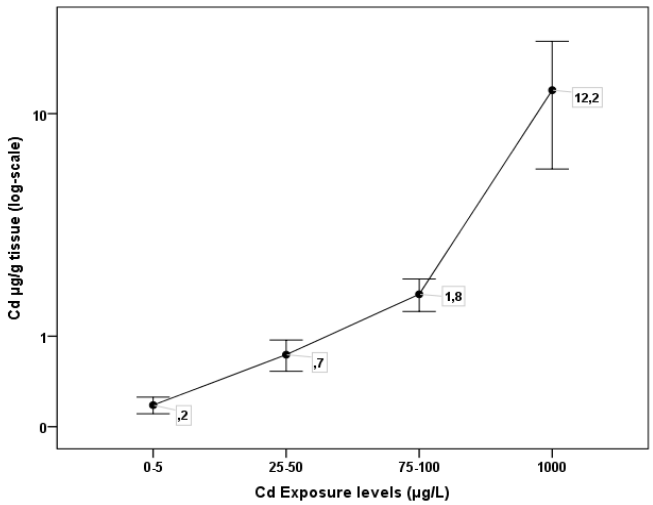

Figure 2. Association of Cd treatments with Cd burden in *Danio rerio* tissue (a), association of group Cd exposure concentrations with Cd burden in zebrafish tissue (b)

Time of exposure does not seem to be a diversification factor for Cd accumulation in zebrafish tissue as it is demonstrated by the association of Cd body burden in zebrafish exposed to Cd levels: 0–5.0(group1), 25.0–50.0(group 2), 75–100.0(group 3) and 1000 µg/L(group 4) to days of exposure (Fig. 3). Although Cd accumulation is clearly dose dependent, it is not significantly affected by duration of exposure.

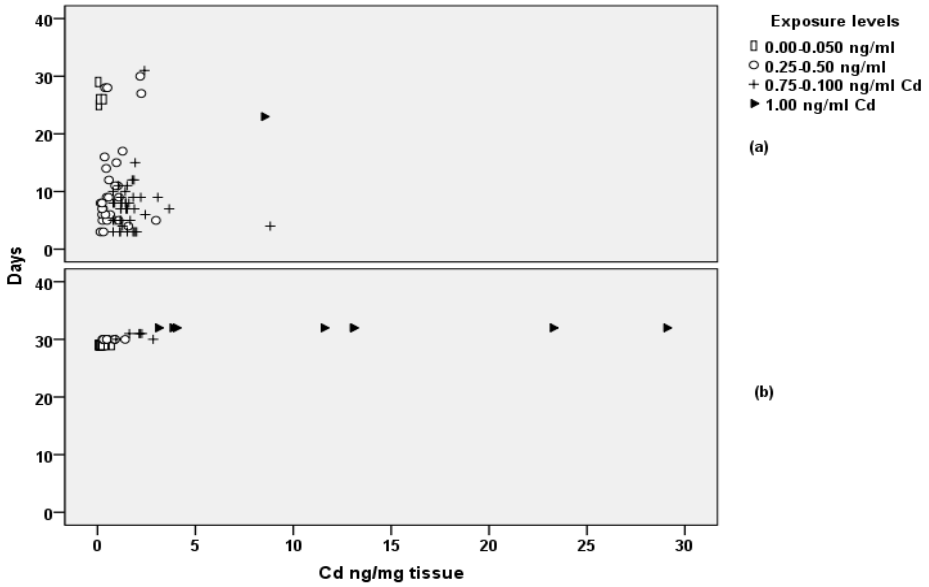

Fig. 3. Cd burden in zebrafish tissue associated with time of exposure for (a) death events before the completion of the experiment and (b) deaths at the end of the experiment for the different Cd exposure levels.

3.2. Histopathology

Study of zebrafish histopathology for most of the fish tissue samples namely gill, muscle, liver and intestinal tube tissues did not show any severe damage for zebrafish exposed to the range of Cd exposures (Fig. 4). However, the most important findings were noted for liver tissue of zebrafish exposed to 75 μg/L that died and was collected on the 15th day of the experiment (Fig. 4e) as well as for liver tissue of zebrafish exposed to the same exposure dead on the 11th day (Fig. 4f). Both of them exhibited lymphocytic infiltration, which is indicative of chronic inflammation. Moreover, liver tissue of zebrafish that died earlier (11th day, Fig. 4f) demonstrates vacuolization in the form of fatty degeneration, which is why the vacuole content does not appear dyed. Another fairly noteworthy observation is that fish exposed to the lowest Cd exposure (5 μg/L) although survived to the end of the experiment (30 days) demonstrated degenerative changes of the epithelium and mild inflammation of the intestinal tube (Fig. 4a). With regard to the remaining Cd exposures, we present indicative results of the study (Fig. 4b,c,d,g,h).

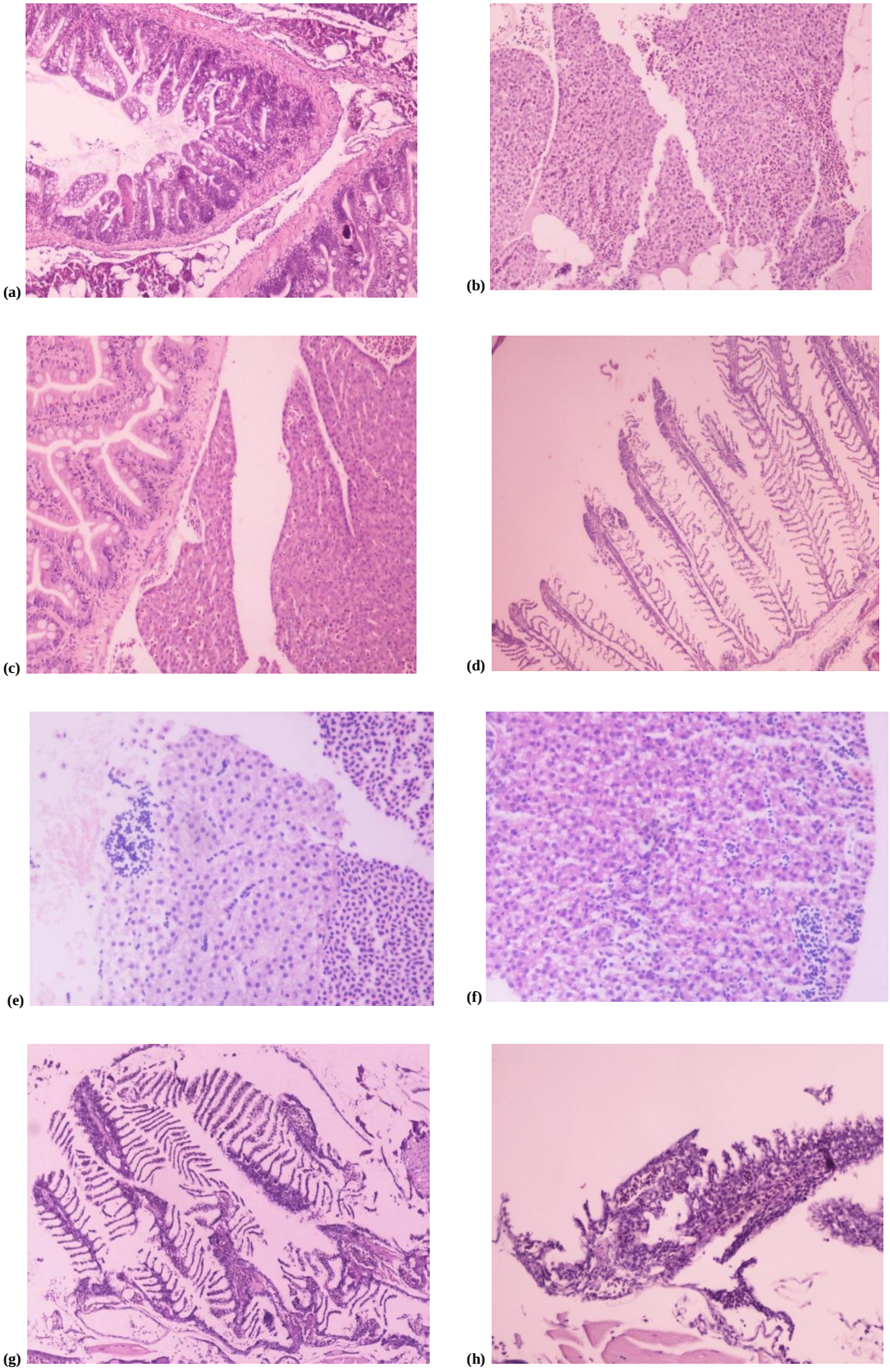

Figure 4. Histology of zebrafish: (a) Intestinal tube, exhibiting regenerative changes of the epithelium and mild inflammation (X100) of zebrafish exposed to Cd treatment = 5 µg/L, (b) Liver tissue with nucleated red blood cells (X200) of zebrafish exposed to Cd treatment = 25 µg/L, (c) Gastrointestinal

tube and liver (X200) of zebrafish exposed to Cd treatment = 50 μg/L, (d) Branchial arches (X100) of zebrafish exposed to Cd treatment = 75 μg/L, (e) Liver tissue of zebrafish exposed to Cd treatment = 75 μg/L dead at 15th day, (f) liver tissue of zebrafish exposed to Cd treatment = 75 μg/L dead at 11th day (g) Branchial arches (X100) of zebrafish exposed to Cd treatment = 100 μg/L and (h) Branchial arches exhibiting nucleated red blood cells in the stroma (X200) of zebrafish exposed to Cd treatment = 1000 μg/L.

4. Discussion

We investigated zebrafish response to a series of Cd exposure levels, aiming to shed light on potential variations across the range of Cd toxicities. The study was primarily concerned with the accumulation of Cd in zebrafish tissue and its association to fish mortality. The additional histopathological analysis was conducted in an attempt to correlate Cd effects with mortality.

4.1. Bioaccumulation

Fish exposed to the different Cd exposures accumulated Cd in their tissues in a linear way and Cd burden determined in zebrafish tissue increased with increasing concentration. However, accumulation was not time dependent for all exposure concentrations. Fish exposed to 1000.0 μg Cd /L seem to have a distinct time-dependent accumulation rate. Fish exposed to the other Cd concentrations accumulate Cd indifferently of time of exposure and in fact, not over a certain level (Fig. 3). Several authors indicate that Cd is accumulated in zebrafish in a dose and time dependent manner both in whole body and individual tissues, following chronic Cd exposure (McGeer et al., 2000; Arini et al., 2015, Cambier et al., 2010; Gonzalez et al., 2006). Nonetheless, Annabi and colleagues, while investigating Cd accumulation in mosquitofish during chronic exposure, revealed two significant patterns of accumulation: Cd accumulation increased significantly until 20 days post-exposure and then decreased by the 30th day of exposure (Annabi et al., 2011). These findings support previous data by Rehwoldt and Karimian- Teherani (1976) who reported plateaus in Cd accumulation after 2 or 3 months in liver and kidney of zebrafish.

In our study, accumulation of Cd was greater for zebrafish exposed to 1000 μg/L with a corresponding median value of 11.6 μg/g (w.w.). We determined the whole body Cd load by ICP-MS, assessing the cumulative Cd burden of all tissues. Although Cd burden in separate zebrafish tissues is estimated by several researchers who evaluate high and low accumulating tissues, it has also been stated that these Cd levels are adjusted following acclimation and detoxification responses. There seems to be a metal transfer between high accumulating

tissues, namely from gills to the liver, where detoxifying mechanisms take over, and it is also suggested that muscles are characterized as low accumulating tissues because of more competent detoxification mechanisms (Cambier et al., 2010, Arini et al., 2015, Gonzalez et al., 2006; Vergauwen et al., 2013, Vergauwen et al., 2013). While the objective is to link the Cd burden to toxic effects, that may often be futile as not all accumulated Cd is reactive and an indefinable proportion could be in detoxified form.

4.2. Mortality

The study of zebrafish survival exposed to a range of Cd concentrations and its association with Cd accumulated in fish tissue across this range revealed a series of unexpected results. We anticipated a linear mortality response of zebrafish with increasing exposure concentration and although this seems to be the case up to a certain exposure level, low rates of mortality at the highest exposure, differentiate the pattern resulting in a nonlinear – non monotonic one.

As indicated by the results of this study, median OS (LT50) for fish exposed to 25 μg/L, is lower (LT50 = 9) than for fish exposed to 50 μg/L (LT50 = 11) illustrating a more severe Cd toxicity at this level of exposure. These findings could be a result of a variation in Cd2+ affinity for specific binding sites at the gills. McGeer et al., 2011 regarding Cd toxicity in fish gill, describe the presence of 2 different types of Cd2+ binding sites. The first type is characterized by high-affinity and low-capacity and is saturable at a relatively low Cd concentration range of exposure ($\leq$ 20 μg/L). The second type exhibits low-affinity and high-capacity sites and is more evident at a Cd exposure concentration of approximately $\geq$ 20 μg/L. Therefore higher mortality rate of zebrafish at 25 μg/L reported in this study reflects the high-affinity of Cd2+ to these sites leading to more adverse effects. As mentioned earlier, an essential Cd toxic mechanism is that Cd2+ antagonizes Ca2+ for gill binding sites leading to acute hypocalcaemia and eventual death (Renieri et al., 2014, McGeer et al., 2011). Ca2+ loss is also described in the work of Alsop and Wood (2011) as an adverse acute Cd toxic effect in zebrafish.

We also inspected mortalities in the different groups of Cd exposure levels and both groups 2 and 3 exhibited mortalities greater than 50% during the experimental period of 30 days. Other studies in the recent literature investigating waterborne Cd toxicity in adult zebrafish, report 28% and 56% mortality for zebrafish exposed to 2 μg/L and 10 μg/L respectively, for 21 days (Cambier et al., 2010). Similar results are presented by Arini et al. (2015) where zebrafish exposed to 35 μg/L for 21 days showed 56% mortality. Reduced

growth performance and survival rate were also reported for zebrafish exposed to 30 μg/L Cd for five weeks (Zheng, a et al., 2016a). The highest mortality rate in this study, was observed for fish exposed to 100 μg/L (mortality = 82.8%, LT50 = 7). Surprisingly fish exposed to the highest Cd exposure (1000 μg/L) exhibited the lowest mortality (11.1%), although they accumulated Cd in their tissues the most (11.6 μg/g). Zebrafish mortality data for an intermediate Cd exposure concentration between 100 and 1000, namely 560 μg/L, present a mortality of 25% (28 days) (Vergauwen et al., 2013b) which is in accordance with our findings. The fact that there are major differences in the reported LC50 in the recent literature should also be considered, for instance: 3,822 μg/L (Alsop and Wood, 2011), 11,510 μg/L (Vergauwen et al., 2013, Vergauwen et al., 2013), 22,482 μg/L (Wang et al., 2015) as an indication of toxicity differentiations.

4.3. Histopathology

Data from this study did not reveal any severe adverse histopathological effects in zebrafish tissue exposed to Cd exposures with the exception of the 75 μg Cd/L exposure. Zebrafish exposed to 75 μg Cd/L demonstrated chronic liver inflammation which could explain the early time of death for these fish as a toxic response. Liver is known to be the tissue accumulating Cd the most (Renieri et al., 2014) and as such, is more susceptible to adverse effects. For a lower Cd exposure Zheng et al. (2016a) report that Cd caused considerable structural changes on zebrafish liver exposed to 30 μg Cd/L for 5 weeks (Zheng, a et al., 2016a). In a previous study, gill epithelial layers of zebrafish were weakened following sub lethal Cd exposure (3 μg/L) while at 10 μg/L lamellar diffusion distance was increased by fluid filling the intermediated space between tissues (Karlsson-Norrgren et al., 1985). Other fish species such as minnows, silver catfish, gilthead seabream etc., exposed to sublethal Cd concentrations (3–22 μg/L) exhibited a variety of histopathological effects: vertebral injuries, gill hypertrophy, hepatic vacuolization, cell necrosis in several tissues (McGeer et al., 2011; Guardiola et al., 2013; Benaduce, 2008). It must be taken into consideration that each fish species demonstrates different sensitivity and responses to Cd, which in turn leads to diverse effects reported in the literature (Renieri et al., 2014).

The nucleated red blood cells are normal in zebrafish physiology (Menke et al., 2011). Nonetheless, fish exposed to 5 μg Cd/L, a concentration level that could represent environmental non polluted areas (McGeer et al., 2011, Cambier et al., 2010), showed degenerative changes of the epithelium and mild inflammation of the intestinal tube. This finding could raise the alarm for fish chronically exposed to sublethal environmental Cd concentrations, yet more evidence is necessary to support this assumption. Also, fish that

showed the lowest survival had no effects as well, which could be explained by early time of death. It is possible that Cd caused quick mortality due to other toxic endpoints, not investigated in this study, before causing histological alterations.

The results of our study indicate that histopathological effects on muscle, liver, intestinal tube and gill tissues, not unlike mortality, show non monotonic responses. Fish exposed to environmental levels demonstrated degenerative alterations, fish exposed to higher levels had no severe adverse effects, fish exposed to Cd concentration with one of the highest mortality rates showed serious toxic effects and fish exposed to the highest Cd concentration did not exhibit any alterations.

Part of our findings, namely mortality data, time independent accumulation as well as histopathological findings were surprising, yet could be related to physiological interactions. The unanticipated responses of lower mortality rates at high Cd exposure concentration could be induced by a modulation in detoxification mechanisms of fish, triggered at higher Cd levels which could result in altered Cd sensitivity (Renieri et al., 2016).

4.4. Overview of mechanisms of Cd toxicity and detoxification in literature

Recent researches on Cd toxicity in zebrafish explore various endpoints both for acute and chronic exposure which reflect the fact that potential toxicity is counteracted by detoxification mechanisms as part of damage-repair-acclimation procedures (McGeer et al., 2011). Primary zebrafish responses to Cd toxicity apart from mortality, are associated with oxidative stress, ion loss and endocrine disruption whereas main detoxification mechanisms involve the Nrf2 (NF-E2-related transcription factor) regulated antioxidant genes pathway and the production of metallothioneins (MTs).

4.4.1. Oxidative stress and detoxification in zebrafish

Oxidative stress induced by Cd exposure is investigated by several authors through its effect on antioxidant enzymes, expression of genes involved in oxidative stress protection and cell function. Wang et al. (2015). report that zebrafish exposure to 1124 μg/L Cd, disrupted the redox state leading to oxidative stress in the liver and finally enzyme misfolding changes in CAT and SOD reducing their activity. In an earlier study Wang and Gallagher (2013) reported for zebrafish exposed to 110 μg/L increased Nrf2 regulated antioxidant genes expression which is also supported by a later study of Arini et al. (2015). In the work of

Vergauwen et al., 2013, Vergauwen et al., 2013 it is shown that long-term Cd exposure increased liver oxidative damage to proteins. Additionally, transcripts of combating oxidative stress genes were elevated in zebrafish liver whereas Cd compartmentalization and protein carbonyl content were increased as well. These findings are supported by the reduction of antioxidant capacity in zebrafish brain (Richetti et al., 2011). Zheng et al. (2016b) also detected reactive oxygen species (ROS), nitric oxide (NO), and malondialdehyde (MDA) increased in a time-dependent manner in the brain and liver of zebrafish following acute Cd exposure. Generation of ROS was attributed to the disturbance of Cu/Zn-SOD and CAT genes at transcriptional, translational, post-translational levels. An increase in Nrf2 mRNA levels was also detected in the liver.

4.4.2. Metal related proteins as biomarkers and adaptive means

MTs as biomarkers of Cd toxicity in zebrafish have also been assessed by a few authors. Gonzalez et al. (2006) report that for zebrafish exposed to 10 μg Cd/L for 21 days, MT gene expression was a late hour biomarker. On the contrary, when zebrafish are contaminated with high amounts of Cd, MT gene expression appears to be quick (Airaksinen et al., 2003). Gonzalez et al. (2006) also report that mt1 genes were still induced after 21 days in liver, although this organ accumulated the highest cadmium concentration. Moreover, the expression of hsp70 (heat shock protein) in gills as an oxidative stress-response was observed along with the expression of genes involved in mitochondrial metabolism and metal detoxification. Expression of hsp70 is reported elsewhere as well as a short-term adaptation to Cd exposure while metallothionein was likely used for long- term detoxification and sequestering of Cd (Blechinger et al., 2002, Chen et al., 2007, Annabi et al., 2011).

4.4.3. Cd as an endocrine disruptor

The act of Cd as an endocrine disruptor (ED) in zebrafish is illustrated by several authors with most of them reporting effects on vitellogenin (vtg) genes. Chen and Chan, 2016a, Chen and Chan, 2016b state that Cd exhibits anti-estrogenic effects in the ZFL cell line, for embryos and adults. They also suggest that Cd effects on vtg1 gene expression in zebrafish are mainly mediated by endoplasmic reticulum (ER). Chouchene et al. (2016) provide further evidence, describing Cd as a potent anti-estrogen in vivo as well as in vitro and suggest that Cd effects on estrogen signalling in the brain could be attributed to the interference with classical nuclear ER subtypes affecting the expression of aromatase B (Aro-B). There are reports on other species as well incriminating Cd as an antiestrogenic (Denslow and Sepúlveda, 2007, Sfakianakis et al., 2015). Ion loss is also considered to occur as an

endocrine stress response (Aslop and Wood, 2013). It should also be stated that EDCs can have effects at low doses that are not predicted by effects at higher doses (Vandenberg et al., 2012).

4.4.4. Acclimation responses in fish

Apart from detoxification mechanisms, we must also take into consideration, possible acclimation responses at higher exposures. As stated above, Cd accumulation can reach plateaus, and a higher depuration rate could result in milder effects. In our study, fish exposed to the highest concentration even though accumulated Cd the most exhibited no histopathological findings, so maybe there could be a modulation in internal metal handling and detoxification after a period. Following an initial shock phase of metal exposure McDonald and Wood (1993) state that fish physiological adaptations take place in order to compensate for ion losses by mucus secretion and gill structure alterations at the cellular and subcellular level. After all, small differences in Cd accumulation may result in large differences in resistance (Xie and Klerks, 2004). It is also suggested that reduced metal uptake mechanisms regulation could be more important than resistance mechanisms. (Van Veld and Nacci, 2008). This could be the case of ion regulation via modification of the Cd^{2+} binding sites mentioned above. Metallothioneins expressed in the gills of Fathead minnows (*Pimephales promelas*) have also been suggested to be a major contributor in physiological acclimation to metals (Benson and Birge, 1985). Recently, Xie and Klerks (2004) evaluated selection for cadmium resistance in multiple generations of laboratory-exposed least killifish (*Heterandria formosa*) and found that development of resistance occurred within one to two generations and was inherited. Metallothionein-like protein levels were elevated in Cd tolerant fish lines and were considered to have contributed to observed tolerance. Although later, Goto and Wallace (2010) investigating Cd detoxification mechanisms in mummichogs (*Fundulus heteroclitus*) revealed that Cd partitioning via sequestration into insoluble granules is more efficient than metal-binding proteins, providing evidence of aquatic organisms living in metal-polluted habitats tolerating elevated concentration of Cd.

Overall, it could be derived from the above that final effects of Cd in zebrafish are a result of a continuous struggle between Cd toxicity, detoxification pathways and acclimation responses which all exhibit various behaviours at different levels of exposure. It is becoming more and more evident in toxicological studies that linear dose response curves do not apply accordingly for low and high doses. Höfer et al. (2010) determined a nonmonotical response of metallothionein expression pS2/TFF1 in the intestine and kidney of female rats. Bain and Kumar (2014) in an in vitro study determined the cytotoxicity of binary mixtures of pharmaceuticals to a rainbow trout cell line and found a non-monotonic concentration–

response relationship. Moreover, Zhang et al. (2016) described a non-monotonic dose–response effect of bisphenol A on rare minnow's (Gobiocypris rarus) ovarian development. Furthermore, different endpoints of toxicity are tested for high and low doses in literature, rendering it difficult to predict a toxic response through a range of doses.

In conclusion, our findings regarding zebrafish mortality, disclose a deviation in toxic responses through a range of Cd concentrations, leading to a nonlinear response. A defence mechanism against Cd toxicity could have possibly been enabled in zebrafish. These differentiated responses, including the data from the literature mentioned above, could be linked to hormesis phenomena. Exposures at high doses resulting in less adverse effects could be attributed to mechanisms stimulated at low doses, inhibiting toxic effects at high doses. For fish exposed directly to high concentrations, the milestone/key point of low exposure could be reached through mechanisms modifying metal uptake after initial metal shock phase, allowing metal accumulation to be gradual or progressive.

Future studies could be targeted towards elucidating counteraction of toxicity by detoxification mechanisms through wider ranges of metal exposures with focus on key concentrations which alter the toxic response profile.

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CHAPTER 2

CHAPTER 3

Cadmium, lead and mercury in muscle tissue of gilthead seabream and seabass: Risk evaluation for consumers

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Abstract

Cadmium (Cd), lead (Pb) and mercury (Hg) presence was investigated in the muscle tissue of gilthead seabream and seabass, collected from various aquaculture sites of the Aegean and Cretan Sea as well as from the fish market (fisheries). Risk for the Greek population through consumption of these species was estimated using two approaches: Target Hazard Quotient (THQ) and Hazard Index (HI). All heavy metal levels in the fish tissue were below the established safe limits for consumption. Metal accumulation was found to differ amongst mode of production, species, location and seasonality. Seabass demonstrated higher Hg and lower Cd concentrations than seabream, Hg and Pb seem to be more accumulated in closed seas and Pb values displayed a linear increasing trend from warmer to colder periods. Regression analysis revealed that the main contributing factor to Cd accumulation is species (beta: −0.28, 95%CI: −0.48 to −0.09); lead is predominately affected by seasonality (beta: 0.44, 95%CI: 0.29 to 0.59), Hg accumulation is mainly affected by location (beta: -0.32, 95%CI: −0.61 to −0.03) while wild seabream accumulates greater levels for Hg and Pb than farmed. Risk analysis demonstrated that consumption of the studied species, is safe for all metals (HI < 0.460 and TTHQ < 0.299).

CHAPTER 3

1. Introduction

Heavy metal presence in marine environments has been under investigation globally, due to potential repercussions on the ecosystem and to the human health. Metals such as cadmium (Cd), lead (Pb) and mercury (Hg) can be introduced into marine systems through atmospheric and coastal depositions, although the primary origin of metal pollution is their discharge as industrial waste. Cd, Pb and Hg are non-essential metals, with no known biological role and could be toxic even in traces for both marine organisms and humans (Islam and Tanaka, 2004; Olmedo et al., 2013).

Due to the fact that these contaminants are not bio-degradable, once accumulated by marine organisms, they can reach humans through the food chain, representing an eminent health hazard. Dietary intake constitutes the major route of human exposure to these contaminants (Rodríguez-Hernández et al., 2016; Renieri et al., 2014; Storelli, 2008). Although several metals have been implicated, marine fish have been reported to contain predominantly Cd, Hg and Pb often in levels exceeding the permissible limits (Bosch et al., 2016).

Health benefits of fish consumption have been well established and attributed to its high nutritional value and rich content in essential ω-3 polyunsaturated fatty acids, which play a major cardio-protective role (Domingo, 2016; Castro-González, Méndez-Armenta, 2008). However, long term consumption of fish, even with a low metal burden, could counterbalance its nutritional benefits (Bosch et al., 2016; Storelli, 2008; Copat et al., 2015).

Health risks arising from the toxicity of Cd are mainly kidney and skeletal damages, neurological disorders and endocrine disruption as well as cardiovascular disfunction and carcinogenic effects, as it has been characterized as a carcinogenic to humans by the International Agency for Research on Cancer (IARC). Pb interrupts the activity of enzymes leading to numerous adverse effects such as neurological problems, hematological effects, nephrotoxicity and hypertension in addition to DNA damage. Pb is also characterized as probably carcinogenic to humans (group 2A) by IARC. Toxic effects of Hg and its most toxic form methyl-mercury (MeHg), include reduced neuronal development and immunodeficiency (Bosch et al., 2016; Buha et al., 2018; Renieri et al., 2014; Copat et al., 2015; Gunnar et al., 2007; Derelanko and Hollinger, 2002). Within the framework of human health protection, health advisories such as the World Health Organization (WHO) and the European Food Safety Authority (EFSA) have established safe consumption levels for these metals, considering metal levels in fish tissue with respect to human intake.

In recent years, many researchers have focused on human dietary exposure to these heavy metals, via fish consumption in Mediterranean countries. Human risk derives not only from the fish metal load, but population dietary habits as well, which vary amongst countries (Olmedo et al., 2013; Kalantzi et al., 2016; Pazi et al., 2017; Copat et al., 2013; Rodríguez-Hernández et al., 2016., Llull et al., 2017; Storelli, 2008; Conti et al., 2012., Copat et al., 2018; Renieri et al., 2014). In Greece, the studies dealing with the issue of human dietary exposure to heavy metals are limited and to the best of our knowledge there are very few studies on metal exposure through consumption of the aforementioned species (Kalogeropoulos et al., 2012; Kalantzi et al., 2016).

Marine fish, and specifically Gilthead seabream (*Sparus aurata*) and Seabass (*Dicentrarchus labrax*), are widely farmed and consumed in Greece, as an integral part of the Mediterranean diet (Kalantzi et al., 2016). Talking into account that fishing degradation has been reported in the Mediterranean Sea (Islam and Tanaka, 2004) as well as globally (Christensen et al., 2014) and that aquacultures' stocks could replenish the consumers' demand, the risk estimation for both fish origins have become essential. Besides that, there have been noted differences in metal levels between farmed and wild (Ferreira et al., 2010).

For the purpose of this study, we collected samples of farmed fish of both species from different aquaculture plants of Greece, as well as wild samples from the local fish market. The specific objectives of the study were: (1) to assess the metal load of Cd, Pb and Hg in fish tissue (2) to estimate the potential health risk for consumers.

2. Materials and methods

2.1. Chemicals – reagents

Cd and Pb standards (100 μg/ml ± 0.5% in 2% HNO3) for inductively coupled plasma mass spectrometry (ICP-MS) and ICP-MS internal standards of Li, Sc, Y, In, and Tb (100 μg/mL ± 0.5% in 2% HNO3) were used from Bruker Daltonics Chemical Analysis (USA). Nitric acid (HNO3) trace SELECT, for trace analysis ≥69%, hydrogen peroxide solution (H2O2) for ultratrace analysis ≥30% and hydrochloric acid (HCL) ≥ 37%, trace SELECT, for trace analysis, were purchased from Sigma Aldrich. Type 1 (18.2 MΩ cm at 25 °C) ultrapure water was used (produced by a Direct-Q® Water Purification System). All glassware and polyethylene vials were kept in 10% HNO3 solution overnight and rinsed thrice with ultrapure water prior to use.

2.2. Sample collection, preparation, and digestion

Fish samples of both species were collected from aquaculture sites as well as the fish market of Heraklion, Crete during the period August 2017–March 2018. All collection sites are located in the Aegean Sea and the Sea of Crete (FAO fishing area 37, subarea 37.3, division 37.3.1). A total of 101 fish of both species namely gilthead seabream (n = 47) and sea bass (n = 54) were collected. More specifically 81 samples (gilthead seabream n = 37, sea bass n = 44) were collected from aquaculture sites and 20 samples from the fish market (10 fish from each species) which were caught in Cyclades and Dodecanese.

There were three distinct periods of collection (months) from fish farms: 21 samples (25.9%) on August (summer), 30 samples (37.0%) on November (autumn) and 30 samples (37.0%) on February–March (winter-early spring).

In the laboratory, length and weight of fish were measured and the condition factor (CF: body weight (g)*100/length3 (cm) was calculated; samples were labeled and stored at −20 °C until dissection. Upon dissection of the fish dorsal muscle tissue was collected in polyethylene vials and stored at −20 °C until further analysis. Fish muscle tissue was homogenized with liquid nitrogen, and 250 mg wet weight (w.w.) of each sample was weighed and placed in acid-cleaned borosilicate glass vials. 6 ml HNO3 ≥ 69% 1:1 H2O was added to each vial and left overnight for pre-digestion. For total dissolution, the predigest with the addition of 0.5 ml H2O2 and 1 ml HCl were placed in Teflon digestion vessels, sealed and placed in a high-pressure microwave digestion system. A speedwave MWS- 3+, BERGHOF microwave digestion system with built in, non-contact temperature and pressure measurement was used for the digestion of the samples in PFA Teflon DAP-60 + pressure vessels. Digested samples were stored in borosilicate glass vials at 4 °C until further analysis. Each sample was diluted with ultrapure water up to HNO3 2% final concentration prior to Inductively Coupled Plasma – Mass Spectrometer (ICP-MS) analysis. All method blanks and spiked samples were prepared using the same protocol. Spiking was conducted before the addition of acids and immediately after homogenization.

2.3. Instrumentation and metal analysis

Analysis of the samples for the determination of heavy metal content was conducted at the shared-access equipment centre, “Industrial Biotechnology”, A.N. Bach Institute of Biochemistry, Research Centre of Biotechnology of the Russian Academy of Sciences,

Moscow, Russia. The ICP-MS measurements were carried out with a quadrupole ICP-MS instrument Aurora M90 (Bruker Corp., USA), equipped with an autosampler and a MicroMist low flow nebulizer. Quantum software (Bruker Corp., v 3.1 b1433) was used for data collection and processing. Limit of detection (LoD) was defined as 3 times the standard deviation of the blank and LoD values (ng/mg) for each metal were Hg = 0.002, Pb = 0.014 and Cd = 0.002 ng/mg. Limit of Quantification (LoQ) was defined as 10 times the standard deviation and LoQ (ng/mg) values determined for each metal were: Hg = 0.008, Pb = 0.046 and Cd = 0.006 ng/mg. A 20-fold dilution was used for all samples. Each sample was measured 5 times.

2.4. Exposure assessment

For the risk assessment of fish consumption two different approaches were used:

(I) Hazard index (HI) estimated as the ratio of the estimated weekly intake (EWI) to the tolerable weekly intake (TWI) proposed by EFSA [Equation (1)].

$$HI = \frac{EWI}{TWI} \qquad (1)$$

The EWI was calculated as follows [Equation (2)]:

$$EWI = \frac{(C_m * WFC)}{BW} \quad (2)$$

where WFC is Weekly Fish Consumption, Cm is the metal concentration in fish tissue (ng/mg), weekly fish consumption for the Greek population is 332 g/week [based on per-capita consumption - live weight equivalent reported by the European Community (EC) = 17.3 kg/year (Eumofa, 2017)] and BW is the average body weight for adult consumer (70 kg).

TWI for Cd is 2.5 μg/kg which replaced the previous value of 7 μg/kg proposed by JEFCA, for MeHg is 1.3 μg/kg BW. Provisional tolerable weekly intake (PTWI) for Pb is 25 μg/kg BW (WHO, 2011).

(II) Target Hazard Quotient estimated as follows based on USEPA method [Equation (3)]:

$$THQ = \frac{(EF * ED * FIR * C)}{RfD * BW * AT} \quad (3)$$

Where EF and ED represent the exposure frequency (365 days/year) and the exposure duration (26 years), respectively; FIR is the fish ingestion rate for the Greek population = 47 gr/day [based on per-capita consumption - live weight equivalent reported by EC = 17.3 kg/year (Eumofa, 2017)], C is the metal concentration (ng/mg) and RfD is the reference oral dose in μg/kg BW/d (0.1 for Hg, 1 Cd and 3.57 for Pb) (USEPA, 2014, USEPA, 2001); BW is the average body weight for adult consumer (70 kg); AT is the average exposure time (EF*LT) for a lifetime (LT) of 70 years. Total target hazard quotient is the sum of THQ of each metal.

We applied both approaches under 2 risk scenarios: Scenario 1- For not detected metals in fish samples, LoD/2 was imputed for purposes of the statistical analysis and Scenario 2- Only positive values were used for metal concentration in fish muscle tissue. Additionally, we estimated the risk for both modes of fish production (wild and farmed) and for each species separately, as well as in total.

2.5. Statistics

Levels of Cd, Pb and Hg were expressed in the form of mean and standard deviation (SD). Median, 3rd quartile and 90th percentile of heavy metals were also calculated as indicators of exposure, in HI and THQ estimations. Two groups or more than two groups' comparisons were made using non-parametric Mann-Whitney and Kruskal Wallis respectively. Multiple linear regression using log scale values of Cd, Pb and Hg as dependent variable and area (closed vs. open seas), species (Seabass vs. Gilthead seabream), collection period (Aug, Sep, Feb–March) and CF as explanatory variables were applied. Bar charts of mean concentrations with 95%CI were used for the graphical representation of heavy metal levels.

Statistical analysis was carried out using IBM SPSS Statistics 24.0 and a level of acceptance of null hypotheses was set at 0.05. A value of 1 was set for HQ, HI and THQ as a margin of (un)safe exposure.

3. Results

A total of 81 fish samples of both species, collected from aquacultures sites located in various regions of Greece, were analysed. An additional group of 20 fish samples of wild captures of seabass and gilthead seabream from two fishing areas were also collected from the open fish market.

The mean length ± SD of farmed Gilthead seabream (n = 37) was 28.9 ± 2.2 while for seabass (n = 44) 32.1 ± 2.1 cm. Mean weight ±SD of farmed Gilthead was 476.7 ± 136.1 gr and for farmed seabass 403.5 ± 61.9 gr. Mean length and weight of wild fish captures were significantly higher in seabass, 34.4 ± 1.4 cm ($p = 0.001$) and 459.9 ± 44.3 gr ($p = 0.002$) respectively, than farmed ones. Wild gilthead seabream had similar dimensions to farmed gilthead seabream, with mean length 29.5 ± 0.8 cm and mean weight 473.2 ± 35.5gr. All fish presented CF values above one, indicative of well growth, with seabass fish samples achieving a significantly lower mean CF value (1.23) than gilthead seabream (1.92) ($p < 0.001$).

3.1. Metal levels in farmed fish and variance between species

Cadmium was detected (>LoD) in 16 samples (19.8%), lead in 31 (38.3%) and mercury in 74 (91.4%). With respect to Cd, mean measured concentration (>LoD) was 0.004 ± 0.002 ng/mg and there were no significant differences found between the two species ($p = 0.957$). Mean Pb for all farmed fish presented the highest levels among metals 0.137 ± 0.169 ng/mg yet there was no significant differences between species ($p = 0.886$). On the other hand, mean Hg concentration in Seabass: 0.050 ± 0.038 ng/mg was found to be significantly higher than in gilthead seabream: 0.032 ± 0.021 ng/mg ($p = 0.012$).

For not detected metals in fish samples, LoD/2 was imputed for purposes of the statistical analysis. Levels of Cd, Pb and Hg imputed with detection values (LoD/2) are presented as mean concentrations with error-bars (95%CI: confidence intervals) in Fig. 1. Pb levels do not differ significantly between Gilthead Seabream 0.058 ± 0.149 ng/mg w. w. and Seabass 0.056 ± 0.094 ng/mg w. w. ($p = 0.983$). However, mean Cd levels in Gilthead seabream (0.002 ± 0.002 ng/mg w. w.) and seabass (0.001 ± 0.022 ng/mg w. w.), demonstrated a significant difference, as did mean Hg levels in Gilthead seabream (0.029 ± 0.022 ng/mg w. w.) and seabass (0.047 ± 0.038 ng/mg w. w.) with p-values <0.001 and 0.029 respectively.

It is worth mentioning that levels of Hg and Pb were correlated weakly yet significantly ($r_s = 0.230$, $p = 0.039$) while assessing metal levels in all farmed fish tissues. Partial correlation analysis did not shown any association when species was considered a controlling factor ($p > 0.05$).

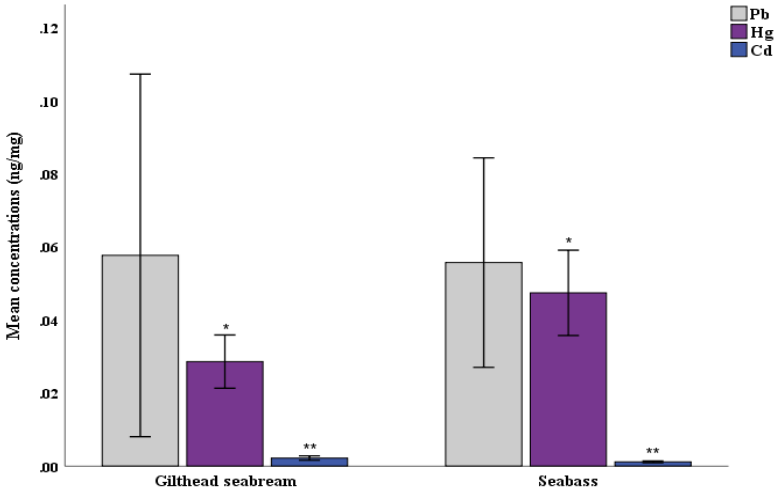


Figure 1. Mean levels with 95%CIs (95%confidence intervals) of Cd, Pb and Hg in muscle tissue of farmed Gilthead seabream and Seabass. Levels expressed as ng/mg w.w. (wet weight) * $p<0.05$, ** $p<0.001$

3.2. Variance/distribution of metals in farmed fish depending on collection site

Distribution of heavy metals was studied for fish of both species and in total, collected from different fish farm sites and results are presented in Table 1. Based on the obtained results it is clear that there is a site effect in most of the comparisons.

Table 1. Concentrations of heavy metals in muscle tissue of both fish species collected from different sites, as means and medians (ng/mg w.w.)

	Cd		Hg		Pb	
Area	**Mean ± SD**	**Median**	**Mean ± SD**	**Median**	**Mean**	**Median**
			Gilthead seabream			
Crete	0.003 ± 0.002	0.002	0.047 ± 0.020	0.047	0.101 ± 0.221	0.039
Mainland	0.001 ± <0.001	0.001	0.019 ± 0.008	0.02	0.010 ± 0.006	0.007
Saronic Gulf	0.003 ± 0.002	0.001	0.025 ± 0.009	0.024	0.042 ± 0.092	0.007
NE Aeagean	0.001 ± <0.001	0.001	0.002 ± 0.001	0.001	0.04 ± 0.057	0.007
Dodecanese	0.002 ± 0.002	0.001	0.010 ± 0.005	0.005	0.025 ± 0.038	0.007
p	**0.032**		**<0.001**		0.134	
			Seabass			
Crete	0.001 ± 0.001	0.001	0.046 ± 0.020	0.045	0.138 ± 0.120	0.122
Mainland	0.001 ± 0.001	0.001	0.034 ± 0.028	0.037	0.009 ± 0.005	0.007
Saronic Gulf	0.001 ± 0.001	0.001	0.047 ± 0.024	0.035	0.007 ± <0.001	0.007
NE	0.001 ± 0.001	0.001	0.105 ± 0.021	0.113	0.007 ± <0.001	0.007

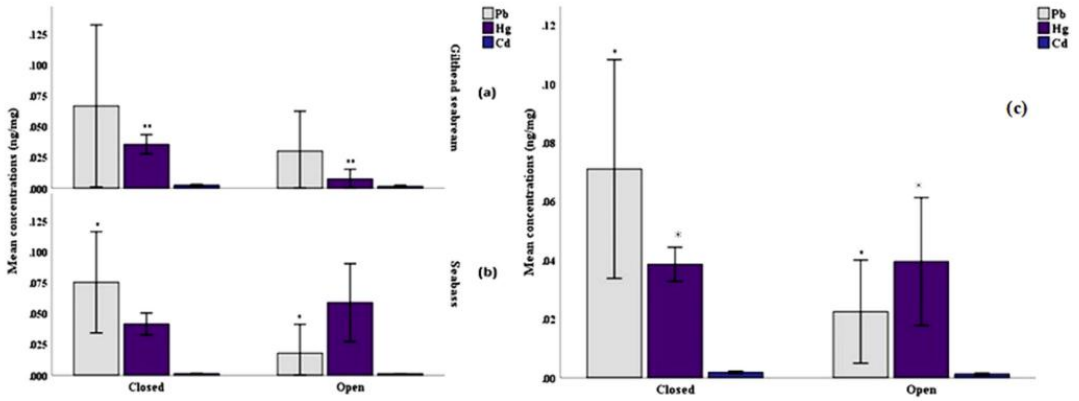
Aeagean						
Dodecanese	0.001 ± 0.001	0.001	0.047 ± 0.058	0.022	0.021 ± 0.047	0.007
p	0.859		0.073		**<0.001**	
			Total			
Crete	0.002 ± 0.002	0.001	0.046 ± 0.019	0.046	0.119 ± 0.176	0.051
Mainland	0.001 ± <0.001	0.001	0.029 ± 0.024	0.023	0.009 ± 0.005	0.007
Saronic Gulf	0.002 ± 0.002	0.001	0.032 ± 0.017	0.029	0.031 ± 0.077	0.007
NE Aeagean	0.001 ± <0.001	0.001	0.053 ± 0.058	0.042	0.024 ± 0.040	0.007
Dodecanese	0.001 ± 0.001	0.001	0.035 ± 0.050	0.019	0.022 ± 0.043	0.007
p	**0.014**		**0.006**		**<0.001**	

Comparison of Cd levels of Gilthead seabream tissues from different sites revealed significant differences ($p = 0.032$). Samples from Crete and the Saronic Gulf displayed the higher means (0.003 ± 0.002 ng/mg w. w.), while median values were higher in Crete 0.002 ng/mg. Correspondingly, with regard to mercury, mean (0.047 ± 0.020 ng/mg) and median (0.047 ng/mg) values are significantly higher in Crete ($p < 0.001$). Pb followed a similar pattern with previous metals for seabass, presenting significantly higher values in the Crete site (mean Pb: 0.138 ± 0.120, median 0.122 ng/mg) than the other farming sites ($p < 0.001$).

For both species in total, all metal levels were significantly different amongst areas. In further detail, Cd levels in fish tissues were significantly higher in Crete and Saronic Gulf farming sites, with means 0.002 ± 0.002 ng/mg ($p = 0.014$). Fish samples from Crete presented the highest Pb levels 0.119 ± 0.176 (mean) and 0.051 (median) ng/mg w. w. amongst sites ($p < 0.001$), while Crete and NE Aegean samples revealed a significant difference in Hg levels with mean Hg values in fish tissues, estimated at 0.046 ± 0.019 and 0.053 ± 0.058 ng/mg w. w., respectively ($p = 0.006$).

Additional analysis for the location factor was made grouping sampling sites into open and closed seas. Differences in metal levels were also studied between open seas (NE Aeagean and Dodecanese) and closed seas (Crete, Mainland and Saronic Gulf). Lead is significantly higher in closed seas ($p < 0.05$) for farmed fish in total (Fig. 2c), as it is for seabass alone ($p < 0.05$) (Fig. 2b). Median mercury level on the other hand, is significantly higher in closed seas for gilthead seabream ($p < 0.001$) and in total as well ($p = 0.029$) (Fig. 2b,c).

Figure 2 (a),(b), (c). Comparison of mean levels (ng/mg w.w.) of heavy metals between open and closed seas in gilthead seabream (a), seabass (b) and in total (c). ***** p<0.05, ******p<0.001



3.3. Variance/distribution of metals in farmed fish depending on seasonality

Another factor that seemed to affect metal accumulation is seasonality. The study of metal levels in fish tissues of both species, as well as in total, for the different months of collection revealed statistical differences for all heavy metals ($p < 0.001$). Levels of Cd in all fish samples were higher in summer (August) and winter to early spring (February–March) (mean: 0.002 ng/mg w. w.) in comparison to autumn (November) (mean: 0.001 ng/mg w. w.) ($p = 0.002$) (Fig. 3c). The above difference in sampling season was revealed in Gilthead seabream ($p < 0.001$) but not in Seabass tissues ($p = 0.620$) (Fig. 3a and b).

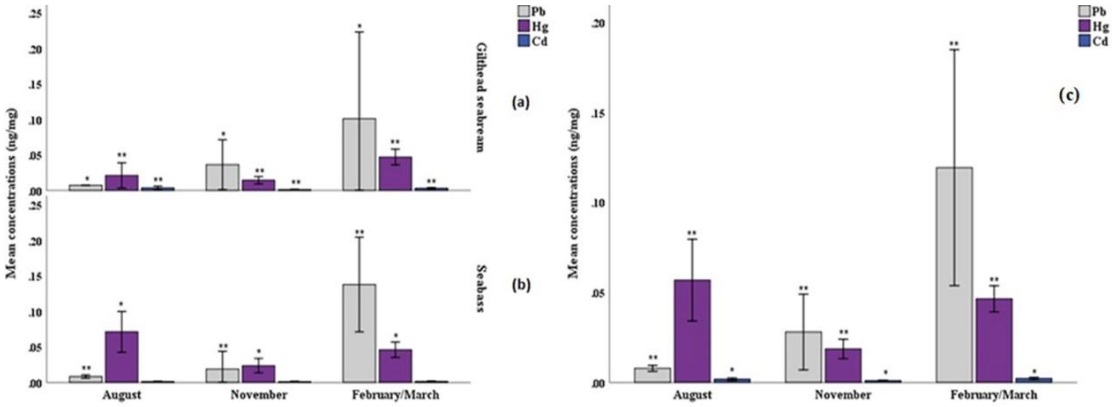


Figure 3 (a), (b), (c). Comparison of mean levels (ng/mg w.w.) of heavy metals among different months of collection in gilthead seabream (a) and seabass (b) and in total (c). ***** p<0.05, ******p<0.001

The estimated Pb levels for fish samples in total showed a statistically significant increase in winter (February/March) with a mean level of 0.119 ± 0.176 ng/mg w. w. ($p < 0.001$). That same seasonal pattern was presented for each species alone. More specifically, seabass samples presented a mean concentration of 0.138 ± 0.120 ng/mg

($p < 0.001$), while Gilthead seabream a mean of 0.101 ± 0.221 ng/mg w. w. ($p = 0.019$). Conclusively, Pb values seem to have a linear increasing trend when assessing metal levels from warmer (August) to colder (February/March) periods.

Hg levels in Gilthead seabream were significantly higher in February/March (mean: 0.047 ± 0.020 ng/mg) ($p < 0.001$), while during August farming period they were higher in seabass samples (mean: 0.071 ± 0.052) ($p = 0.004$) (Fig. 3 a, b). In the total of samples both summer and winter farming periods have increased Hg levels in fish tissues ($p < 0.001$) as it is presented in Fig. 3 (c).

3.4. Analysis of main effects on heavy metal levels in fish tissues

Multiple linear regression models using log-scaled Pb, Cd, and Hg levels as dependent variables were applied using species, seasonality (from "warmer" periods to "colder" periods), farming areas (closed/open seas) and condition factor of fish.

The analysis revealed that Cd levels seem to have only a species effect presenting lower levels in Seabass as indicated from the estimates slope (beta: −0.28, 95%CI: −048 to −0.09). Within the same regression model open seas showed a decreasing level of Hg (beta: -0.32, 95%CI: −0.61 to −0.03). Finally, when moving from warmer to colder periods an increasing level was observed for Pb (beta: 0.44, 95%CI: 0.29 to 0.59) (Table 2).

Table 2. Adjusted Beta coefficient and 95 CIs using Cd, Pb, and Hg as dependant variables.

Log (Cd)				
From	**To**	**B**	**95%LL to 95%UL**	**p**
Gilthead seabream	**Seabass**	-0,28	-0,48 to -0,09	0,005
Closed	**Open**	-0,09	-0,22 to 0,03	0,141
Aug	**Nov, Feb-Mar**	0,00	-0,07 to 0,08	0,929
CF		-0,14	-0,38 to 0,09	0,233
Log (Hg)				
From	**To**	**B**	**95%LL to 95%UL**	**P**
Gilthead seabream	**Seabass**	0,18	-0,27 to 0,62	0,437

Closed	**Open**	-0,32	-0,61 to -0,03	0,032
Aug	**Nov, Feb-Mar**	0,11	-0,06 to 0.27	0,211
CF		-0,16	-0,71 to -0,40	0,575

Log (Pb)

From	**To**	**B**	**95%LL to 95%UL**	**P**
Gilthead seabream	**Seabass**	0,01	-0,40 to 0,42	0,956
Closed	**Open**	-0,07	-0,33 to 0,20	0,609
Aug	**Nov, Feb-Mar**	0,44	0,29 to 0.59	0,000
CF		-0,17	-0,67 to 0,33	0,491

3.5. Distribution of metals in farmed fish and wild fish

For the assessment of differences in the metal load between farmed and wild fish, samples of wild seabass were compared to farmed seabass collected from the same area, namely Dodecanese, while wild gilthead seabream from Cyclades was compared to farmed seabass from open seas (N.E. Aegean and Dodecanese).

Wild gilthead seabream showed significantly greater levels for Hg and Pb than farmed seabream($p < 0.05$). Similarly, wild seabass presented significantly higher mean values of Pb than farmed (Table 3).

Table 3. Mean and median values (ng/mg ww.) of Cd, Pb and Hg in the tissues of farmed and wild Gilthead seabream and seabass.

		Farmed			**Wild**			
		Mean	**SD**	**Median**	**Mean**	**SD**	**Median**	**p**
Gilthead seabream	**Cd**	0.001	0.001	0.001	0.007	0.008	0.001	0.114
	Hg	0.007	0.010	0.003	0.037	0.022	0.044	**0.008**
	Pb	0.030	0.042	0.007	0.219	0.189	0.185	**0.009**
Seabass	**Cd**	0.001	0.000	0.001	0.001	0.000	0.001	0.361
	Hg	0.047	0.058	0.022	0.034	0.016	0.035	0.235
	Pb	0.021	0.047	0.007	0.039	0.049	0.021	**0.026**

3.6. Dietary intake and risk assessment for humans through fish consumption

For both approaches used for the exposure assessment of the Greek population to heavy metals via fish consumption, HI and THQ, all risk indexes are far below one, which indicates that gilthead seabream and seabass from the Greek seas are safe for consumption (Table 4).

Table 4. Estimated Hazard Indexes (HIs) and Target Hazard Quotients THQs for each metal in farmed and wild fish under 2 scenarios ([a] (Scenario 1): Imputed values were used, [b] (Scenario 2): Only positive values were used for metal levels).

		HI[e]				THQ[f]			
		HQ50	**HQ75**	**HQ90**	**HI**	**THQ50**	**THQ75**	**THQ90**	**TTHQ**
Gilthead seabream[a,d]	Cd	0.002	0.009	0.016		0.000	0.001	0.002	
	Hg	0.106	0.172	0.201	0.270	0.073	0.118	0.139	0.160
	Pb	0.001	0.020	0.053		0.000	0.007	0.020	
Gilthead seabream[b,d]	Cd	0.009	0.017	0.033		0.001	0.002	0.004	0.179
	Hg	0.113	0.175	0.201	0.330	0.078	0.121	0.139	
	Pb	0.020	0.047	0.096		0.007	0.018	0.036	
Seabass[a,d]	Cd	0.002	0.002	0.002		0.000	0.000	0.000	
	Hg	0.131	0.228	0.360	0.392	0.090	0.157	0.248	0.260
	Pb	0.001	0.010	0.030		0.000	0.004	0.011	
Seabass[b,d]	Cd	0.008	0.000	0.000		0.001	0.000	0.000	0.299
	Hg	0.136	0.237	0.402	0.460	0.094	0.163	0.277	
	Pb	0.018	0.029	0.058		0.007	0.011	0.022	
Total [a,c,d]	Cd	0.002	0.002	0.009		0.000	0.000	0.001	
	Hg	0.117	0.185	0.272	0.322	0.081	0.127	0.188	0.204
	Pb	0.001	0.013	0.041		0.000	0.005	0.015	
Total[b,c,d]	Cd	0.009	0.013	0.032		0.001	0.002	0.004	
	Hg	0.131	0.189	0.278	0.385	0.090	0.130	0.192	0.224
	Pb	0.020	0.036	0.075		0.007	0.013	0.028	
Wild [a,c]	Cd	0.002	0.002	0.032		0.000	0.000	0.004	
	Hg	0.139	0.175	0.220	0.341	0.096	0.121	0.152	0.189
	Pb	0.006	0.038	0.089		0.002	0.014	0.033	
Wild [b,c]	Cd	0.030	0.035	NE		0.004	0.005	NE	
	Hg	0.146	0.177	0.226	0.321	0.101	0.122	0.156	0.191
	Pb	0.028	0.054	0.095		0.011	0.020	0.035	

[a] Imputed values were used, [b] Only positive values were used, [c] both species, [d] farmed, NE: not estimated,

[e] HQ50, HQ75 and HQ90, indices based on median, 3rd quartile and 90th percentile of Cd, Hg, Pb concentrations

[f] THQ50, THQ75 and THQ90, indices based on median, 3rd quartile and 90th percentile of Cd, Hg, Pb concentrations

Farmed fish consumption for each species separately as well as in total, poses no risk to humans and more specifically, HI for seabream is slightly lower that for seabass (0.33 vs. 0.46), whereas for both species in total HI is 0.38. The metal that seems to be contributing the most is mercury. Risk of consuming farmed fish (HI = 0.38) is of the same magnitude as in consuming wild fish (HI = 0.32). Matching results are obtained when using the second approach (TTHQ), although the risk is even lower, probably due to the fact that this method estimates exposure for a fragment of time and (ED/LT) and that there are differences between TWI values set by EFSA and RfDs values set by EPA. Again, farmed fish seem to pose an analogous risk to wild ones (TTHQ farmed = 0.22 and TTHQ wild = 0.19).

4. Discussion

4.1. Metal levels in fish muscle tissue

Heavy metal concentrations determined in fish muscle of both species, in all cases, are below the established safe limits for food consumption (EFSA, 2012; FAO, 2011). Existing regulatory limit for Cd in muscle meat of fish is 0.050 mg/kg w. w., while for Pb is 0.3 mg/kg w. w. and 0.5 for Hg mg/kg w. w. Our results are in agreement or comparable with the recent literature on Cd, Hg and Pb concentrations in the muscle tissues of gilthead seabream and seabass, collected from various regions in the Mediterranean Sea (Table 5). To be more specific, Cd levels in fish collected for this study, from the Aegean Sea and the Sea of Crete, were similar or lower than fish collected from adjacent seas (Creti et al., 2010) and somewhat higher than fish collected from the same FAO division (FAO 37.3.1) (Kalantzi et al., 2016). This is the case for Cd in farmed seabass as well; our findings are similar or lower compared to other studies within the same region (Squadrone et al., 2016; A. Iamiceli et al., 2015; Dalman et al., 2006; G. Dugo et al., 2006; Kalantzi et al., 2016). With regard to Pb in farmed seabream our results are quite similar to the ones obtained by Minganti et al. (2010), yet higher that others (Kalantzi et al., 2016; A. Iamiceli et al., 2015), while for seabass our results are lower than levels reported in other studies (Iamiceli et al., 2015; Dugo et al., 2006; Dalman et al., 2006). Hg levels estimated in our study for both species are within the range reported in the relevant literature (Table 5).

CHAPTER 3

Table 5. Heavy metal concentrations in the muscle tissues (expressed as means and range ng/mg ww) of gilthead seabream and seabass, in the Mediterranean sea, reported in the recent literature and this study

		Cd		Pb		Hg			
Species	**season**	**mean**	**range**	**mean**	**range**	**mean**	**range**	**REGION**	**REFERENCE**
gilthead seabream [a]		bdl		bdl			0.02-0.1	Greece	Kalantzi et al. 2016
gilthead seabream [a]		<0.01		<0.1				Italy	Cretì et al., 2010
gilthead seabream [a]			<0.003 -0.022		<0.013 -0.139	0.12	0.07-0.16	Italy	Minganti et al. , 2010
gilthead seabream [a]	all year	<0.01		<0.02				S Adriatic, Tyrrhenian, Ionian Sea	Iamiceli et al. 2015
gilthead seabream [a]	August	0.003		0.007		0.021		FAO zone 37.3.1	This study
gilthead seabream [a]	November	0.001		0.036		0.014		FAO zone 37.3.1	This study
gilthead seabream [a]	Feb-March	0.003		0.101		0.047		FAO zone 37.3.1	This study
seabass [a]		bdl		bdl			0.06-0.07	Greece	Kalantzi et al. 2016
seabass [a]	February	<0.010		<0.010		0.036		Ligurian Sea	Squadrone et al. 2016
seabass [a]	all year	<0.01		<0.02				S Adriatic, Tyrrhenian, Ionian Sea	Iamiceli et al. 2015
seabass [a]	all year	<0.01		0.274				S Adriatic, Tyrrhenian, Ionian Sea	Iamiceli et al. 2015
seabass [a]	July		0.08- 0.13		0.18-0.32			Tyrrhenian Sea	Dugo et al. 2006
seabass [a]	July		<0.9-0.11		0.12-0.35			Sea of Sicily	Dugo et al. 2006
seabass [a]			<0.01-0.04		<0.02-0.4			SE Aegean Sea (Turkey)	Dalman et al. 2006
seabass [a]	August	0.001		0.008		0.071		FAO zone 37.3.1	This study
seabass [a]	November	0.001		0.024		0.024		FAO zone 37.3.1	This study
seabass [a]	Feb-March	0.002		0.019		0.046		FAO zone 37.3.1	This study
seabass [b]	all year						0.017-0.108	Serbia	Djinovic-Stojanovic et al. 2015
seabass[b]		0.002		0.004		0.079		Murcia, Spain	Olmedo et al. 2013
gilthead seabream [b]	all year						0.017-0.108	Serbia	Djinovic-Stojanovic et al. 2015
gilthead seabream [b]		0.001		0.004		0.037		Murcia, Spain	Olmedo et al. 2013
gilthead seabream [c]			<0.004 - 0.007		<0.013-0.027	0.54	0.29-0.72	Italy	Minganti et al. / 2011
gilthead seabream [c]	summer	0.11		0.16				E. Mediterranean Sea.	Ersoy and Çelik, 2010
gilthead seabream [c]	automn	0.05		0.58				E. Mediterranean Sea.	Ersoy and Çelik, 2010

gilthead seabream [c]	winter	0.2	0.15		E. Mediterranean Sea.	Ersoy and Çelik, 2010
gilthead seabream [c]	spring	0.19	0.19		E. Mediterranean Sea.	Ersoy and Çelik, 2010
gilthead seabream [c]		<0.09	<0.02	<0.06	SE Sicilian coast(FAO zone 37 1.3)	Nacarri et al., 2015
gilthead seabream [c]		0.073	<0.02	0.455	SE Sicilian coast(FAO zone 37 1.4)	Nacarri et al., 2015
gilthead seabream [c]	Feb- March	0.007	0.289	0.037	FAO zone 37.3.1	This study
seabass [c]	Feb-March	0.001	0.039	0.034	FAO zone 37.3.1	This study

[a]farmed, [b]fish market, [c]wild, bld: below detection limit

Cd in wild gilthead seabream in our study, is lower than values reported by Ersoy and Çelik, 2010 for the same species and the same season of collection. It is worth mentioning that Pb was lower yet close to the maximum level safe for consumption (0.289 ng/mg) and higher than values published by other authors (Olmedo et al., 2013; Minganti et al., 2010; Naccari et al., 2015). Hg levels estimated for wild gilthead seabream are within the range reported in the relevant literature (Table 5).

It has been reported by many authors, that metal accumulation in fish tissue is affected by a number of factors such as species, physiologic condition, diet, season, habitat, metal concentration etc. (Renieri et al., 2014; Ferreira et al., 2010; Rodríguez - Hernández et al., 2017; Martignago, R.et al., 2009; Dural et al., 2006; Copat et al., 2018; Storelli et al., 2008; Renieri et al., 2017). We investigated such relationships between the aforementioned factors and metal levels detected in our study and focused our study on muscle tissue, since it is typically the edible part and the primary concern of fish consumption risk assessment. It should also be taken into consideration, that muscle tissue, being the less active metabolically, accumulates metals in lower levels than other tissues (Renieri et al., 2014; Nasyitah Sobihah et al., 2018; Squadrone et al., 2016) however, metals are transported to muscles through other tissues and muscles can serve as indicators of an implemented chronic exposure (Kalantzi et al., 2016).

4.2. Species specific metal accumulation in fish muscle tissue

Although both fish species inhabit similar depths (demersal species) and samples of each species were collected from the same farming sites, differences in Hg and Cd levels were revealed. Our results suggest that heavy metal accumulation is species dependant. Mercury levels in seabass muscle tissue exceeded significantly Hg levels in gilthead seabream tissue, which is in consistency to results reported by Kalantzi et al. (2016). On the other hand, cadmium mean concentrations in seabass were found to be significantly lower than in gilthead seabream muscle tissue. Similar differences have been depicted by numerous authors, attributing species specific metal accumulation to various factors such as natural habitat, diet and feeding behavior, lipid content and metabolic activity among others (Renieri et al., 2014; Ferreira et al., 2010; Storelli et al., 2008; Á. Rodríguez-Hernández et al., 2017; Copat et al., 2018). In the present study, samples of both species were collected from each aquaculture site, exposed to the same environmental metal background, implying an alternate factor liable for different metal accumulation.

A possible interpretation could be the differences in size, condition factor and lipid composition between species. We have determined that seabass fish samples showed a significantly lower mean CF than gilthead seabream ones. According to the literature, CF is positively correlated to the total lipid content of fish (Mozsár et al., 2015) and seabass muscle tissue has a lower lipid and higher protein content than gilthead seabream (Nasopoulou C et al., 2011; Erkan and Ozden, 2007). Moreover, metal distribution in tissues has been reported to be dependent on the tissue lipid and protein content; some metals are accumulated more in tissues with low fat and high protein content (Kalantzi et al., 2016). It has also been suggested that high lipid content might lead to less accumulated Hg (Bosch et al., 2016). While the concentrations of Cd and Pb were found to positively relate to lipid contents in farmed fish in the work of Y.W. Qiu et al. (2011) concentrations of Hg did not. Additionally, mercury has been found to be accumulated more in gilthead seabream muscle rather than other tissues (Ferreira et al., 2008; Kalantzi et al., 2016) whereas Cd is more accumulated in the gills and kidney (Creti et al., 2010). Therefore we could argue that higher Hg and lower Cd concentrations in seabass found in our study is linked to differences in size and body composition between species and different metal behavior.

Another explanation could be lying in the fact that each species was administered different aquafeed. Fish feeds have been implicated in contributing to the metal load in farmed fish muscle and differences have been attributed to variations in the metal concentrations among feeds (Rodríguez-Hernández et al., 2017; Yildiz, 2008; Creti et al., 2010; N. Nasyitah Sobihah et al., 2018). It has also been reported that cadmium accumulates mainly through the diet (R. Martignago et al., 2009) and this is supported by L.D. Rozon-Ramilo et al., (2011), who suggested that Cd behavior in the fish body is dependent on route of exposure, with diet exposure leading to greater levels that waterborne. Moreover, metals are distributed to different tissues depending on the route of exposure (Ferreira et al., 2008). Accordingly, these factors may contribute to the fact that Cd and Hg levels are diversified between gilthead seabream and seabass. It is also worth mentioning, that a recent study has revealed potentially different metal detoxification mechanisms between the two species in vitro, suggesting that gilthead seabream cells were more sensitive to metals than sea bass cells (P. Morcillo et al., 2018).

4.3. Metal distribution in fish muscle tissue depending on farming site

Distribution of metals in fish is a reflection of metal occurrence in their habitat; micro-element composition and pollution of sea water is of high importance in fish metal bioaccumulation (G. Dugo et al., 2006). Cadmium, lead and mercury end up in marine ecosystems through both natural and anthropogenic origins as well. Aquaculture sites located

in the Aegean and the Cretan Sea receive metal loads through atmospheric and onshore waste origins, although metals in aquacultures may also occur from agricultural runoff, antifouling paints on the nets of fish cages, sediment diffusion as well as waste feed and fecal production (Islam and Tanaka, 2004; Alhashemi et al., 2012; J. Castritsi-Catharios et al., 2015; Kalantzi et al., 2016; Weng and Wang, 2014; Ranjbar Jafarabadi et al., 2017). However, metal bioavailability and potential toxicity to organisms is determined by their chemical form, salinity, pH value, hardness (Alonso Castillo et al., 2013) and dissolved metals are more bioavailable (Ranjbar Jafarabadi et al., 2017). The dissolved form is favoured in low pH values of seawater and low dissolved oxygen levels.

Our results showed that metal levels vary amongst fish collected from different fish farms. Distribution appears to be site dependent for all metals analysed and in particular, Pb demonstrates the greatest variance of mean concentrations, with fish from Crete showing the higher values. Pb levels in Crete could be attributed to the existing harbour in the bay where the farming site is located (Pb is often used in coating in shipping activities), aside from the possible Pb release of antifouling in fish cages (Dean et al., 2007). Fish samples collected from the NE Aegean, exhibited the highest mercury levels. The aquaculture site located in the NE Aegean, being close to Dardanelles is affected by the continuous transport of contaminants originated from the Black Sea and in addition it is located near the industrialized Aliaga and Izmir Bays. The mean Hg levels, in fish samples collected from the adjacent Aliga Bay, in 2009 were above the acceptable values in fresh fish according to the FAO limits due to industrial inputs into the Aliaga Bay (I. Pazi et al., 2017). Additionally, a biomonitoring study in microalgae from eastern Aegean coastal areas revealed high Hg levels in algae from the Izmir Bay (Akcali & Kucuksezgin, 2011), while a simulation of the fate of the pollutants, transported from the Dardanelles into the North Aegean Sea by Kopasakis et al. (2012) predicts that the ecosystem may be seriously threatened in the future decades. In addition, the entire Mediterranean Sea is characterized by variations in Hg distribution, creating zones with high mercury concentrations (Damiano et al., 2011). Taken together, higher mean Hg values in the NE Aegean found in our study could be justified. With regard to Cd distribution, Cd was found to be higher in fish collected from Crete and the Saronic Gulf, both fish farms located in closed seas. Higher levels in the Cretan aquaculture site could be explained by agricultural washouts from adjacent farms through the river which flows nearby, taking into account that a major source of Cd release into the environment is the production and use of phosphate fertilizers (McGeer et al., 2011). The Saronic Gulf on the other hand, is burdened by industries and shipyards in addition to urban waste effluents. However the existing biological wastewater treatment plant counterbalances the pollution rates.

Attempting to further elucidate our findings, we also studied differences in metal distribution between samples from open seas (NE Aeagean and Dodecanese) and closed seas (Crete, Mainland and the Saronic Gulf). Lead and mercury are significantly higher in closed seas for farmed fish in total. Closed seas retain a low capacity of water interchange which may affect metals' biochemical cycle and environmental fate which is evident also in a greater scale, for instance, fish from the Atlantic have lower metal levels than their Mediterranean counterparts (Ferreira et al., 2008).

In addition, our results regarding site specific variations, disclose a species effect as well. With respect to Cd levels in gilthead seabream alone, fish from Crete and Saronic Gulf displayed the highest values. Correspondingly, mean mercury values were significantly higher in Crete. For seabass samples, Pb followed a similar pattern with previous metals, presenting significantly higher values in Crete than in the other farming sites.

4.4. Metal distribution in fish muscle tissue depending on seasonality

Seasonality as a modulator of metal accumulation in fish muscle tissue has been illustrated by several authors (Renieri et al., 2014; Giannakopoulou, L., & Neofitou, C., 2014; B. Ersoy, M. Çelik, 2010; Aksu et al., 2011; Cardinal et al., 2011). Our results revealed differences in metal levels among the different farming seasons, suggesting a season effect of metal accumulation.

More specifically, Cd displayed the lowest levels for all fish samples in autumn and higher levels at the end of summer and winter. The above difference in sampling season was also revealed in Gilthead seabream but not in Seabass tissues. This is in agreement to the results published by Ersoy, M. Çelik (2010), who reported that the maximum Cd value was detected in Gilthead seabream in winter seasons. This could be explained by differences in fat content among seasons, since it has been shown that sea bream present a substantial fatty acid variation according to the season with the highest fat level observed in October and that the richer in fat of fish muscle, the lower the affinity for certain metals (Cardinal et al., 2011; Grigorakis, 2007). Contrastingly, although (Giannakopoulou & Neofitou, 2014) determined that the seasonality factor is significant for Cd concentrations in muscle tissue of *Pagellus erythrinus*, reported higher Cd values in autumn and the lowest in summer, associating the variations with differences in polluting sources, apart from each fish species characteristics.

Moreover we determined that estimated Pb levels for all fish samples showed a statistically significant increase in winter. That same seasonal pattern was presented for each species alone. Pb values displayed a linear increasing trend when assessing metal levels from warmer to colder periods whereas investigation of differences in fish CF showed a statistically significant decrease in seabass from summer to winter. This is in contrast to the results of Giannakopoulou & Neofitou (2014) who stated that Pb was more concentrated in June for *Pagellus erythrinus*. This could reflect a species effect since we analyzed different fish species.

Furthermore, we observed that for all fish both summer and winter farming periods have increased Hg levels in fish tissues and lower levels are presented in autumn. Hg levels in Gilthead seabream were significantly higher in winter, while during the summer farming period Hg was higher in seabass samples. In the work of Aksu et al. (2011), Hg levels in *Merluccius merluccius* displayed lower concentrations in August and slightly higher in winter that was linked to the fact that the total lipid content increases in fish during colder periods. Possibly, this is another case of species specific variations and differentiated metal accumulation due to body composition.

It must also be taken into consideration that gilthead seabream reproduction occurs during autumn, while seabass during the end of the winter and early spring. This suggest variations in metabolic condition, body composition and feeding behaviour, all factors affecting metal accumulation (Renieri et al., 2014).

4.5. Analysis of main effects on heavy metal levels in fish tissues

We attempted to clarify the variability of our results by applying linear regression analysis models. Log transform of metals' concentrations was made for normalizing the distribution, while linear regression was applied to avoid biases due to not systematic sampling. The results of the analysis highlighted the main contributing factor to metal accumulation for each metal. Cadmium levels in particular, seem to be subjected only to species effect presenting lower levels in Seabass. This is consistent to what has been in discussed in the respective individual study assessing species specific variations and was related to the fact that the two species differ in lipid composition which is additionally affected by different reproductive periods. Mercury levels on the other hand were mostly affected by location and as depicted by the analysis, open seas present lower levels as closed ones. This could be linked to the fact that closed seas have a slower interchange rate of waters, leading to higher levels of metals in the water, sediment and biota. As revealed by the same model, lead is predominately affected

by seasonality and presents higher levels in muscle tissue levels when moving from warmer to colder periods. Again, this could be linked to the seasonal changes in lipid composition of fish and the way it affects this metal's accumulation.

4.6. Comparison of metal distribution between farmed fish and wild fish

Potential variations in the metal load of fish muscle, between farmed and wild fish in the Mediterranean Sea, have been studied by a number of authors, in the light of assessing the hazard of fish consumption (Creti et al., 2010; Ferreiera et al., 2008; Rodríguez-Hernández et al., 2017; Minganti et al., 2010; S. Vizzini et al., 2010; Grigorakis, 2007). Our findings show that wild gilthead seabream tissue accumulates greater levels for Hg and Pb than farmed gilthead seabream. Our results are in agreement with Ferreira et al. (2008) who reported that wild white seabream showed higher accumulation than cultured ones. It is worth mentioning that farmed fish have higher lipid levels than their wild counterparts (Grigorakis, 2007; Rodríguez-Hernández et al., 2017; Cirillo et al., 2009; Ferreira et al., 2008a). Moreover out study revealed that, wild seabass tissue presented higher mean values of Pb than farmed ones. Ferreira et al. (2010) established that wild seabass has shown higher metal accumulation than cultivated species and argued that it was a result of feeding behaviour, since adult seabass are top predators and their diet may contain higher metal loads than fish feeds, highlighting the importance of diet exposure as the main source of metal accumulation in European seabass. It could also be argued that, higher Pb levels in wild fish are a reflection of breeding patterns. Seabass early life stages are pelagic, occupying more shallow waters that adults (demersal), where temperature could be higher during summer. Higher temperature values usually render fish more susceptible to intoxication (Sfakianakis et al., 2015). Pb detected in wild fish could be a result of early life accumulation. On the other hand, Minganti et al., (2010) found that only Hg showed significant differences between farmed and wild gilthead seabream, linking these results to the fact that molluscs the main diet of wild S. aurata, and have been found to contain high Hg levels. Diet composition could also affect antioxidant responses of gilthead sea bream as has been reported (Kokou et al., 2017) which could ultimately lead to variations in metal accumulation. Rodríguez-Hernández et al., 2017 reported higher Cd and Hg levels in wild whitefish, yet they could not define a pattern, acknowledging the fact that elements in marine environment have a more complex distribution than organic pollutants, as a result of local anthropogenic inputs, natural sources and hydrological conditions. It has also been suggested that lower metal levels in farmed fish maybe due to the effect of bio dilution because of faster growth rates of farmed fish (Kelly et al., 2008). On the whole, we could suggest that differences in metal levels found in our study between the muscle of farmed and

wild fish, are the combined result of different feeding behaviour, growth rate and therefore metabolic rate, aside from the effects of waterborne exposure.

4.7. Risk assessment for humans through fish consumption

Our study of potential health risks for Greek consumers revealed that the risk involved in consumption of the studied species is minimal. TTHQ values in all cases were less than 0.3 indicating safe levels of heavy metal dietary intake through fish consumption, since for HQ values lower than 0.1 no hazard exists, while for values between 0.1 and 1.0 the hazard is considered low (Kalogeropoulos et al., 2012). Moreover, taking into account the alternate approach of the HI, deriving as the ratio of the estimated to the tolerable intake, heavy metals measured in the fish consist no threat to humans as well, since the ratios are below 0.5. The highest HI values were estimated for Hg in seabass under the worst case scenario (0.4), yet still remaining on the safe side. Using a conservative approach, we intentionally used the TWI value for methyl-mercury (MeHg instead) of total mercury (THg) (assuming that 100% is MeHg), the most toxic form of THg, as it is estimated that approximately 90% of the total mercury (THg) in fish and shellfish is present in the form of MeHg and that they are the main diet contributor for this metal (EFSA CONTAM Panel, 2012).

Similar results to ours for cadmium, lead and mercury, were reported for fish from the Greek seas, describing low to non-existing risks for Greek consumers for the same (Kalantzi et al., 2016) as well as various other fish species (Kalogeropoulos et al., 2012). In the broader area of the Mediterranean Sea, numerous authors have evaluated the risk of dietary heavy metal intakes, using different hazard estimation approaches (Marti-Cid et al., 2008; Vieira et al., 2011; Conti et al., 2012; Pastorelli et al., 2012; Copat et al., 2013; Ersoy and Celik, 2010; I. Pazi et al., 2017). In a recent study from the western Mediterranean, consumption of lean fish by the Spanish population, although considered safe, contained a higher risk with regard to THg (EWI = 50% of PTWI), than that estimated in our study, while risk using values for MeHg was alarming (EWI = 150% of PTWI) (Llull et al., 2017). Nonetheless, we must take into consideration that the Spanish population consumes fish from other areas f.i. Canary Islands and the Atlantic Ocean in general (Rodríguez-Hernández et al., 2016). On the other hand, the maximum intake values set by European regulations for Hg, Cd and Pb were never exceeded for farmed seabass from Italy (Squadrone et al., 2016). In the Eastern Mediterranean, EWI for Pb and Cd through the consumption of gilthead seabream among other species was far below the PTWI (Ersoy and Celik, 2010).

With respect to data obtained with the use of THQ method, some results that raise concern were reported from the Eastern Mediterranean recently. Although the THQ values for Cd, Pb in fish samples collected from the coasts of Turkey were lower than 1.0, the THQ for Hg was higher than 1.0 for most of the samples and the consumption of certain species from Aliaga Bay was considered potentially hazardous to human health due to the Hg concentrations (Pazi et al., 2017). In a comparative study aiming to evaluate changes in the health risk of Italian consumers due to fish and shellfish consumption between 2012 and 2017, the risk for Cd was slightly higher because of higher Cd levels in molluscs but still very low (THQ = 0.007) whereas Pb-THQ obtained in 2017 (0.003) was equal to Pb-THQ in 2012 and Hg-THQ (0.2) in 2017 lower than in 2012 (Copat et al., 2018). Bonsignore et al. (2018) reported high Hg-THQ (≥1) for fish, crustaceans, molluscs and echinoderms from the Tuscany coast and warned that TTHQ values suggested that the local population could experience adverse health effects due to local seafood consumption, mainly of demersal and benthic species.

On the whole, studies from the Mediterranean focusing on the health risk resulting from fish consumption report quite diverse data, since it is affected by a number of miscellaneous parameters, such as consumption habits of each population, metal levels in fish, fish species selected and relationships between metals among others. It is noteworthy that several studies have showed that selenium (Se) may hold a protective role against MeHg toxicity whereas others argue that it may even exacerbate MeHg toxicity; in any case it has been signified that the Hg:Se ratio could be a useful tool to better assess the risk linked with fish intake (P. Olmedo et al., 2013; Kalantzi et al., 2016; Renieri et al., 2014). With regard to consumption, an EU report on Consumer Habits Regarding Fishery and Aquaculture Products (Eumofa, 2017) demonstrated the preference fluctuations between wild and farmed fish as well as fresh or processed seafood based on criteria such as country of origin, socioeconomic status and age as well as supply and demand dynamics.

Although we assessed the risk arising from the metals considered to be more toxic in two of the most consumed species, for an under - represented country in the literature such as Greece, we have to acknowledge certain limitations to our study. The dietary exposure to Cd, Pb and Hg is a result not only of fish consumption but other foodstuff as well (various seafood, cereals, milk etc.) which must be factored in risk assessment analysis. It must be underlined however, that seafood is the main contributor to heavy metal dietary intake. We did not include the Hg:Se ratio in our analysis, but we do not consider it likely that it would have affected our results. In addition, although heavy metal adverse effects in humans have

been described widely in the literature, long term low dose effects such as those that could be induced by fish consumption, could be the object of further investigation and risk analysis. Furthermore, current established limits on metal exposure have been set talking into consideration the single-stimulus exposure f. i. cadmium, lead or mercury alone, whereas this is far from the truth, since exposure to a combination of stressors usually occurs in real life scenarios. (Kostoff, R. N., Goumenou, M., & Tsatsakis, A.,2018). Acknowledging the fact that it would be nearly impossible to obtain a comprehensive optimization over all potential combinations of toxic stimuli in order to mitigate combination enhancement effects, biomonitoring and epidemiological studies on cumulative exposure remain essential in order to set more realistic exposure limits (Kostoff, R. N., Goumenou, M., & Tsatsakis, A., 2018; Tsatsakis A.M. et al., 2016; Tsatsakis A.M. et a., 2017; Hernandez A.F. & Tsatsakis A.M., 2017; Hernandez A.F. et a., 2013).

5. Conclusions

Cadmium, lead and mercury concentrations determined in the muscle tissue of gilthead seabream and seabass collected from two modes of production, aquaculture as well as fisheries showed levels far below the safe limits for consumption set by authorities. Differences in Hg and Cd levels between species were depicted and more specifically seabass demonstrated higher Hg and lower Cd concentrations, which can be attributed to differences in fish size and body composition as well as different metal behaviour, besides potential variations in the metal concentrations among feeds. In addition site specific variations in metal levels were revealed, which can be difficult to read as each metal displayed diverse distribution, however Hg and Pb seem to be more accumulated in closed seas. Seasonality is also a factor that weighs in metal accumulation in fish tissue as indicated by results obtained from this study as Pb values displayed a linear increasing trend from warmer to colder periods. Increased Hg levels in fish tissues for all fish were observed both in summer and winter farming periods while lower levels are presented in autumn. A species effect is evident while assessing seasonality as well, since Hg levels in Gilthead seabream were significantly higher in winter, while during the summer farming period higher Hg levels were detected in seabass. Regression analysis highlighted that the main contributing factor to Cd accumulation is the species; lead is predominately affected by seasonality while Hg accumulation is mainly affected by location. Cd and Pb accumulation patterns could be linked to the seasonal and species-specific changes in lipid composition of fish. Moreover, this study provides evidence of differences in metal levels between fish from the two modes of production which can be linked to different feeding behaviour, metabolic rate in combination to effects of waterborne

exposure. Finally, health risks for Greek consumers revealed that the risk involved in consumption of the studied species, farmed and wild, is minimal for all metals. Further investigation is essential in order elucidate metal accumulation issues in frequently consumed fish species as well as the development of more comprehensive models for risk assessment of the dietary intake of heavy metals.

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CHAPTER 4

Indicator PCBs in farmed and wild fish in Greece - Risk assessment for the Greek population

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Abstract

Health benefits of fish consumption could be counterbalanced by the intake of contaminants after long term fish consumption, burdened even in trace levels. The presence of the indicator PCBs (NDL-PCBs and PCB 118) in farmed and wild Gilthead seabream and Seabass was evaluated. For the determination of PCB, a GC-MS method was developed and evaluated. The association of PCB accumulation in fish with seasonality, locality, production mode and species was also investigated. A new approach for the risk characterization after exposure to NDL-PCB through fish consumption in Greece was developed, based on the real exposure and the permitted maximum levels of both aggregated dietary exposure and exposure through fish consumption. PCB levels determined in fish were below established permitted limits (6.24 ng/g 95th percentile), while PCB levels and congener distribution varied significantly between farmed and wild fish (p=0.001). Seasonality was highlighted as an important factor affecting NDL-PCBs accumulation, with high levels coinciding with the reproduction period of each species. Differences were also depicted for sampling sites, with PCB 118 presenting significantly higher values in open seas while NDL-PCB congeners in closed seas. Risk assessment of NDL-PCB intake through fish consumption corrected for the aggregated exposure revealed no risk for the consumers.

1. Introduction

Fish consumption has been well established as part of a healthy diet, mainly due to the rich content in essential nutrients and ω-3 polyunsaturated fatty acids (PUFA) of fish meat. Numerous health benefits have been linked to the intake of PUFA and high quality protein found in fish, with protection against cardiovascular diseases being the most

important. However, diet exposure to various toxicants and xenobiotics bioaccumulated in fish, could counterbalance these beneficial effects (Storelli, 2008; Domingo, 2016).

Wild, as well as farmed fish, are exposed to a set of various organic contaminants, such as Polychlorinated biphenyls (PCBs), Polychlorinated dibenzo-p-dioxins (PCDDs), Poly- chlorinated dibenzofurans (PCDFs) and Polycyclic aromatic hydrocarbons (PAHs) however, PCBs exhibit sharper biomagnification in the food chain (Çakiroğullari, Kiliç and Uçar, 2010; Paiano *et al.*, 2013; Costopoulou, Vassiliadou and Leondiadis, 2016a). Moreover, PCB levels in fish tissue increase in relation to the fish fat content. PCBs are a synthetic group of persistent organic contaminants (POPs), which were manufactured to be used as plasticizers, heat exchanging fluids, additives in pesticides and were adopted in electrical equipment and pigment industry as well. Their release in the environment was of anthropogenic origin and although their use and production has been banned, their occurrence in the environment and food chain is ubiquitous due to their low elimination rate and high resistance to metabolic degradation (Schrenk and Chopra, 2017).

PCBs constitute a class of 209 congeners which are categorized under 2 major groups based on their toxic potential: the dioxin-like PCBs (DL-PCBs), which share a common toxicity mechanisms with dioxins, and the non- dioxin-like PCBs (NDL-PCBs) (Arnich *et al.*, 2009; Schrenk and Chopra, 2017). Both groups are described as potential food contaminants, yet the sum of seven congeners (ΣPCB-7) (PCBs 28, 52, 101, 118, 138, 153, and 180) is commonly used as a gauge to determine the total PCB burden in environmental as well as tissue and food samples. Six of these seven are NDL- PCBs (PCB 28, PCB 52, PCB 101, PCB 138, PCB 153 and PCB 180), and one is a DL-PCB (PCB 118).These seven PCBs, often called "indicator PCBs" were identified by international bodies such as the European Commission (European Commission, 2011) and the International Commission for the Exploration of the Seas (ICES). The indicator PCBs have been since, routinely monitored by researchers and are also included in the Water Framework Directive (European Commission, 2002) for their monitoring (Schrenk & Chopra, 2017; Squadrone et al., 2015; St-Gelais, Aeppli, Burnell, & Costa-Pierce, 2017; Storelli & Perrone, 2010).

According to EFSA (2012), for a large portion of food samples, the sum of six indicator PCBs represents about 50% of the total NDL PCB load. Moreover, EFSA reported in 2012 that the highest levels of NDL-PCBs were observed in products derived from aquatic animals and specifically for fish muscle cited a mean of 23.3μg/kg w/w, while average exposure to NDL-PCB indicators ranged from 4.3 to 25.7 ng/kg bw per day and the 95th percentile between 7.8 and 53.7 ng/kg bw per day. Consumption of fish, was one of the highest contributing food groups to dietary exposure. Additionally, the EU has set maximum

tolerable levels (MLs) for the sum of the six indicators NDL-PCBs (ΣPCB-6) to 75 ng/g wet weight (EU, 2011). However, there is no valid established value for tolerable intake (TI) or guidance value for ΣPCB- or the ΣPCB-7 by international legislating authorities, since their toxicities for humans have not yet been fully characterized (EFSA, 2012b; WHO, 2016). An ADI value of 10 ng/kg bw/day has been proposed by the National Institute for Public Health and the Environment (RIVM 2001) for the sum of six NDL-PCB indicators (Baars *et al.*, 2001) and has been employed in certain risk assessment studies (Arnich *et al.*, 2009; Giandomenico *et al.*, 2016).

PCB burden in wild and farmed fish strongly depends on fish species, as well as area of sampling, demonstrating wide differentiations among farmed and wild-caught (Hayward, Wong and Krynitsky, 2007). Lundebye and colleagues (2017) reported higher PCB levels in wild fish than farmed while Carubelli et al., 2007 showed that farmed sea bass was 2 times more burdened than wild and similar results were published by Ferreira et al., 2010 who reported higher levels in farmed than wild.

According to FAOSTAT total fishery aquaculture and capture in Greece, in 2015, was 105.969 and 65.188 tons respectively, while per capita supply was 19.3 kg in 2013, 8.8 kg of which was demersal fish and 3.2 kg pelagic fish. Greek consumers demonstrate different preferences as to farmed or wild-caught fish, depending on various criteria such socioeconomic status and area of residence; however, there are no official published data.

Human health implications associated with PCBs include, endocrine disruption, reproductive defects, neurological disorders and potentially cancer (Schantz, Widholm and Rice, 2003; Buck Louis *et al.*, 2005; Meeker and Hauser, 2010; Boas, Feldt-Rasmussen and Main, 2012; IARC, 2015; Paliwoda *et al.*, 2016; Petrakis *et al.*, 2017) There is also evidence of PCBs synergistic effects during co-exposure with other substances, such as the ones with Cd on thyroid function (Buha *et al.*, 2013). NDL-PCBs in particular, seem to adversely affect dopamine neurotransmitter levels, calcium homeostasis and induce CYP enzymes in the liver (Fattore *et al.*, 2008; Schrenk and Chopra, 2017). It has also been suggested that CYP polymorphisms could be responsible for adversities caused by exposure to PCBs (Docea *et al.*, 2017). Regarding the presence of PCBs in marine fish from the Greek market, to the best of our knowledge, there are 2 studies assessing PCB levels in fish tissue, one of which focuses on DL-PBCs alone (Papadopoulos *et al.*, 2004) while the second reports levels of both DL-PBCs and NDL-PBCs in farmed fish and wild caught fish (Costopoulou, Vassiliadou and Leondiadis, 2016a).

The aim of this study is to investigate the burden of the indicator PCBs (ΣPCB-7) in farmed and wild-caught fish from the Greek market as well as to assess the risk of human exposure through fish consumption for the Greek population.

As far as we know, there is not a previously described methodology for the risk characterisation of exposure from a specific food item. All the current methodologies refer to risk characterisation due to aggregated dietary intake. In our study we aimed in the estimation of risk due to exposure to NDL-PCB but only through fish consumption. For this purpose, we used a newly developed approach which is based on the classic hazard index of unit but corrected for the intake due to a specific food.

2. Materials and methods

2.1. Chemicals – reagents

Standard solution PCB-Mix 3, containing all the investigated congeners (PCBs 28, 52, 101, 118, 138, 153, 180) at 10 ng/μL, was purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Diethyl ether (for analysis) and *n*-hexane (95%) were supplied from PanReac AppliChem ITW Reagents. SiliaFlash® Irregular Silica Gels, 70-230 mesh, 60 Å (R10140B) was supplied from SiliCycle. Hexachlorobenzene (HCB) was used as an internal standard (Dr. Ehrenstorfer GmbH). Aluminium oxide 15 μm was supplied from Agilent.

2.2. Sample collection

Fish samples of both species (gilthead seabream and sea bass) were collected from aquaculture sites as well as the fish market of Heraklion, Crete during the period August 2017–March 2018. All collection sites are located in the Aegean Sea and the Sea of Crete (FAO fishing area 37, subarea 37.3, division 37.3.1). A total of 101 fish of both species namely gilthead seabream (n = 47) and sea bass (n = 54) were collected. More specifically 81 samples (gilthead seabream n = 37, sea bass n = 44) were collected from aquaculture sites and 20 samples from the fish market (10 fish from each species) which were caught in Cyclades and Dodecanese, Greece.

There were three distinct periods of collection (months) from fish farms: 21 samples (25.9%) on August (summer), 30 samples (37.0%) on November (autumn) and 30 samples (37.0%) on February–March (winter-early spring).

In the laboratory, length and weight of fish were measured; samples were labelled and stored at −20 °C until dissection. Upon dissection of the fish, dorsal muscle tissue was collected in glass vials and stored at −20 °C until further analysis. An amount of 4 gr of fish

muscle tissue wet weight (ww) was collected from each sample and all fish samples were freeze-dried.

2.3. Sample extraction and clean-up

Each freeze-dried fish sample was placed in a glass vial with a screw cap and crushed with a spatula. In each vial the internal standard HCB (20 ng) and 9 ml of n-hexane were added. Vials were hermetically sealed and placed in an ultrasonic bath for 1 hour at 50°C. Silica gel (4.5 g) and aluminium oxide (3.6 g) were placed in a Schott filter used as a short glass column. Each column was washed with 25 ml of a mixture of hexane-diethyl ether (95:5). Subsequently, the extract obtained after the ultrasonic bath was passed through the sorbents and washed twice with 9 ml of hexane-diethyl ether (95:5). The purified extract was collected in a 100 ml glass flask and was evaporated to a volume of approximately 1 ml before being transferred to a 2 ml vial. The flask was washed with 600 µl of a mixture of hexane-diethyl ether (95:5) and combined with the previously evaporated extract. The obtained extract was evaporated to dryness under a gentle stream and reconstructed in 100 µl of hexane.

2.4. GC-MS equipment and analysis

Instrumental analysis was performed with a gas chromatograph coupled to a mass spectrometer (GC-MS Shimadzu QP2010 Ultra) equipped with an AOC-20i/s autosampler. An HP-5 MS column (30 m ×0.25 mm, film thickness: 0.25 µm) was used for separation. The initial oven temperature was set at 100°C, and then raised to 110°C with a heating rate of 4°C/min. The temperature was finally raised to 280°C at 15°C/min and held for 15 min. Injection volume was 2 µl in splitless mode and helium was employed as carrier gas (purity ≥ 99,999%) with a constant flow of 1.0 ml/min. The temperature of the ion source was 230°C. The MS was operated in single ion monitoring (SIM) mode and the mass traces acquired (m/z) for each congener and IS are reported in Table 1.

2.5. Method Evaluation

All blanks and spiked samples were prepared as described. Method validation was carried out and the examined analytical parameters were linearity, limits of quantification (LOQ), % recovery, inter-day precision (%RSD) and % accuracy. The quality standards were set to meeting ISO/IEC 17025 standards (ISO/IEC 17025:2005).

The standard and spiked curves were constructed using the ratio of each compound area to IS area and used of the study of instrument response as well for the quantification of the target compounds in fish muscle tissue. The LOQ (S/N>10) was determined from the lowest spiked blank sample. The % recovery, the % inter-day precision (%RSD) and the %

accuracy were determined at 4 spiked levels: 0.125 (lower limit of quantification, LLOQ), 0.625 (quality control low level, QCL), 1.25(quality control medium level, QCM), 2.5(quality control high level, QCH) ng/gr) using 3 repetitions (n=3) and the obtained data are presented in Table 1. For the determination of % recovery, PCB values obtained for a QCL sample spiked before extraction were compared to values obtained for a standard low level (STD-L) sample spiked after extraction. For the investigation of the carryover effect, the blank sample was injected immediately after the spiked sample at the upper limit of quantitation. The chromatogram of the blank sample was evaluated by comparing the peak area of the analyte in LLOQ samples and the analyte area in the blank sample.

2.6. Statistical methods

Levels of each PCB congener as well as of the sum of the 6 NL-PCBs (ΣPCB-6) and the sum of the 7 indicator PCBs (ΣPCB-7|) were expressed in the form of mean and standard deviation (SD) and median. Levels and % of detection of individual PCB congeners, ΣPCB-6 and ΣPCB-7 were estimated using only samples with levels >LOQ values. Statistical analysis and exposure assessment were conducted using the following approach: Detected values <LOQ were replaced with LOQ/2 and not detected with LOQ/6.

Median, 3rd quartile and 90th percentile of PCBs were also calculated as indicators of exposure, for the dietary exposure assessment. Analysis of two or more groups were performed using non-parametric Mann-Whitney and Kruskal Wallis, respectively.. Multiple linear regression using log scale values of PCB congeners as well as for the sums of PCBs, namely ΣPCB-6 and ΣPCB-7 as dependent variable and area (closed vs. open seas), species (Seabass vs. Gilthead seabream), collection period (August, September, February–March) as explanatory variables were applied. Stacked bar charts of mean concentrations as % contribution of each PCB congener to ΣPCB-7 were used for the graphical representation of PCB levels. Statistical analysis was carried out using IBM SPSS Statistics 24.0 and a level of acceptance of null hypotheses was set at 0.05.

2.7. Exposure assessment

For the exposure assessment, we considered only the sum of the 6 NDL-PCBs (ΣPCB-6) since they share a common mode of action (MoA) and we regarded it as an assessment group. This corresponded to a whole-mixture approach and it is consistent with the current globally agreed practice for the NDL-PCBs (EFSA 2012; WHO 2016). We conducted the exposure assessment by calculating the estimated daily intake (EDI) (ng contam /kg bw/day) of the ΣPCB-6 from the measured occurrence (contamination) in the studied fish and the daily fish consumption in Greece.

The EDI from fish consumption (EDI_f) was calculated as follows:

$$EDI_f = \frac{Cf \times Occ}{BW}$$

Where:

Cf is the daily fish consumption for the Greek population (g/person),

Occ is the ΣPCB-6 occurrence (contamination) in fish tissue expressed as the 95th percentile (ng contam/g fish) of ΣPCB6 determined in this study and

BW is the mean body weight for an adult consumer (70 kg).

The daily fish consumption value for the Greek population was considered from two consumption databases: FAOSTAT (http://www.fao.org/faostat/en/) and DAFNE-ANEMOS software (http://www.hhf-greece.gr/DafnesoftWebV2/). According to FAOSTAT Cf is 24,1 g/person/day (value for demersal fish), whereas DAFNE-ANEMOS reports 38 g/person/day (value for all fish).

2.8. Risk characterization

For the risk characterization, a newly developed approach was used. According to classic approach of HI (US-EPA, 2007) the ratio of aggregated exposure to the ADI (or the sum of EDI/ADI for mixtures in the component-based approach) should be less than the HI of one. However, in our study we aimed to evaluate the risk through fish consumption and not all food. For this purpose, we proceeded to the refinement of the HI based on an appropriate correction factor (F_C), determining the corrected fish specific hazard index HI_f. The Fc expresses the contribution of fish to the total ΣPCB-6 daily intake and it is equal with the ratio of the maximum permitted daily intake through fish consumption, $MPDI_f$ (fish consumption * permitted occurrence in fish) to the maximum permitted daily intake through the whole diet, $MPDI_A$ (SUM of MPDIi = SUM ($food_i$ consumption * permitted occurrence in the $food_i$),where *i* represents each food group considered for the dietary intake).

$$MPDI_A = \sum MPDIi$$

$$F_C = \frac{MPDIf}{MPDI_A}$$

For the calculation of PMDI*i* in ng/kg bw per day for each relevant food group (*i*) we used the current EU Maximum levels (European Commission, 2011) and the consumption data from the aforementioned databases. The food groups we considered were: cheese, eggs,

demersal fish (FAOSTAT), pelagic fish(FAOSTAT), fish (DAFNE-ANEMOS), meat and products, milk and products and total added lipids since they represent the food sources responsible for almost the total intake of ΣPCB-6 (WHO, 2016).

Since for the moment there is no ADI value for the ΣPCB-6 officially set by the regulatory bodies, we used as the existed proposed guidance value (ADIg) of 10 ng/kw bw per day reported in the literature (Baars *et al.*, 2001).

For considering no-risk it should be:

$$HIf > \frac{EDI_f}{ADI_g}$$

where $HI_f = Fc\ x\ HI$.

The results were also normalized to produce a different way of expression (Goumenou et al., 2019).

3. Results

3.1. Method validation

The developed method was validated in terms of linearity, accuracy, quantification limits, precision, recovery and carry over effect. The results are presented in Table 1. For quality assurance and quality control of the PCB quantification method, contamination was evaluated by blank controls and results were always below the detection limit. The accuracy was evaluated by analysis of spiked samples and recoveries for the analysed congeners ranged between 82.64 and 99.41%. The precision was calculated on replicate analysis giving an overall variability of 0.3- 14.9%. LLOQ for individual PCBs is 0.125 (ng/g). Spiked curves were linear with r^2>0.99 (Table 1). The detector's response to the analyte retention time in a blank sample was less than the response in the LLOQ sample and the effect on the retention time of the internal standard was lower than 5% of IS mean. Thus, no carry-over effect was observed.

Table 1. Analytical and validation parameters of the applied method.

			PCB 28	PCB 52	PCB 101	PCB 118	PCB 138	PCB 153	PCB 180
m/z			256, 186	292, 220	326, 256	326, 256	360, 290	360, 290	396, 324
Rt *(min)*			16.20	16.69	17.88	18.69	18.97	19.36	20.38
		C(ng/g)							
Inter-day precision (%RSD)	*LLOQ*	*0.125*	14.9	7.2	9.7	1.7	10.3	6.8	10.7
	QCL	*0.625*	3.2	5.9	4.2	6.9	1.6	0.9	0.6
	QCM	*1.25*	6.8	2.2	8.3	9.3	6.7	6.1	5.2
	QCH	*2.5*	1.4	1.3	1.8	1.7	0.6	0.3	1.7
		Mean (±SD)	6.6±6.0	4.1±2.8	6.0±3.6	4.9±3.8	4.8±4.5	3.5±3.4	4.5±4.5
% Accuracy	*LLOQ*	*0.125*	90.0	86	116	116	116	112	112
	QCL	*0.625*	97.6	102.4	95.2	95.6	96.4	96	98.8
	QCM	*1.25*	98.2	101.0	102	100.4	97.8	99.4	96.2
	QCH	*2.5*	101.3	100.8	102.4	103	102.1	102.4	104.6
		Mean (±SD)	96. 8±4.8	97.5±7.7	103.9±8.7	103.7±8.7	103.1±8.9	102.4±6.9	102.9±7.0
% Recovery	*QCL*		99.4	96.3	89.2	82.6	84.9	84.1	87.4

** LLOQ: lower limit of quantification. QCL: quality control low level. QCM: quality control medium level. QCH: quality control high level*

3.2. PCB levels in fish muscle tissue.

PCB levels in fish muscle tissue were far below the maximum permissible limits set by the EU for both approaches used as it is presented in Table 2.

Table 2. Mean ± SD, median and range (ng/g) of ΣPCB-6 and ΣPCB-7 for farmed and wild fish for imputed and not imputed values.

			Mean ± SD	**Median**	**Range**
Imputed	**ΣPCB-6**	Farmed	1.96 ±1.55	1.68	0.11-7.17
		Wild	2.43±1.92	1.82	0.16 -6.85
	ΣPCB-7	Farmed	4.68±6.94	2.97	0.18-57.7
		Wild	3.14±2.29	2.84	0.41-7.93
Not imputed	**ΣPCB-6**	Farmed	1.95±1.54	1.68	0.13-7.09
		Wild	2.47±1.92	1.70	0.21-6.83
	ΣPCB-7	Farmed	4.73±7.02	2.96	0.13-57.6
		Wild	3.04±2.33	2.74	0.21-7.92

The % of detection for each PCB congener in farmed and wild fish samples is presented in Figure 1 for gilthead seabream (a) and seabass (b). Although the two species present a similar pattern of detection for farmed samples, they exhibit distinct differences for wild fish samples. More specifically, PCB 52 and PCB 180 are detected in wild gilthead seabream in 60% and 40% of the samples respectively and are absent in all of the wild seabass samples. On the other hand, PCB 138 and PCB 153 were more often detected in wild

fish for both species. PCB 138 was present in 100% of wild gilthead seabream and 90% of wild seabass samples, while PCB 153 in 90% of wild gilthead seabream and 80% of wild seabass samples.

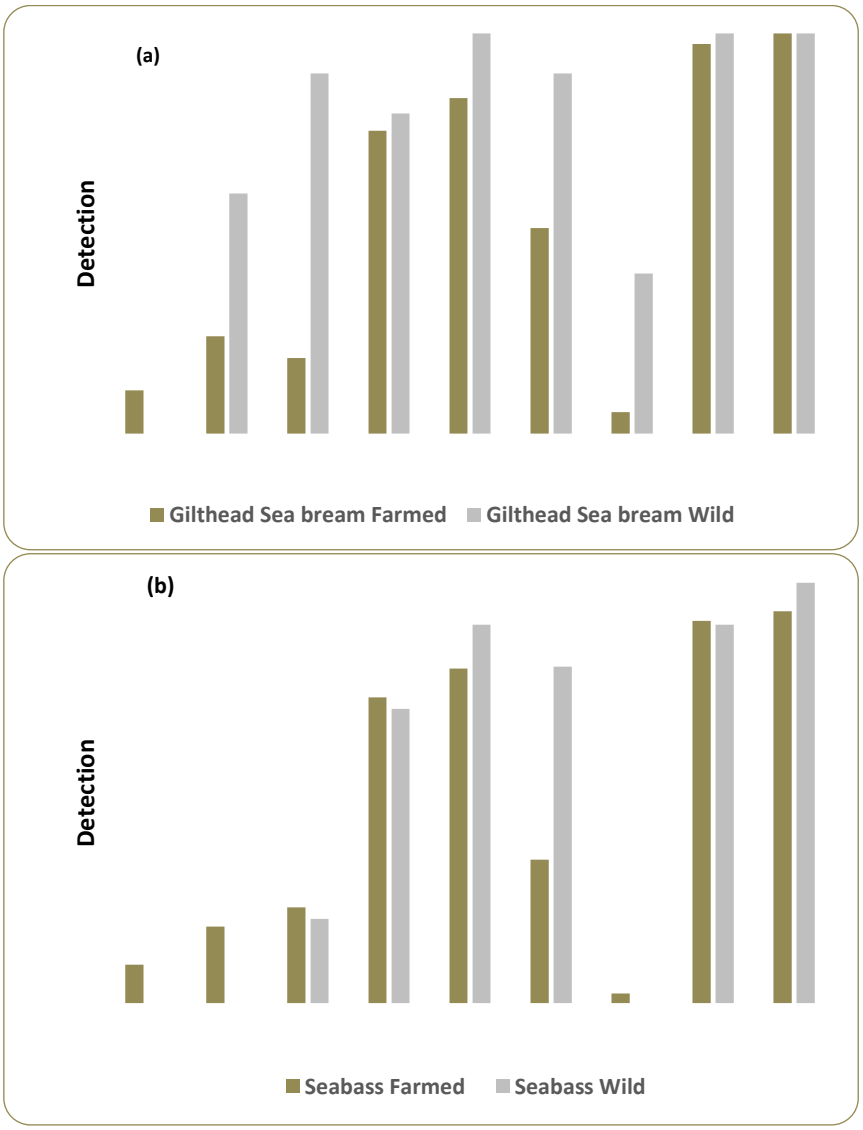

Figure 1. % Detection of each PCB congener for gilthead seabream (a) and seabass (b).

For all farmed fish in total, PCB 138 was the predominant congener, followed by PCB 118 and PCB 153. PCB 138 was the most abundant for wild fish as well followed by PCB 153 and then PCB 118.

Regarding gilthead seabream, when considering imputed values, statistical analysis revealed significant differences for PCB 101 ($p<0.001$), PCB153 ($p=0.015$) and PCB180 ($p=0.015$) between farmed and wild fish, with wild fish presenting higher mean values. Wild seabass showed significantly higher values for PCB 153 than farmed seabass ($p=0.014$). Moreover, mean detected values of PCB congeners for farmed gilthead seabream decreased in

the following order: 118>138>153>52>101>180>28 and for wild gilthead seabream in a different order: 138>153>118>101>52>180>28. Farmed seabass showed a similar pattern to farmed seabream: 118>138>153>52>101>28>180 and wild seabass one resembling more to wild seabream: 138>118>153>101>52>180>28.

3.3. Distribution of PCB levels in fish muscle tissue depending on seasonality.\

PCBs distribution was studied in relation to the sampling season for farmed gilthead seabream and seabass and the results are presented in Figure 2. In all cases PCB 118 seams to contribute the most to the ΣPCB-7 and the distribution of congeners seams to differentiate according to the sampling season. Further statistical analysis revealed that for gilthead seabream median PCB 28 is significantly higher during the summer season: 0.06 ng/g (August) ($p=0.036$) compared to the other periods. PCB 138 is significantly higher in November with a median value of 2.14 ng/g ($p=0.003$) while median PCB 153 in late winter-early spring: 0.65 ng/g ($p=0.002$). For seabass samples, PCB52, PCB 101 and PCB153 showed significantly higher median values in late winter-early spring: 0.06 ng/g ($p=0.016$), 0.33 ng/g ($p<0.001$) and 0.44 ng/g ($p<0.001$), respectively.

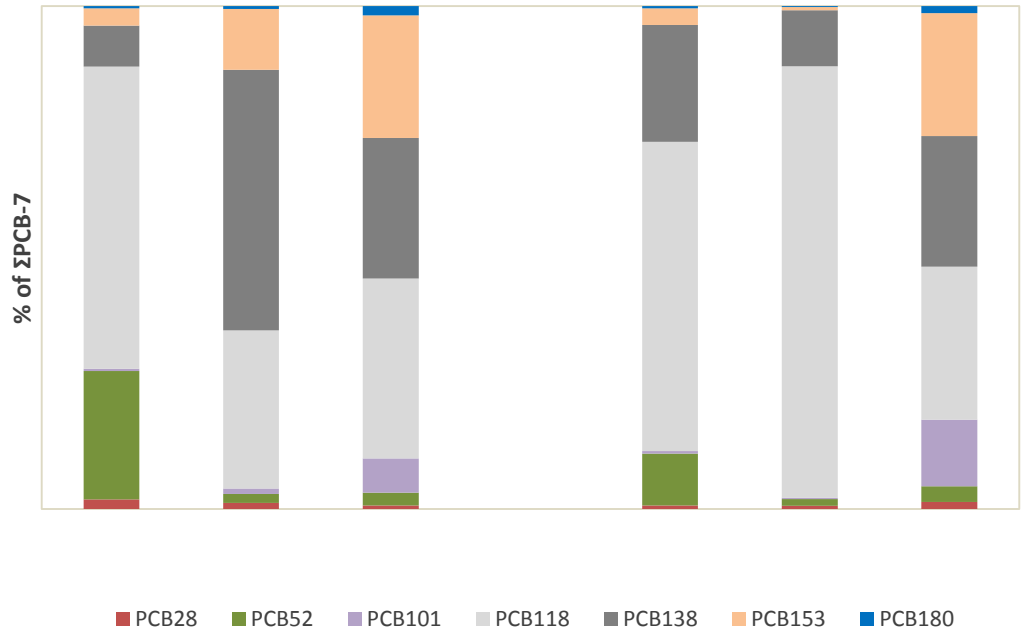

Figure 2. % contribution of each PCB congener to ΣPCB-7 in relation to sampling season for farmed gilthead seabream and farmed seabass.

3.4. Distribution of PCB levels in fish muscle tissue depending on collection site.

PCB distribution was additionally studied in relation to the collection site for farmed gilthead seabream and seabass and more specifically for fish collected from open seas (NE Aegean and Dodecanese) compared to fish obtained from closed seas (Crete, Mainland and Saronic Gulf). There is a clear variation between farmed seabass collected from open seas and seabass collected from closed seas and more specifically median PCB 118 is significantly higher in fish obtained from open seas (p=0.023), whereas PCB28 and PCB52 present significantly higher medians in fish collected from closed seas (p=0.021 and p=0.004 respectively).

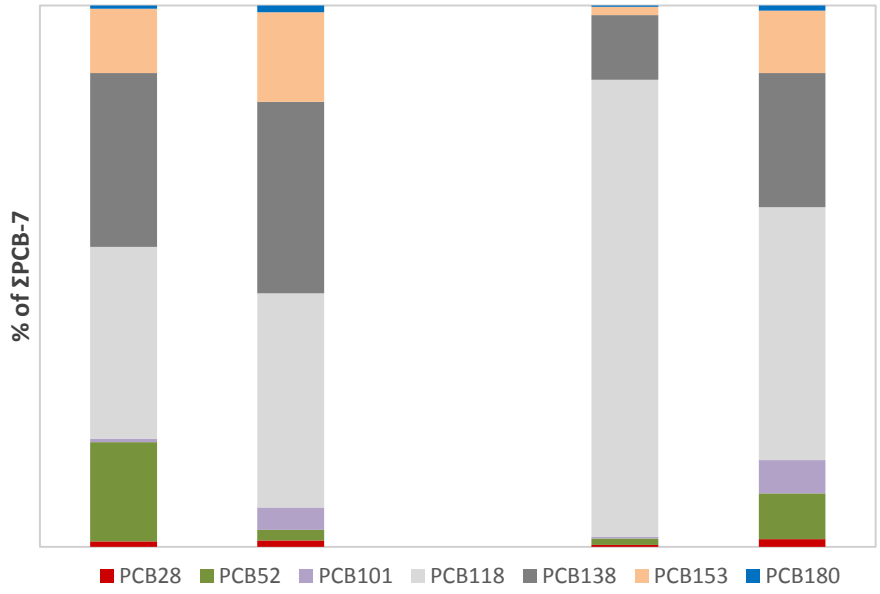

Figure 3. % contribution of each PCB congener to ΣPCB-7 in relation to sampling site (open or closed seas) for farmed gilthead seabream and farmed seabass.

3.5. Analysis of main effects of PCB levels in fish tissues

Multiple linear regression models using log-scaled PCB levels as dependent variables were applied using species, origin (farmed or wild) seasonality (from "warmer" periods to "colder" periods) and farming areas (closed/open seas). Species appears to be the main factor affecting both ΣPCB-6 and ΣPCB-7 levels (p=0.037 and p = 0.001 respectively) as can be seen in Table 3. With respect to factors affecting specific PCB congeners, statistical significance was demonstrated for PCB 153, PCB 28 and PCB 101. Specifically, for PCB 153

and PCB 101 primary factor proven to affect their levels was sampling season ($p<0.001$), while for PCB 28 farming site and sampling season as well ($p<0.05$).

Table 3. Adjusted Beta coefficient and 95 CIs using ΣPCB-6 and ΣPCB-7 as dependant variables.

		B	95%LB	95%UB	p	R^2
Log (ΣPCB-7)	Species	-0.18	-0.35	-0.01	**0.037**	0.09
	Farming site	-0.19	-0.41	0.03	0.097	
	Origin	-0.17	-0.48	0.14	0.278	
	Sampling Season	-0.06	-0.19	0.07	0.335	
Log (ΣPCB-6)	Species	-0.251	-0.402	-0.100	**0.001**	0.124
	Farming site	-0.081	-0.282	0.119	0.422	
	Origin	0.051	-0.225	0.328	0.714	
	Sampling Season	-0.023	-0.140	0.094	0.700	

3.6. Exposure assessment

Exposure assessment, through the EDI calculation, was based on the occurrence (contamination) of NDL-PCB in the Greek fish as determined in the current study, and fish consumption data in Greece from the FAOSTAT and DAFNE-ANEMOS databases (Table 4). The 95^{th} percentile of occurrence in our samples, determined in fish tissue, was 6.24 ng PCB/g fish. Exposure assessment in the 95^{th} percentile of occurrence revealed that the NDL-PCBs intake values for the Greek population through the consumption of demersal fish (EDI_f) range between 2.15 and 3.38 ng/kg bw/day.

Our estimations regarding fish consumption for the Greek population indicate that demersal fish, such as gilthead seabream and seabass, contribute to about 3-8% to the whole diet, while the exposure to NDL-PCB through fish consumption climbs to 43-72% of the total dietary intake, when considering as occurrence in these fish and the various dietary products (contamination) the maximum levels set by EFSA (2012).

Table 4. Consumption data for the NDL-PCBs related food and their respective Maximum Permitted Daily Intakes.

	CONSUMPTION (g food/person/day)		MPDI (ng contam/g bw/d)	
DATABASE	FAOSTAT	DAFNE-ANEMOS	FAOSTAT	DAFNE-ANEMOS
Food group				
Cheese	92.0	63	9.65	6.61
Eggs	24.8	17	1.57	1.08
Demersal Fish	**24.1**	**-**	**25.8***	
Pelagic fish	9.00	-	9.36	
Fish	**-**	**38**		**40.7***
Meat and products	371	164	7.42	3.28
Milk	219	162	5.01	3.70
Milk Products	-	37	-	1.40
Total Added lipids	2.42	0.88	1.38	0.50
SUM	**742**	**468**	**60.2****	**57.29****
% contribution of fish	3%	8%		

*MPDEf. ** $MPDE_A$

3.7. Risk characterization

According to the used approach the EDI for fish, the $MPDE_A$, MPDEf, Fc and HIf were calculated (Table 5). The ratio EDIf to ADI with data consumption from FAOSTAT and DAFNE-ANEMOS (0.22 and 0.34 respectively) are well below the respective HIf (0.43 and 0.72 respectively) indicating no-risk for the Greek population after exposure to NDL-PCB through fish consumption. Normalizing our results to HIf equal to 1 we ended up to the normalised EDIf/ADI ratios being 0.50 for FAOSTAT and 0.48 for DAFNE-ANEMOS (50% and 48% risk respectively).

Table 5. Hazard characterisation parameters

	FAOSTAT	DAFNE-ANEMOS
EDI_f (ng/kg bw/day)	2.15	3.38
ADI (ng/kg bw/day)	10.0	10.0
EDIf/ADI	**0.22**	**0.34**
MPDEf (ng/kg bw/day)	25.8	40.7
$MPDE_A$ (ng/kg bw/day)	60.2	57.3
F_C = HIf	**0.43**	**0.72**
Risk %	**50%**	**48%**

$MPDE_A$: Aggregated Maximum Permitted exposure, MPDEf : Maximum Permitted Exposure from fish, HIf: Hazard Index for fish

4. Discussion

4.1. PCB levels in fish muscle tissue of farmed and wild fish.

With regard to PCB congener distribution in fish, results obtained from this study, demonstrate that for farmed fish in total, PCB 138 was the predominant congener followed by PCB 118 and PCB 153, whereas PCB 138 was the most abundant in wild fish as well followed by PCB 153 and then PCB 118. This is in accordance with results reported in the relevant literature where PCB 138 and PCB 153 are described as the most common and at higher levels detected congeners(Antunes and Gil, 2004; Baptista, Pato, Pereira, *et al.*, 2013) In several cases, PCB 153 has been reported as the predominant congener, followed by PCB 138 (Giandomenico et al., 2016; Trocino, Majolini, and Xiccato 2009; Vuković et al., 2018). Nevertheless, both congeners (PCB 138 and PCB 153) appear to be the most frequently detected due to their high persistence and stability which are attributed to their chemical structure as di-ortho substituted and highly chlorinated congeners. Additionally, these congeners are amongst the major components of commercial PCB mixtures (Rodríguez-Hernández *et al.*, 2017). Their resistance to degradation and lipophilicity leads to high levels of accumulation in fish tissue.

In our study, although the two species present a similar pattern of detection for farmed samples, they exhibit distinct differences for wild fish samples. Besides the fact that wild fish present higher mean values for the sum of NDL-PCBs (ΣPCB-6) (2.43 ng/g) than farmed (1.96 ng/g), differences have been depicted in congener distribution as well. More specifically, for gilthead seabream PCB 153, 101 and 180 differed significantly between farmed and wild samples, while for seabass a difference was disclosed for PCB 153. On the other hand, for the sum of all PCBs analysed (ΣPCB-7) values were higher for farmed fish, suggesting that PCB 118 presence is higher in farmed fish (2.73 ng/g) compared to wild ones (0.71 ng/g). Similar differences between farmed and wild fish have been illustrated by several authors. Serrano et al. (2008) reported higher PCB levels in wild gilthead seabream in relation to its farmed counterpart and such variations were published in other studies as well (Ferreira *et al.*, 2010). Conversely, seabass collected from natural environment presented lower PCB levels than cultivated seabass according to Antunes and Gil (2004). Several other authors determined higher values in farmed fish (Carubelli *et al.*, 2007; Henríquez-Hernández, Montero, Camacho, Ginés, Luis D. Boada, *et al.*, 2017; Lundebye *et al.*, 2017) and it has generally evolved into a debate on account of conflicting results. Most authors ascribe

differences in PCB levels between farmed and wild fish to variations in lipid content, suggesting that higher lipid content in farmed fish justifies for higher PCB levels (Antunes and Gil, 2004; Trocino, Majolini and Xiccato, 2009). Moreover, many authors associate feed composition administered to farmed fish with differences in PCB levels and PCB distribution between farmed and wild fish (Carubelli *et al.*, 2007; Serrano, Blanes and López, 2008; Çakiroğullari, Kiliç and Uçar, 2010; Cirillo *et al.*, 2014; Henríquez-Hernández, Montero, Camacho, Ginés, Luis D Boada, *et al.*, 2017; Ginés *et al.*, 2018). It has also been suggested that variations can be attributed to biological effects as fish growth and metabolic activities of fish, especially for various stages of fish farming (Ferreira *et al.*, 2008). However, we tried to eliminate this factor in our study by selecting fish samples of commercial size in all cases. Variations between farmed and wild fish in our study could be explained by diversified types of diet exposure due to aquafeeds.

Our results on PCB levels determined in fish muscle tissue, for both species and mode of production were far below the maximum permissible limits set by the EU (2011). This is in accordance with levels reported for these species in the Mediterranean, as it is presented in Table 6.

4.2. Factors affecting PCB levels and congener distribution in fish tissues

Our results highlight seasonality as an important factor affecting PCB accumulation and distribution in fish muscle tissue. ΣPCB-6 for farmed gilthead seabream is higher in autumn; while for farmed seabass higher mean ΣPCB-6 value is recorded in winter-early spring (Table 5). In both cases, seasons presenting the highest values coincide with the beginning of the reproduction period of each species. This association could be explained by higher lipid content before reproduction season, due to increased food intake, as it is discussed by other authors as well (Serrano, Blanes and López, 2008; Blanes, Serrano and López, 2009). Seasonal alterations in PCBs levels on that context are also species dependent, as PCB accumulation is dictated by each species ecology and biological cycle (Baptista, Pato, Tavares, *et al.*, 2013; Henríquez-Hernández, Montero, Camacho, Ginés, Luis D Boada, *et al.*, 2017). Moreover, different levels between these species were presented by a recent study in Greece, where higher values were observed in sea bream in comparison to seabass, a fact which was attributed to gilthead seabream's higher lipid content(Costopoulou, Vassiliadou and Leondiadis, 2016b). In our study, the species effect was further underlined by the multiple linear regression models, which revealed that both ΣPCB-6 and ΣPCB-7 levels are primarily affected by the fish species.

Moreover, our results regarding ΣPCB-7 reveal a differentiated distribution of PCB congeners in relation to sampling season for each species, which is significant for PCB 28, 138 and 153 for gilthead seabream, counter to PCB 52, 101 and 153 for seabass. The predominant congener detected in our study (PCB 138) follows the concentration fluctuation dictated by the reproduction hypothesis, whereas PCB 153, one the most persistent congeners is significantly higher for both species in winter-early spring. This is hard to interpret, yet a possible explanation could be the influx of contaminant loads into the marine environment in early spring, due to the water cycle. Nevertheless, regression analysis revealed the sampling season to be the main factor affecting PCB 153 levels, in accordance with results reported by Serrano, Barreda, and Blanes 2008.

As regards to the collection site effect, interestingly, PCB 118 presented significantly higher values in fish collected from open seas, while PCB 28 and PCB 52 present significantly higher values in fish collected from closed seas. Furthermore, PCB 28 appeared to be mainly influenced by farming site, besides sampling season, in the regression analysis models carried out in our study. PCB congener distribution and levels are area dependent according to other authors as well, who report that location of fish growth and collection is of great importance to PCBs accumulated in fish tissues (Baptista, Pato, Pereira, *et al.*, 2013; Paiano *et al.*, 2013; Henríquez-Hernández, Montero, Camacho, Ginés, Luis D Boada, *et al.*, 2017). Additionally, PCB 28, PCB 118, PCB 52 and 101 are considered as indicators of recent contamination (Arnich *et al.*, 2009; Giandomenico *et al.*, 2016). Therefore, distinctions in PCB congeners between open and closed seas could be attributed to different types and sources of contamination.

4.3. Exposure assessment

Exposure assessment for the Greek population showed that NDL-PCB intake through fish consumption (2.15 - 3.38 ng/kg bw/day) is comparable to other European countries, reaching about 50% of the total dietary intake (EFSA 2012; WHO 2016). A WHO report recently published (WHO 2016) reviewing the relevant literature, describes a range of whole dietary exposure between 8 and 45 ng/kg bw/ day.

In relation to the aggregated dietary exposure to NDL-PCBs for the general population or subgroups a number of studies have been published in the literature (Arnich *et al.*, 2009; Cirillo *et al.*, 2009; Cimenci *et al.*, 2013; Mihats *et al.*, 2015; Perelló *et al.*, 2015; Costopoulou, Vassiliadou and Leondiadis, 2016b; Giandomenico *et al.*, 2016; Rodríguez-Hernández *et al.*, 2016) .

As previously mentioned the fish consumption data obtained from the two different databases led to slightly divergent exposure results (2.15 and 3.38 ng/kw bw/day), underlying the importance of dietary habits to exposure. Indicative to the importance of consumption data use, is additionally the fact that although the contamination levels in fish determined in our study represents approximately the 8% of the maximum established NDL-PCB maximum permitted limits, and thus they may considered safe, this was translated in approximately 50% of the fish contribution in the overall intake from the NDL-PCBs in the Greek population. Moreover, a recent biomonitoring study in Greece, assessing PCB occurrence and levels in the hair of two Greek population groups (residents of different agricultural regions) revealed significant differences in PCB levels between the two groups, as well as differences in congener distribution which could be a reflection of divergent dietary habits among other reasons (Barbounis *et al.*, 2012). Similar differences regarding PCB prevalence and congener distribution and were found between hair of children from rural and urban regions(Tzatzarakis *et al.*, 2014)

Comparison with other studies on exposure assessment and risk characterization becomes complicated for reasons which include the different approaches used. However, based on our results NDL-PCB intake for the Greek population through consumption of fish is estimated at 150-237 ng/person/day, higher the intake of the Spanish population (87 ng/person/day) recently reported (Rodríguez-Hernández *et al.*, 2016) for the same group of fish.

4.3. Risk characterisation

Risk characterization for NDL-PCB is challenging on account of the fact that there are no established values for acceptable daily intake due to lack of determined NOAELs. Although, recent studies on PCB effects on hepatotoxicity through oxidative stress induction, support the use of benchmark dose (BMD) concept in the prediction of health risks associated with PCBs exposure, instead of the NOAEL approach(Buha *et al.*, 2015). Moreover, a comparative approach using the minimum effect doses from available studies was developed in order to estimate margin of exposure MOEs and to provide guidance on human health risk (WHO, 2016), however, this approach has not been conclusive or finalized.

In our study we estimated the NDL-PCBs intake of the Greek population from fish consumption only, aiming to assess the weighted risk arising from fish consumption. Up to date, the classical HI approach (HI=1) has been used extensively in risk assessment studies dealing with exposure from single food items (Tsakiris *et al.*, 2013, 2015, Renieri *et al.*, 2014, 2019). With a view to refine current risk assessment methods, we chose instead of using the classical HI to use a newly developed food specific HI approach. Based on this approach fish

contribution to the maximum permitted aggregated dietary exposure was considered, arriving to a lower value for the HI.

It must be noted that however realistic we aspire to be in risk assessment, true risk is difficult to identify for numerous reasons and existing methods assessing the risk from PCB intake contain a rather ample amount of uncertainty. Besides the lack of ADI values, dietary exposure falls in the context of long term-low dose exposure, whose adverse effects have not been yet characterized. Moreover, human behaviour including dietary habits varies greatly based on various criteria such as locality and socioeconomic status. Furthermore when considering specific single chemicals or assessment groups, such as NDL-PCBs in our case, we can determine just a part of the overall risk and cannot provide an integrated assessment of the multiple risks triggered by exposure to different toxic stimuli (Tsatsakis, Docea and Tsitsimpikou, 2016; Hernández and Tsatsakis, 2017; Tsatsakis *et al.*, 2017, 2018; Docea *et al.*, 2018; Kostoff, Goumenou and Tsatsakis, 2018)

Finally, we have to accept certain limitations to our study such as the fact that we considered only adult consumers, not taking into account children or sensitive groups due to lack of available data. Additionally, we estimated the NDL-PCB contamination in two fish species. Regardless, these are the most frequently consumed fish by the Greek population and the ones more likely to contribute to the PCB intake. Although we applied a new risk characterisation approach, further refinement is possible, with more targeted and updated consumption data which are currently lacking and contamination data in more fish species as well. Even though we covered an appreciable range of areas of the Greek seas that we collected fish from, more sites could be represented in the future. Our study provides data on the contamination of frequently consumed fish in Greece and further elucidates the risks involved in fish consumption, regarding the NDL-PCB intake which could contribute to the efforts of researchers, health advisories and regulative authorities to balancing health benefits and risks of fish consumption.

5. Conclusions

The levels of the sum of indicator PCBs and congener distribution determined in fish muscle tissue were found to vary depending on fish species, seasonality, sampling area and mode of production. In most cases differences were attributed to variations in lipid content, fish biology and PCB occurrence in aquafeeds. Results obtained from this study show that PCB levels in gilthead seabream and seabass were well below the established maximum permitted limits in fish tissue and risk assessment for the NDL-PCBs revealed no risk for Greek consumers. It is important that in the future we work with a view of improving the dissemination of consumption and contamination data in order to build readily accessible

databases which could assist the advancement of more integrated risk assessment methods for aggregated and cumulative exposures.

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Table 6. Sum of concentrations of indicator PCBs concentrations (ΣPCB-6, ΣPCB-7) in the muscle tissues (expressed as means and range ng/g ww) of gilthead seabream and seabass in the Mediterranean Sea, reported in the recent literature.

Species	Season	ΣPCB-6	ΣPCB-7	Origin	REGION	REFERENCE
gilthead seabream		8.02		farmed	Greece	Costopoulou, Vassiliadou and Leondiadis, 2016 [b]
	May	22.1		farmed (1)	Turkey	Çakiroğullari, Kiliç and Uçar, 2010
	September	3.94		farmed (2)		
	March	14.9		farmed (4)		
	winter		3.7	farmed red muscle	Spain	Serrano, Barreda and Blanes, 2008
	automn		34	farmed red muscle		
	automn		2.4	farmed white muscle	Spain	Serrano, Barreda and Blanes, 2008
	winter		<LoQ	farmed white muscle	Spain	Serrano, Barreda and Blanes, 2008
	automn		2.2	farmed white muscle		
	summer	**1.71**	**4.3**	farmed	Greece	This study
	autumn	**2.54**	**3.71**	farmed		
	winter	**2.31**	**3.6**	farmed		
	winter	**3.35**	**3.14**	wild		
	winter		11	wild red muscle	Spain	Serrano, Barreda and Blanes, 2008
	winter		<LOQ	wild white muscle		
	automn		23	wild red muscle		
	automn		<LOQ	wild white muscle		
	automn		0.15	wild white muscle		
Seabass		5.24		farmed	Greece	Costopoulou, Vassiliadou and Leondiadis, 2016 [b]
	October to January		2.2	farmed (extensive)	Italy	Trocino et al., 2009
	May	8.01		farmed (1)	Turkey	Çakiroğullari, Kiliç, and Uçar 2010
	September	8.29		farmed (2)		
	October	3.05		farmed (3)		
	March	9		farmed (4)		
		7.02± 2.79		farmed	Italy	Carubelli et al., 2007
	October to January		12.4	farmed (intensive concrete tanks)	Italy	Trocino et al., 2009
	October to January		8.8	farmed (Sea-cages)		

October to January	10.6		farmed (Semi-intensive ponds)		
	3.69± 2.38		farmed (1)	Italy	Paiano et al., 2013
	10.73± 8.27		farmed (2)		
summer	**1.67**	**4.3**	farmed	Greece	This study
automn	**1.36**	**9.5**	farmed		
winter	**1.94**	**2.78**	farmed		
winter	**1.31**	**1.96**	wild		
spring and autumn		192 ± 159	wild	France	Bodin et al., 2014 [c]
automn		242 ± 169	wild		
spring	122 ± 136		wild		
June-Jyuly	3.85± 2.35		wild	Italy	Carubelli et al.,2007

[a] mean, [b] upperbound,[c] dry weigh

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CHAPTER 5

General Discussion and Conclusions

1. Nonlinear responses to waterborne cadmium exposure in zebrafish. An in vivo study

The *in vivo* study was planned with aim to investigate zebrafish responses to a range of Cd exposure levels, spanning from environmental to toxic. The main interest of the study was to research potential variations of toxic responses, concerning primarily metal accumulation and mortality, across the range of Cd exposures. Further histopathological analysis was conducted, in an attempt to correlate Cd effects with mortality.

The results of the study show that zebrafish exposed to this range of Cd concentrations, manifest deviations from the anticipated linear or monotonic toxic responses. Documented responses regarding mortality rate were non-linear, supporting the increasingly gaining ground hypothesis of non-monotonic and not linear responses to gradient exposures to toxic stimuli. It is progressively becoming evident in the field of toxicology, that dose response curves do not apply accordingly to low and high doses for various effects. Although some adversities exhibit linearity according to dosage, there are effects that deviate from that norm. The fact that fish exposed to the most toxic Cd level survived more than fish exposed to less toxic and almost as long as fish exposed to environmental levels is very interesting in terms of detoxification pathways and acclimation responses. Although Cd accumulation in their tissues was the highest and was also dose dependant, fish not only survived, but did not exhibit severe adversities regarding histopathology either. Meanwhile, there was a surprisingly high mortality rate at the second of the six exposure levels, which draws the attention to that specific Cd concentration. This level could hold a key to the elucidation of the toxicity coping mechanisms, since modifications of these mechanisms beyond this level are implied by our results. Moreover, histopathological findings were more severe at the fourth exposure level, adding to the deviating responses along with the fact that fish exposed to the first level, corresponding to environmental, demonstrated histopathological effects. It was also derived from our findings that accumulation was not time dependant.

These observations illustrate a constant struggle of defence mechanisms against Cd toxicity, which apparently diverge in effectiveness according to the exposure level. It could moreover be suggested that there are milestone levels, which lead to the modification of defence pathways and deserve further investigation. This could also be a manifestation of hormesis phenomena where mechanisms stimulated at low doses, inhibit toxic effects at high

doses. Another hypothesis is that at very high exposures, following an initial metal shock phase, there are key exposure points, which trigger pathways that modify metal uptake, allowing metal accumulation to be gradual and consequently allowing for acclimation responses.

In this study we focused on the toxicity of specific toxic stimuli, which is very important for toxicity characterization. However, in the grand scheme of things, this is not realistic, since all natural exposure occurs in the form of mixtures. Of course characterization of individual substances is a prerequisite for investigating their behavior in mixtures. At this point, the issue of inconsistent LC_{50} and the respective no-observed-adverse-effect-levels (NOAEL) throughout the literature should be mentioned as well. Identification of specific NOAELs may need retrospect and reconsideration, keeping in mind the nonlinearity of certain effects and the fact that long term low dose exposures could result in unanticipated adversities.

2. Cadmium, lead and mercury in muscle tissue of gilthead seabream and seabass: Risk evaluation for consumers.

The aims of this study included the evaluation of the heavy metal load in the edible part of frequently consumed fish and the investigation of the main factors affecting the heavy metal accumulation in the fish muscle tissue. Moreover, risk assessment for the Greek population from fish consumption was conducted based on the determined heavy metal concentrations.

Based on results obtained from our study, heavy metal levels in the frequently consumed fish, were determined at levels far below the safe limits for consumption set by authorities, for each metal individually as well as for their sum. The investigation of the factors affecting metal accumulation, namely origin of production, seasonality, locality and species revealed some interesting results. The two species analyzed demonstrated significant differences in Hg and Cd levels which can be attributed to a number of reasons such as differences in intrinsic factors (body composition, lipid content and reproduction cycle) between species, different metal behavior, different aquafeed metal load or a combination of all the above. With regard to locality, Hg and Pb seem to be more accumulated in closed seas which can be difficult to read since each metal displays diverse distribution in tissues, however it could imply that these metals have a similar distribution pattern in the medium of exposure (water), or that they share the same origin of dispersion, possibly waste disposals from human activity. Metal levels were clearly affected by seasonality as was illustrated by our results. Lead accumulation displayed a linear increasing trend from warmer to colder

periods whereas mercury accumulation displays a low point in autumn. Furthermore, season dependent variations disclose a species effect as well, since significant differences in metal accumulation amongst seasons were recorded between species. With regard to origin of production this study provides evidence of differences in metal levels between fish from the two modes of production which can be the combined result of different feeding behavior, growth rate and therefore metabolic rate, aside from the effects of waterborne exposure.

The risk evaluation we conducted for Greek consumers based on the metal levels we determined in the most frequently consumed fish, both farmed and wild indicates minimal risk for all metals. However, it is important to acknowledge that the risk was assessed only for metal intake through fish consumption, not considering dietary intakes from other foodstuff. Although seafood is the main contributor to heavy metal dietary intake, it does not account for the cumulative exposure. It must also be underlined that current established limits on metal exposure have been set talking into consideration the single-stimulus exposure f.i. cadmium, lead or mercury alone, whereas this is not realistic, since exposure to a combination of stressors usually occurs in real life scenarios. To elaborate, co exposure to multiple metals through consumption could lead to increased susceptibility to toxic effects or alleviation of them, or could result in impairment of the capacity to compensate for exposure to additional stressors. Moreover dietary intake of metals, is a case of long term-low dose exposure and metal effect on humans through this kind of exposure have not yet been characterized. An additional limitation to risk characterization as it is carried out today, is that consumption data are difficult to utilize properly, since consumption habits vary in many levels even within same groups, f.i. different preferences are exhibited based on criteria such as socioeconomic for adult consumers. Databases on consumption of the Greek population are lacking and it is essential that they are updated promptly.

This study provided monitoring data on metal load in fish that could be used in further ecotoxicity studies in addition to human intake evaluations. The principal factors affecting Cd, Hg and Pb accumulation in fish were also described and risk assessment with current means and methods for the Greek population was conducted, filling the gap of the scarce relevant literature in Greece.

3. Indicator PCBs in farmed and wild fish in Greece - Risk assessment for the Greek population

Through this study, the occurrence and burden of the indicator PCBs (ΣPCB-7) in frequently consumed fish in Greece was determined, via a developed and evaluated GC-MS method. Additionally, the association of the ΣPCB-7 accumulation in fish to seasonality, locality, production mode and species was investigated. Furthermore, a more elaborate risk assessment method, than those previously used, was developed for the characterization of the hazard of PCB intake through fish consumption, for the Greek population.

Results regarding the occurrence and levels of the ΣPCB-7 in the muscle tissue of farmed and wild gilthead seabream and seabass, reveal that for both species and mode of production levels were far below the maximum permissible limits set by the EU (2011). Moreover, ΣPCB-7 congener distribution as was determined in this study, depicted that more highly chlorinated congeners such as PCB 138 and PCB 153 were more abundant and more often detected in fish tissue, most probably due to higher resistance to degradation and lipophilicity. This is evolving to a common assumption throughout the literature with regard to fatty tissues.

The investigation of PCB levels and congener distribution in muscle tissue of the aforementioned species, in relation to particular biotic and abiotic parameters disclosed certain associations. With regard to mode of production, wild fish presented higher levels of the ΣPCB-6, while farmed fish accumulated PCB 118 at higher levels. This could be linked to differences in mode of action between NDL and DL PCBs yet it is very hard to pin down since different accumulation patterns between farmed and wild fish could be a result of a combination of factors namely variations in lipid content, feed composition administered to farmed fish as well as habitat.

Moreover both ΣPCB-6 and ΣPCB-7 levels are primarily affected by the fish species which is in close relationship to the fact that our results highlighted seasonality, as an important factor affecting PCB accumulation and distribution in fish muscle tissue, as well. The association becomes apparent when considering that, levels and distribution vary between species and seasons presenting the highest values coincide with the beginning of the reproduction period of each species. Seasonal alterations in PCBs levels seem to be dictated by each species ecology and biological cycle. In addition to the above, distinctions in PCB congeners between open and closed seas were also demonstrated, which could be attributed to different types and sources of contamination.

Finally, we estimated the NDL-PCBs intake of the Greek population from fish consumption only, aiming to assess the weighted risk arising from fish consumption. Although the contamination levels in fish determined in our study represents approximately the 8% of the maximum established permitted limits for ΣPCB-6, and thus may be considered safe, this was translated in approximately 50% of the fish contribution in the overall dietary intake of the NDL-PCBs for the Greek population. The exposure assessment showed that NDL-PCB intake through fish consumption is comparable to other European countries. However, the use of consumption data from the two different sources resulted in slightly divergent exposure results underlying the importance of dietary habits to exposure.

Based on gained experience in risk assessment from the previous study on heavy metal exposure and acknowledging the fact that risk characterization methods need refinement, a new food specific HI approach was developed. For this approach, fish contribution to the maximum permitted aggregated dietary exposure was considered, arriving to a lower value for the HI. Risk characterization revealed no risk for Greek consumers. This new approach is a step towards more realistic risk characterization. However, there is a lot more ground to cover in terms of setting ADIs, determine long term-low dose dietary exposure effects, update and disseminate consumption and contamination data and ultimately, developing methods for more integrated assessment of the multiple risks arising from exposure to multiple different stressors under real-life scenarios.

This study extends the narrow current knowledge on the contamination of frequently consumed fish in Greece and at the same time illustrates biotic and abiotic factors affecting PCB accumulation in fish. Finally, this study advances current risk assessment methods with the proposed use of a weighted hazard index when assessing exposure from single food items.

Conclusions

- Zebrafish exposed to a range of Cd concentrations, spanning from environmental to toxic accumulate Cd in their tissues in a dose dependant manner but not time dependant.
- Mortality rate responses were non-linear, supporting the increasingly gaining ground hypothesis of non-monotonic and not linear responses to gradient exposures to toxic stimuli.
- Histopathological findings also deviate from anticipated dose dependant responses, revealing more severe effects in lower exposures and adverse effects occurring even at environmental levels.
- There are is low exposure level with surprisingly high mortality rate which draws the attention and this level could hold a key to the elucidation of the toxicity coping

mechanisms, since modifications of these mechanisms beyond this level are implied by our results.

- Heavy metal levels in the frequently consumed fish by the Greek population were determined at levels far below the safe limits for consumption set by authorities, for each metal individually as well as for their sum.
- Gilthead seabream and seabasss demonstrated significant differences in Hg and Cd levels which can be attributed to a number of reasons such as differences in intrinsic factors between species, different metal behavior, different aquafeed metal load or a combination of all the above.
- Hg and Pb seem to be more accumulated in closed seas which could imply that these metals have a similar distribution pattern in the medium of exposure (water), or that they share the same origin of dispersion, possibly waste disposals from human activity.
- Metal levels were clearly affected by seasonality and season dependent variations disclose a species effect as well, since significant differences in metal accumulation amongst seasons were recorded between species.
- Differences in metal levels between farmed and wild were demonstrated which can be the combined result of different feeding behaviour, growth rate and therefore metabolic rate, aside from the effects of waterborne exposure.
- The risk evaluation we conducted for Greek consumers based on the metal levels we determined in the most frequently consumed fish, both farmed and wild indicates minimal risk for all metals.
- Occurrence and levels of the ΣPCB-7 in the muscle tissue of farmed and wild gilthead seabream and seabass, reveal that for both species and mode of production levels were far below the maximum permissible limits set by the EU (2011).
- More highly chlorinated congeners such as PCB 138 and PCB 153 were more abundant and more often detected in fish tissue, most probably due to higher resistance to degradation and lipophilicity.
- Wild fish presented higher levels of the ΣPCB-6, while farmed fish accumulated PCB 118 at higher levels
- Both ΣPCB-6 and ΣPCB-7 levels are primarily affected by the fish species which is in close relationship to the fact that our results highlighted seasonality, as an important factor affecting PCB accumulation and distribution in fish muscle tissue, as well. Seasonal alterations in PCBs levels seem to be dictated by each species ecology and biological cycle.
- Distinctions in PCB congeners between open and closed seas were also demonstrated, which could be attributed to different types and sources of contamination.

- The exposure assessment showed that NDL-PCB intake through fish consumption for the Greek adult population is comparable to other European countries.
- The use of consumption data from the two different sources resulted in slightly divergent exposure results underlying the importance of dietary habits to exposure.
- A new food specific HI approach was developed, for which fish contribution to the maximum permitted aggregated dietary exposure was considered, arriving to a lower value for the HI. Risk characterization revealed no risk for Greek consumers.

Future perspectives

Our results on Cd toxicity to zebrafish demonstrated nonlinear toxic responses and have shed light on deviations with regard to exposure level. Further investigation is required in order to elucidate counteraction of toxicity by detoxification mechanisms through wider ranges of metal exposures with focus on key concentrations which alter the toxic response profile.

Future *in vivo* studies investigating exposure to mixtures of contaminants are fundamental in order to evaluate synergistic or antagonistic effects and elucidate contaminant accumulation issues.

Moreover, long term low dose effects such as those that could be induced by fish consumption, could be the object of further investigation and risk analysis. Current established limits and guidance values on environmental contaminant exposure have been set talking into consideration the single-stimulus exposure which is not realistic. Biomonitoring and epidemiological studies on cumulative exposure remain essential in order to set more realistic exposure limits.

Finally, it's imperative to work towards the development of more comprehensive models for risk assessment and risk characterization regarding not only the dietary intake of contaminants but cumulative exposure as well.

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About the author

Elisavet Maria Renieri was born in Heraklion Crete on 20 February 1985. She completed secondary education at the 2nd Lyceum of Heraklion in Crete in 2002 and in the same year she started studying in National and Kapodistrian University of Athens, School of Science, Faculty of Biology. After completing her studies, in 2011 she became a member of Centre of Toxicology Science & Research (CTSAR) and was involved in a number of research projects. In 2013 she started her PhD at CTSAR and continued participating in local and international research projects, where she acquired knowledge on different analytical chemical and biological techniques as well as the ways of conducting *in vivo* and *in vitro* experiments. Elisavet has a strong interest in various fields of toxicology, involving the impact of environmental contaminants in public health and marine ecosystems as well.

List of Publications

1. Renieri, Elisavet A, Marina Goumenou, Dmitry A. Kardonsky, Valery V. Veselov, Athanasios K. Alegakis , Aleksandra Buha, Manolis N. Tzatzarakis, Alexander E. Nosyrev, Valerii N. Rakitskii, Maroudio Kentouri, Aristidis M. Tsatsakis 'Indicator PCBs in farmed and wild fish in Greece - Risk assessment for the Greek population',*Food and Chemical Toxicology* (2019) *in press*
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1. Fragkiadoulaki, I., C. Mamoulakis, A. Alegakis, M.N. Tzatzarakis, V. Karzi, A. Stratidakis, and others, 'Natural Antioxidants Prevent Contrast-Induced Nephropathy by Enhancing Nitric Oxide Synthesis in an Animal Model', *Toxicology Letters*, 295 (2018), S243 <https://doi.org/10.1016/J.TOXLET.2018.06.1007>
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