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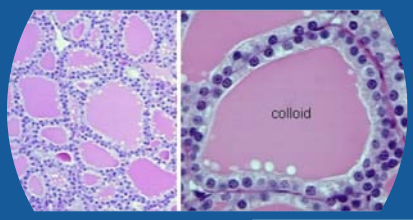

Approaches for assessing the presence and impact of thyroid hormone disrupting chemicals in sea bass (*Dicentrarchus labrax*) from European coasts

Doctoral dissertation by

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Approaches for assessing the presence and impact of thyroid hormone disrupting chemicals in sea bass (*Dicentrarchus labrax*) from European coasts

by

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"What is research but a blind date with knowledge?"

— Will Harvey

Silicon Valley entrepreneur

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List of abbreviations

Cd	cadmium
CEMAGREF	*Institut de recherche pour l'ingénierie, de l'agriculture et de l'environnement*
CF	Condition Factor
Cu	copper
DDT	p,p'-dichlorodi-phenyltrichloroethane
DHA	docosahexaenoic acid
DNA	Deoxyribose Nucleic Acid
DW	dry weight
EPA	eicosapentaenoic acid
FAO	Food and Agriculture Organization
FT_3	Free Triiodothyronine
FT_4	Free Thyroxine
GABA	gamma-aminobutyric acid
HCH	hexachlorocyclohexanes
Hg	mercury
HPT axis	hypothalamic-pituitary-thyroid axis
I_2	Iodine
ICES	International Council for the Exploitation of the Sea
ICPMS	*Inductively Coupled Plasma Mass Spectrometer*
IFREMER	*Institut français de recherche pour l'exploitation de la Mer*
INBO	*Institut voor Natuur- en Bosonderzoek*
IRD	Inner-Ring-Deiodinase
IUPAC	International Union of Pure and Applied Chemistry
LOD	Limit of Detection
LOQ	Limit of Quantification
LW	lipid weight
Mn	Manganese
MRL	Maximum Residue Limit
Ni	nickel
ORD	Outer-Ring-Deiodinase
Pb	lead
PCB	Polychlorinated biphenyl
RDA	Recommended Dietary Allowance
RNA	Ribo Nucleic Acid
rT_3	reverse T_3= 3,3',5'-triiodo-L-thyronine
Se	selenium
SGR	Specific Growth Rate
SULT	sulfotransferase
T_2	3,3'-diiodo-L-thyronine
T_3	triiodothyronine
T_4	Thyroxine
TRH	Thyrotropin-releasing-Hormone
TSH	Thyroid-Stimulating-Hormone
TT_3	Total Triiodothyronine
TT_4	Total Thyroxine
TTR	Transthyretin
UGT	UDP-glucuronosyltransferases
WHO	World Health Organization
WW	wet weigth
Zn	zinc

Chapter 1

General introduction

Accumulating evidence over the last two decades indicates that a wide range of anthropogenic chemicals have the ability to alter endocrine function in humans and wildlife. Endocrine disruptors are exogenous substances that interfere with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis (normal cell metabolism), reproduction, development, and/or behaviour (Damstra et al. 2002). In recent years, a number of man-made chemicals have been shown to be able to mimic endogenous hormones, and it has been hypothesized that alterations in the normal pattern of reproductive development seen in some populations of wildlife are linked with exposure to these chemicals. Of particular importance are those compounds that mimic thyroid hormones, because of their central role in growth and development (Boas et al. 2006).

Overview of the thyroid cascade

Thyroidal biosynthesis, secretion, and metabolism of L-thyroxin (T_4; central control)

The thyroid endocrine system is highly conserved throughout evolution and has been described in all vertebrate species studied so far (Brown *et al.* 2004a). The structures of thyroid hormones are the same in all taxa, as is the mechanism by which they are synthesized. The biosynthesis of thyroid hormones, the central regulation of thyroid gland activity, peripheral metabolic pathways of thyroid hormones and thyroid function, are very similar in fish, amphibians, reptiles, birds and mammals (Blanton and Specker 2007).

Like other endocrine glands, the thyroid gland is highly vascularised, mainly to support the secretion of thyroid hormones produced by the thyroid, as well as to support the supply of iodine and energy. The functional subunit of the thyroid system in all vertebrates is the follicle; which consists of epithelial cells called thyrocytes (Figure 1). Unlike mammals, the thyroid follicles of most fish do not form

a single organ, but are dispersed along the afferent artery; also ectopic thyroid tissue has been reported for several species (Raine 2005).

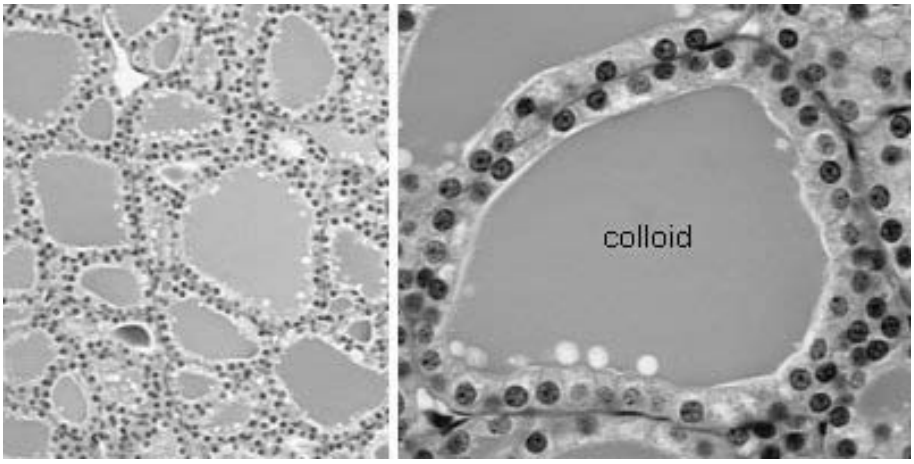

Figure 1 Histology of the thyroid gland
(Source: http://www.vivo.colostate.edu/hbooks/pathphys/endocrine/thyroid/anatomy.html)

The production of thyroid hormones is regulated by thyroid-stimulating-hormone (TSH) secreted by pituitary (Figure 2). A hypothalamic factor controlling TSH secretion is unknown in fish; thyrotropin-releasing-hormone (TRH), which stimulates TSH releasing in mammals and birds, is not effective in fish (Yamano 2005). TSH increases the uptake of iodide into thyroid cells, moving to the apical border where it's oxidized to its reactive form iodine (I_2) (Blanton and Specker 2007). Unlike higher vertebrates, the shortage of iodine in the diet is not a problem for fish because iodine is taken predominantly from ambient water through the gills (Yamano 2005).

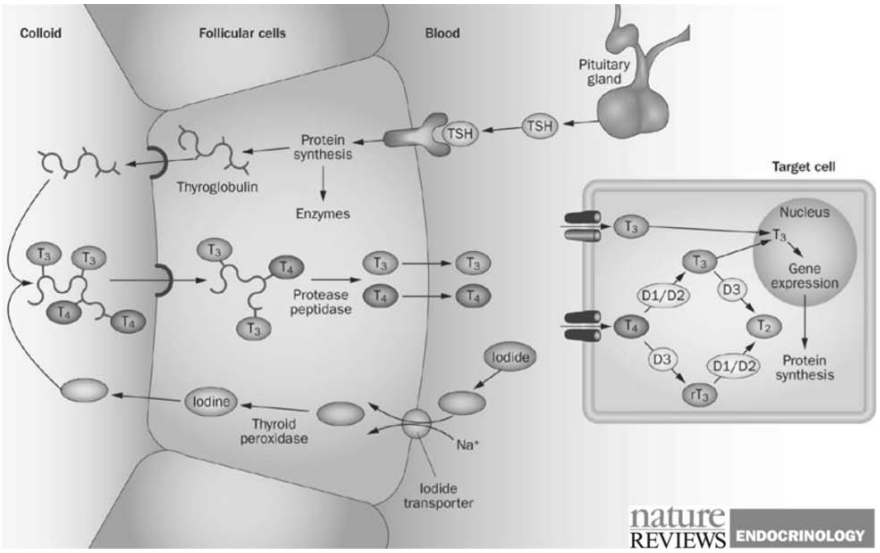

Figure 2 Synthesis of thyroid hormones. Thyroid hormones are synthesized in follicular cells of the thyroid gland from tyrosine residues within the thyroglobulin molecule. T_4 and T_3 molecules are then cleaved and released into the circulation. T_3, the physiologically active form of thyroid hormone, can also be formed from the deiodination of T_4. T_4 is converted to T_3 predominantly by type I iodothyronine deiodinase. Abbreviations: D1, type I iodothyronine deiodinase; D2, type II iodothyronine deiodinase; D3, type III iodothyronine deiodinase; rT_3, reverse T_3 (After Cohen-Lehman, J. et al. (2009) Nat. Rev. Endocrinol. doi:10.1038/nrendo.2009.225)

Concomitantly, amino acids are assembled into polypeptides on ribosomes of the endoplasmic reticulum, then transported to the Golgi complex where occurs the glycosylation (Bloom and Fawcett 1975). The obtained glycoprotein called thyroglobulin is transported in small vesicles to the apical surface of the cell where the iodination occurs. The iodinated tyrosyl residues within thyroglobulin form either monoiodotyrosyl or diiodotyrosyl residues, which pair and couple then covalently and either bound to form tetraiodothyronyl and triiodothyronyl residues, still incorporated into thyroglobulin and stored in the colloid (Banks 1986).

The mobilization of thyroglobulin is essential for the secretion of the thyroid hormones. Thyroglobulin must move back into the cell by endocytosis and the resulting cytoplasmic colloid droplets fuse with lysosomes to form a phagolysosome in which proteolysis of thyroglobulin release T_4, which then diffuses to the blood (Brown et al. 2004a).

Less than 1% of plasma total T_4 is free with 99% reversibly bound to plasma proteins. Plasma free T_4 has a strong negative feedback action on the brain-pituitary-thyroid axis and TSH secretion (Brown et al. 2004a). Free T_4 enters cells partly by simple diffusion and mainly by transport systems. Intracellular T_4 binds reversibly to cytoplasmic proteins and may be metabolized enzymatically by deiodination or by sulphate and glucuronide conjugation pathways. Deiodination mainly occurs in the endoplasmic reticulum of liver and other tissues. Iodine is removed by outer ring T_4 deiodination (T_4ORD) to form T_3 (a more active thyroid hormone) or by inner-ring T_4 deiodination (T_4IRD) to form 3,3′,5′-triiodo-L-thyronine (reverse T_3 = rT_3; an inactive thyroid hormone form) (Figure 3). The rT_3 is in turn degraded by a separate outer-ring deiodinase to 3,3′-diiodo-L-thyronine (3,3′-T_2). The T_4 conjugation mainly occurs in liver and the inactivated and more water-soluble conjugates are excreted in bile. Sulfation requires a cytoplasmic sulfuryltransferase and glucuronidation requires an endoplasmic-reticulum glucuronyltransferase. Enterohepatic recycling of the biliary-excreted T_4 or its conjugates is negligible.

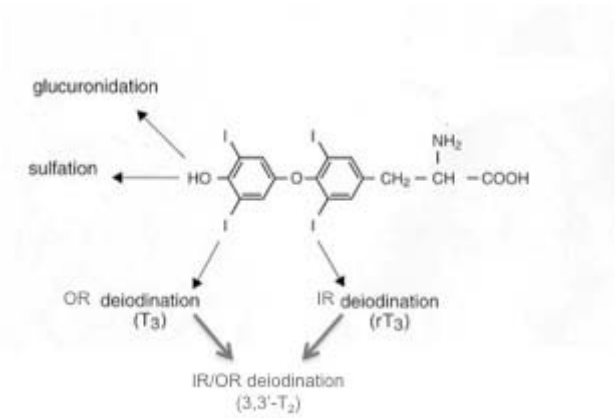

Figure 3 Pathways of Thyroid Hormone Metabolism (www.thyroidmanager.org)

T_3 production and metabolism (peripheral control)

Most T_3 is produced extrathyroidally, therefore the primary control of T_3 levels occurs in peripheral organs or tissues and may be limited to specific cell types. In liver, and some other peripheral tissues, T_3 is formed from T_4 by T_4ORD due to the activities of one or more deiodinases (Figure 4). The T_3 can then enter the plasma T_3 pool, a systemic source of T_3 for target tissues. However, T_3 formed in other (target) tissues may represent a T_3 source for their local needs. The T_3 itself is degraded by removal of one of its inner-ring iodine (T_3 inner-ring deiodination; T_3IRD) to form the presumed inactive 3,3′-T_2. The T_3IRD activity is relatively high in brain and retina. Thus, the balance between the activities of T_4ORD, T_4IRD, and T_3IRD pathways may regulate the amount of T_3 in plasma (systemic supply) or regionally in particular tissues (local supply).

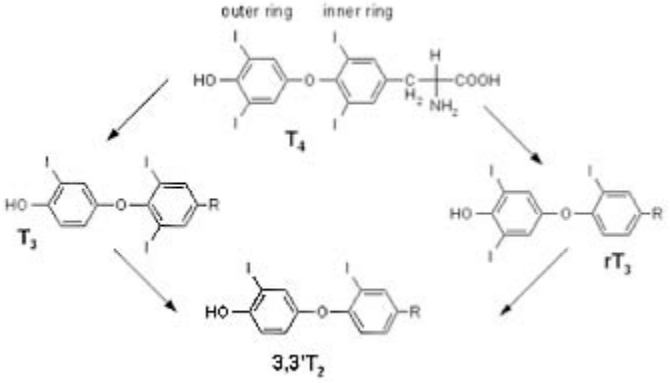

Figure 4 Deiodinase pathways Thyroxin (T_4) is deiodinated in the periphery to produce 3,5,3'-triiodothyronine (T_3) and 3,3',5'-triiodothyronine (reverse T_3) (http://flipper.diff.org/app/pathways/info/1238)

The proportion of total T_3 (TT_3) that is free in plasma (FT_3) is usually less than that for T_4. The T_3 binds to some plasma proteins that bind T_4 and FT_3 enters cells by simple diffusion or by active transport. Kinetic studies show that about 80% of the T_3 in salmonids may reside in a slowly exchanging reserve pool, mainly represented by skeletal muscle (Brown *et al.* 2004a). The T_3 conjugation mainly occurs in liver

and the products excreted in bile, recycling of the biliary-excreted T_3 or its conjugates are negligible.

Thyroid hormone actions in fish

The major actions of thyroid hormones in all vertebrates involve mainly bioactive T_3 binding to nuclear receptors, which then bind in pairs to adjacent paired thyroid hormones response elements, each consisting of a specific five base pair region of DNA (Figure 5). This whole complex acts as a transcription factor to regulate RNA transcription of a nearby gene. The resulting RNA is then edited to mRNA and translated on ribosomes to form proteins.

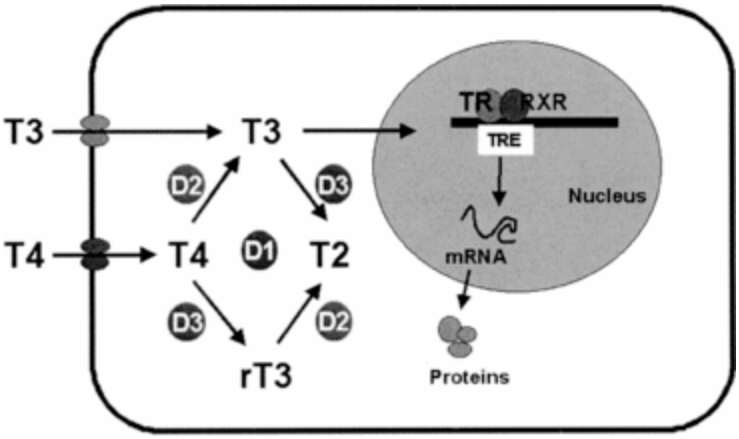

Figure 5: T3 bind to nuclear receptor and thyroid hormones response elements, acts as a transcription factor to regulate RNA transcription

Thyroid hormones have been found in eggs at similar or slightly higher levels than those in blood of adult fish (Tagawa et al. 1990b). The origin of thyroid hormones in eggs is apparently maternal. Thyroid hormones are considered to enter into eggs through the blood circulation, either by passive diffusion (Raine and Leatherland 2003), entry with vitellogenin (Monteverdi and Di Giulio 2000), or by involvement of other transport proteins (Tagawa and Brown 2001).

The presence of a considerable amount of thyroid hormones in eggs together with the evidence that the levels of thyroid hormones in eggs decrease during embryonic development, suggest its significant roles in fish embryogenesis (Tagawa and Hirano 1987; Leatherland et al. 1989). Experiments of thyroid hormone administration enhance in some cases survival and growth of the fish (Brown et al. 1988; Brown et al. 1989; Reddy and Lam 1992; Ayson and Lam 1993). These results support the meaningful role of thyroid hormones during early development in fish.

Small thyroid follicles appear first at the early stages of the larval period. Depending on species, approximately 3-4 weeks after fertilization, the developing embryos begin to produce their own thyroid hormones (Greenblatt et al. 1989). Then the follicles increase gradually in number and size and the thyroid hormone levels are detectable, though the concentration is relatively low (Tagawa et al. 1990a). Thyroid hormones have an effect on the larva-juvenile transition since the concomitant increase of thyroid hormone levels with metamorphosis or larva-juvenile development was remarked in conger eel (Yamano et al. 1991) and flounder (de Jesus and Hirano 1992; Schreiber and Specker 1998). The fish undergoing faster transformations seems to show clearer elevation of thyroid hormone levels.

The action of thyroid hormones on the metamorphosis was further verified experimentally: thyroid hormone administration to larvae induces precocious metamorphosis whereas anti-thyroid drugs caused developmental retardation in various fish (Tagawa et al. 1990a; de Jesus and Hirano 1992; Inui et al. 1995; Schreiber and Specker 1998). In addition to morphological changes in their external appearances, the development is accompanied by behavioural, functional and biochemical alterations. Thyroid hormones stimulates the transition of muscle proteins (Inui et al. 1995), skin pigmentation (de Jesus and Hirano 1992), development of gastric glands (Inui et al. 1995) and scale and fin formation (Reddy and Lam 1992; Brown 1997).

Thyroid disrupting chemicals

The thyroid system is a major target of the so-called endocrine disrupting chemicals. Today there are around 116 environmental compounds, which are suspected to disrupt the thyroid function (Howdeshell 2002). The sea act as final sink for persistent semivolatile contaminants and aquatic organisms accumulate high concentrations of these endocrine disrupting compounds (Looser et al. 2000). Numerous environmentally relevant chemicals, including polychlorinated hydrocarbons, polycyclic aromatic hydrocarbons, organochlorine pesticides, chlorinated paraffins, organophosphorous pesticides, carbamate pesticides, cyanide compounds, methyl bromide, phenols, ammonia, metals, acid loads, sex steroids, and pharmaceuticals, exert acute or chronic effects on the thyroid cascade in the approximately 40 teleost fish species tested to date (Brucker-Davis 1998; Rolland 2000; Brown et al. 2004a). The following paragraphs presents the relevant pollutants treated in this thesis.

Polychlorinated biphenyl mixtures

A possible 209 polychlorinated biphenyl (PCB) structures (congeners) based on the degree and position of chlorination exist (Figure 6). Various commercial mixtures of PCB congeners, sold under trade names such as Aroclor (Monsanto, St. Louis, MO, USA), Clophen (Monsanto), or Kanechlor (Kanegafuchi, Osaka, Japan), were used mainly as dielectric fluids in electrical products such as transformers, and in hydraulic fluids, printing inks, adhesives, and paints (Ramade 2000). The PCBs are highly stable. They persist in the environment and are passed up food webs with the highest levels accumulating in top predators. Since the mid-1970s, strict control of PCB manufacture, import, and use reduced environmental levels markedly, but in recent years they have remained constant (Ramade 2000).

Effects of PCB mixtures on the thyroid cascade have been examined extensively in other vertebrates. Brouwer et al. has compiled an excellent review on the

interactions of persistent organohalogens, including PCBs, on thyroid status in mammals and birds. In general, PCB mixtures increase the metabolism and excretion of thyroid hormones and lower the circulating T_4 levels (Brouwer et al. 1998).

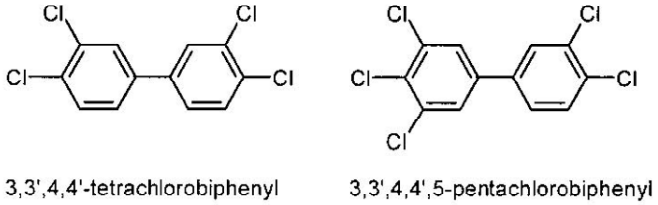

Figure 6: Chemical structure of PCBs

Research on fish reported changes in thyroid histological appearance and plasma thyroid hormone levels in coho salmon (*Oncorhynchus kisutch*), chinook salmon (*Oncorhynchus tshawytscha*), Rainbow trout (*Oncorhynchus mykiss*) and flounder (*Platichthys flesus*) (Leatherland and Sonstegard 1978; Leatherland and Sonstegard 1980; Leatherland 1993; Besselink et al. 1996). Overall, these studies suggest that PCB mixtures can alter indices of thyroid status in fish but that their mode of action is not well understood.

Coplanar PCBs are ubiquitous environmental contaminants arising mainly from anthropogenic activities. They are formed as by-products in manufacture of commercial PCB mixtures (e.g., Aroclor, Clophen, Kanechlor) and arise from diverse combustion sources and are the most toxic (Safe et al. 1987). In fish, coplanar PCB congeners, such as 77 or 126 affect plasma thyroid hormone levels, thyroid histology, liver T_4ORD activity and hepatic T_4 glucuronidation (Schreiber and Specker 1998; Adams et al. 2000; Palace et al. 2001; Soffientino 2001; Brown et al. 2002; Brown et al. 2004b). They may also cause a dose-dependent increase in hepatic retinoic acid hydroxylation and oxidation (Boyer et al. 2000). Because T_3 and retinoic acid and their receptors can bind as heterodimers to DNA response

element sites, alterations in availability of either ligand (T_3 or retinoic acid) for their receptors may alter downstream gene transcription (Boyer et al. 2000).

Organochlorine pesticides

Organochlorine pesticides can alter thyroid function. These pesticides include p,p′-dichlorodi-phenyltrichloroethane (DDT), and γ-hexachlorocyclo-hexane (lindane).

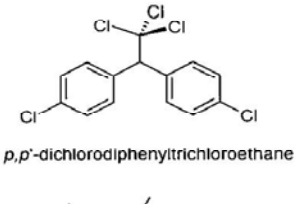

Figure 7: Chemical structure of DDT

The DDT (Figure 7), an organochlorine insecticide that alters Na+ channel function in nervous tissue, has been used widely, particularly to control typhus and malaria vectors. Tilapia (*Sarotherodon mossambicus*) exposed to DDT showed greater thyroid epithelial cell height and nuclear (Shukla and Pandey 1986). Mullet (*Liza parsia*) exposed to DTT showed a decrease in thyroid epithelial cell height, degeneration of epithelial cells, and depletion of colloid (Pandey et al. 1995).

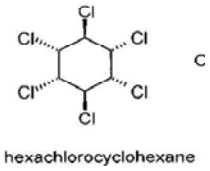

Figure 8: Chemical structure of hexachlorocyclohexane

Lindane, the gamma isomer of hexachlorocyclohexane (γ-HCH) (Figure 8), acts by interfering with class of receptors that respond to the neurotransmitter gamma-aminobutyric acid (GABA) of insect neurons, and is used as an insecticide and fumigant on a wide range of soil-dwelling and plant-eating insects as well as in

personal-care products for the control of lice and mites. Exposure of *Heteropneustes fossilis* increased the plasma T_4 level but decreased the plasma T_3 level but no differences in serum T_4 could be observed in rainbow trout (Aldegunde et al. 1999). Toxicity experiments also have been carried out with the β-isomer of hexachlorocyclohexane in medaka (*Oryzia latipes*) where the epithelial cells showed hypertrophy and diminished colloid content; moreover, the number of thyrotropic hormone-producing cells in the pituitary was increased (Wester and Canton 1988)

We conclude that both short-term and long-term treatments with high doses of organochlorine pesticides may alter fish thyroid activity and impair thyroid hormone synthesis.

Metals

Metals above normal environmental background levels often derive from mining or manufacturing by-products. Aquatic vertebrates and birds (particularly waterfowl and fish-eating species) are the most likely to be contaminated by heavy metals that can disrupt thyroid function (Gupta and Kar 1998; Gupta and Kar 1999). Metals such as mercury (Hg), cadmium (Cd), and lead (Pb) show a strong affinity for sulfhydryl or thiol groups and, therefore, tend to inhibit enzymes with such functional groups (Pavia J'unior et al. 1997).

Cadmium enters the aquatic environment mainly from atmospheric fallout and in effluents from smelting and refining industries Exposure of fish to cadmium reduced thyroid epithelial cell height and lowered plasma thyroid hormone concentrations in Clarias batrachus (Gupta et al. 1997). Acute Cd exposure of juvenile rainbow trout increased plasma T_4 levels, while subacute exposure decreased plasma T_4 levels. Neither dosage altered plasma T_3 levels (Hontela et al. 1996; Ricard et al. 1998). In Corydoras punctatus, cadmium decreased thyroidal T_4 content (Bhattacharya et al. 1989). Long-term exposure (6–9 months) of lake trout to Cd decreased thyroid follicle epithelial cell height (Scherer et al. 1997).

Lead enters the environment from its mining but mainly from the refining and smelting of Pb and other metals. Pb caused thyroid epithelial cell hypertrophy and reduced thyroid colloid content in C. batrachus (Katti and Sathyanesan 1987). Pb reduced plasma T_3 levels and liver ORD activity in H. fossilis (Chaurasia and Kar 1999) and C. batrachus (Chaurasia et al. 1996) and reduced plasma T_4 levels in rainbow trout (Spieler et al. 1990).

Mercury cycles in the environment as a result of both natural and anthropogenic activities. Its release to the environment has increased with industrialization mainly due to emissions from combustion of waste and fossil fuels. Once in the aquatic environment, Hg can be methylated and then accumulates in predators in aquatic food webs. In juvenile rainbow trout exposed to mercurial compounds both plasma T_4 and T_3 levels increased (Bleau et al. 1996). However, in C. punctatus exposed to mercury, plasma T_4 levels decreased (Bhattacharya et al. 1989). Exposure of C. punctatus or mullet (L. parsia) to an ambient $HgCl_2$ concentration caused thyroid follicular epithelial cells to become columnar and the colloid to exhibit varying degrees of vacuolization (Pandey et al. 1993). Hg-based compounds caused thyroid epithelial cell hypertrophy, reduction in colloid content, and decreased plasma T_4 and T_3 levels in C. batrachus (Kirubagaran and Joy 1989; Kirubagaran and Joy 1994).

Based on the above limited evidence, we conclude that exposure to several metals influences the vertebrate thyroid system and that deiodination activity may be especially susceptible.

European Sea bass

Regarding the fact that the thyroid endocrine system is highly conserved throughout the vertebrates, it is quite interesting to study this problematic in a fish species. We selected the European sea bass (*Dicentrarchus labrax* L.; Moronidae; Perciformes)

(Figure 9), a marine species of great economic importance, particularly in Mediterranean aquaculture, for our study. Sea bass were historically cultured in coastal lagoons and tidal reservoirs before the race to develop the mass-production of juveniles started in the late 1960s. Fish culture was initially associated with salt production in coastal evaporation pans and marshes. The salt was harvested during the high evaporation season of summer and autumn, and fish were cultured during winter and spring. The supply for this culture came from trapping schools of fish that lived in these estuarine areas (Pickett and Pawson 1994).

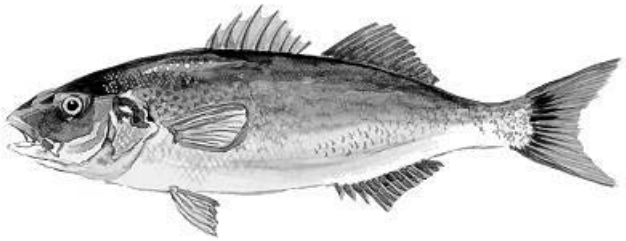

Figure 9: The Sea bass *Dicentrarchus labrax*

During the late 1960s, France and Italy competed to develop reliable mass-production techniques for juvenile sea bass and, by the late 1970s, these techniques were well enough developed in most Mediterranean countries to provide hundreds of thousands of larvae (Pickett and Pawson 1994). The European sea bass (*Dicentrarchus labrax*) was the first marine non-salmonid species to be commercially cultured in Europe and at present is the most important commercial fish widely cultured in Mediterranean areas. Greece, Turkey, Italy, Spain, Croatia and Egypt are the biggest producers (Lloris 2002).

The European sea bass are eurythermic (5-28 °C) and euryhaline (3 to full strength sea water); thus they are able to frequent coastal inshore waters, and occur in estuaries and brackish water lagoons (Wheeler 1975; Smith 1990). Sometimes they venture upstream into freshwater. There is only one breeding season per year, which takes place in winter in the Mediterranean population (December to March), and up to June in Atlantic populations. Sea bass spawn small (1.02-1.39 mm) pelagic eggs in

water with salinities lower than 35, near to river mouths and estuaries or in littoral areas where the salinity is high (≥30‰) (Pickett and Pawson 1994). Being not particularly sensitive to low temperature some fish may over-winter in coastal lagoons instead of returning to the open sea. Sea bass are predators and their feeding range includes small fish, prawns, crabs and cuttlefish (Wheeler 1975; Smith 1990).
By bringing together all these characteristics, the European sea bass revealed to be a study species of choice. Indeed, as high predator, they accumulate considerable amounts of organic chemicals by biomagnification through trophic transfer (Table 1).

Table 1: Organochlorine pollutant concentrations in muscle of European sea bass (ng g^{-1} lipid)

Location	Sum PCB	Sum DDT	Source
Sicily, Italy	150	100	Lo Turco *et al., 2007*
Ebro Delta, Spain (n=10)	800 ± 50	513 ± 97	Pastor *et al.,*1996
Orbetello Lagoon, Italy (n=13)	369 ± 195		Carbuelli *et al.,* 2007
Ria de Aveiro, Portugal (n=10)	155 ± 49 to 294 ± 104	108 ± 43 to 336 ± 132	Antunes *et al.,*2004
Kavalla wild, Greece (n=13)	806 ± 514	615 ± 348	Schnitzler *et al.*, 2008
Thassos aquaculture, Greece (n=17)	487 ± 136	180 ± 95	Schnitzler *et al.*, 2008

Juvenile sea bass can be considered as sedentary fish, since they grow in nursery areas within estuaries and, only after they reached a mean length of 36 cm disperse in coastal marine environment (Pawson *et al.* 2007). So sea bass below 36 cm should be a good bio indicator organism reflecting the local water environmental pollution in their tissues. Finally, their eurythermic and euryhaline capacities facilitate their laboratory maintenance for experimental studies.

Objective and outline of this thesis

Surprisingly little research has been performed on the thyroid effects of endocrine disrupting chemicals of this economically important fish species. Particularly when considering that the actions of thyroid hormones on metabolism, growth and development are important issues in aquaculture.

The objective of the research presented in this thesis is to investigate the different aspects of endocrine disruption of the thyroid system in sea bass. Specific objectives include (i) the concentration levels of potential endocrine disrupting compounds commonly found in European coastal waters, (ii) the environmental factors affecting thyroid function of wild sea bass from European coasts and (iii) the underlying mechanisms and effects of polychlorinated biphenyls on thyroid hormone physiology and metabolism.

The concentrations of organochlorine pesticides, PCBs and trace elements in muscles of European Sea bass originating from the major North-Western European estuaries, namely, the Scheldt, the Seine, the Loire, the Charente and the Gironde are presented in **chapter 2**. The sedentary habits of juvenile sea bass allowed us to compare the local pollution of the different regions. The conception of the study, assessing simultaneously organic pollutants, such as PCBs and organochlorine pesticides as well as non-essential and essential elements is genuine. We identified location-specific accumulation patterns, evaluated the benefit and risk of consumption and emitted a risk-based consumption advice for these fish.

To assess the ability of occurring pollutants in the European coastal areas to disrupt the thyroid system in sea bass, we describe in **chapter 3** the thyroid functional status of these wild sea bass. To this end, we investigated the thyroid status of wild sea bass collected near major estuaries of European coastlines: the Scheldt, the Seine, the Loire, the Charente and the Gironde. In order to examine the status of thyroid function at multiple levels, we have studied simultaneously different endpoints. The centrally controlled thyroidal secretion of T_4 was monitored from muscular T_4 levels and thyroid gland histology. The peripherally controlled conversion of T_4 to T_3 was monitored by *in vitro* deiodination activities, and muscular T_3 levels were measured to reflect peripheral thyroidal (T_3) status. In addition, two biochemical pathways i.e. sulfation and glucuronidation, involved in thyroid hormone metabolism and phase-2 response to toxicants, were assayed. We applied

multivariate statistical analysis to identify associations between chronic exposition to organic pollutants and thyroid function in wild sea bass.

To gain an integrated insight of the underlying mechanisms and effects of polychlorinated biphenyls on thyroid hormone physiology and metabolism, **chapter 4** describes the effects of a 4-month *in vivo* exposure to various environmentally relevant doses of commercial mixtures of polychlorinated biphenyls (PCBs) on the thyroid system of *Dicentrarchus labrax*. Again several endpoints were analyzed simultaneously: thyroid gland histology, hepatic 5'-deiodination (or outer ring deiodination, ORD) activities that convert the thyroid prohormone T_4 to the bioactive hormone T_3, and muscular T_4 and T_3 levels. In addition, two biochemical pathways i.e. sulfation and glucuronidation, involved in thyroid hormone metabolism and phase-2 response to toxicants, were assayed. This approach allowed us to determine underlying mechanisms and dose dependency of the effects of these pollutants on the thyroid system of these fish, and to examine the consequences of a potential disruption of the thyroid system on growth performance and condition factor in these commercially important fish species.

Finally, **chapter 5** provides a more integrative view of this thesis by summarizing the obtained results of both, field and experimental, studies. We will thoroughly discuss these observations and propose accurate interpretations, by reviewing the different stages of teleost thyroid function and regulation.

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Chapter 2

Organochlorine pesticides, polychlorinated biphenyl and trace element residues in wild sea bass (*Dicentrarchus labrax*) off European estuaries

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Abstract

Polychlorinated biphenyls (PCBs) and organochlorine pesticides like Dichloro-Diphenyl-Trichloroethane (DDTs), Hexachlorocyclohexanes (HCHs), aldrin, dieldrin and trace elements (Cd, Cu, Se, Pb, Zn and Hg) were analysed in the muscle of sea bass (*Dicentrarchus labrax*) sampled in coastal regions near several important European river mouths (Gironde, Charente, Loire, Seine and Scheldt). High contamination levels of organochlorinated compounds were measured in the muscles of European sea bass sampled in the coastal regions near the Scheldt, Seine, Loire, Charente and Gironde. The Scheldt and the Seine are still among the most contaminated estuaries in Europe. Each region presented their specific contamination patterns reflecting different sources due to the input of the respective rivers. As fish and fishery products are the main contributors of the total dietary intake of organochlorinated pollutants, regular consumption of sea bass with the reported contamination levels may represent a significant exposure route for the general human population.

Keywords

Dicentrarchus labrax, Polychlorinated biphenyl, Organochlorine pesticides, consumption, trace elements, estuaries

Introduction

Sea bass (*Dicentrarchus labrax*) is a euryhaline species that inhabit estuaries, lagoons and coastal waters. This fish species is carnivorous, feeding on fish, crustaceans and cephalopods (Pickett and Pawson, 1994) and thus accumulate through its food major organic pollutants, such as PCBs and organochlorine pesticides (Loizeau et al., 2001) as well as non-essential and essential elements (Dural et al., 2006; Türkmen et al., 2005). This species displays both economic and environmental importance. Indeed, human consumption of sea bass through fishing (8 528 000 t in 2008) and aquaculture (66 738 000 t in 2008) is very important and the species is considered as a bioindicator of marine pollution (Loizeau, 2001). Moderate to high levels of organochlorine compounds (Antunes and Gil, 2004; Carubelli et al., 2007; Pastor et al., 1996; Schnitzler et al., 2008) and trace elements (Dugo et al., 2006; Dural et al., 2006; Durrieu et al., 2005; Miramand et al., 2001) were previously described in sea bass, which were below the European norm. It is noteworthy that the norm of PCBs for meat products (200ng g^{-1} lw; (Belgisch_Staatsblad-Moniteur_Belge, 1999)), does not apply to fish, but instead a norm of 75 ng g^{-1} ww is used.

Sea bass use estuaries and coastal bay as nursery area, after they reached a mean length of 36 cm, they disperse in coastal marine environment (Pawson et al., 2007). So sea bass below 36 cm should be a good bioindicator organism reflecting the local water environmental pollution in their tissues. Meanwhile, the Current Minimum Legal size for fishing sea bass is 36cm and any fish below this size should be returned into sea.

For many years seafood such as fish, molluscs and crustaceans has often been the focus of attention in nutritional and toxicological work. Nutritionists consider these products to be an important source of high-quality proteins, essential elements and (omega-3) fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Toxicologists tend to regard seafood as a major vector for toxic substances such as toxic metals and persistent organic pollutants (Domingo and Bocio, 2007;

Voorspoels et al., 2008). It is thus interesting to evaluate the nutritional benefits of nutritive elements in food and the health risks related to toxic substances, as high rates of pollutants in fish might counterbalance the health benefit derived from the fatty acid in fish lipid. A recent study revealed that sea bass contributes to 7.12% of the recommendation for fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) for inhabitants of French coastal areas (Le Blanc et al., 2006)

There is a need for more information on contaminant levels in European fish. Whereas many studies consider the pollution levels of sea bass in the Mediterranean (Antunes and Gil, 2004; Carubelli et al., 2007; Dugo et al., 2006; Dural et al., 2006; Lo Turco et al., 2007; Trocino et al., 2007), there are almost no studies from the Atlantic coasts. The present study aimed to assess and compare the levels of organochlorinated compounds and trace elements measured in muscle of sea bass near the mouth of several European rivers (Gironde, Charente, Loire, Seine and Scheldt). The sedentary habits of juvenile sea bass allowed us to compare the local pollution of the different regions. The conception of the study, assessing simultaneously organic pollutants, such as PCBs and organochlorine pesticides as well as non-essential and essential elements is genuine. Benefits and potential risk linked to human consumption of this edible fish will be discussed.

Methods

Sampling

Eighty-seven sea bass were collected between September 20th and November 1st in 2007 during different scientific missions of CEMAGREF (Institut de recherche pour l'ingénierie de l'agriculture et de l'environnement), IFREMER (Institut Francais de Recherche pour l'Exploitation de la Mer) and INBO (Instituut voor Natuur- en Bosonderzoek). Fish were caught in the coastal region near these European rivers: Gironde, Charente, Loire, Seine, and Scheldt.

The caught fish were immediately dissected. Total length and the weight were measured. Gender and maturity stage were assessed through gross examination of the gonads. Approximately 30 g of skeletal muscle was excised from the area behind the head, dorsal to the lateral line and anterior to the dorsal fin. The muscle samples were stored frozen at -70°C until analysis.

Chemical analysis

All solvents were of pesticide grade; n-hexane and acetone (Burdick & Jackson brand) were purchased from Fluka (Buchs, Switzerland). The Mirex (Dodecachloropentacyclo-[5.3.0.0.0.0]decane) used as internal standard, the pure PCB congeners (IUPAC nos. 28, 44, 52, 66, 70, 87, 95, 101, 105, 110, 118, 128, 138, 149, 153, 156, 170, 180, 183, 187, 194, 195, 206, 209) and the congener used as surrogate (IUPAC no. 112), were obtained from Ultra Scientific® and Dr Ehrenstorfer®. All other chemicals used were of analytical grade.

Extracts of 10 g of dorsal muscular tissue of each fish were analyzed for Polychlorinated biphenyls (PCBs), Dichloro-Diphenyl-Trichloroethane (DDTs), Hexachlorocyclohexanes (HCHs), aldrin and dieldrin by gas chromatography using a Thermo Quest Trace 2000 gas chromatograph equipped with a 63Ni ECD (Thermo Quest, Trace 2000). The details of sample preparation and clean-up as well as quality assurance are provided in Schnitzler *et al*, 2008.

Trace element analysis

After being weighed and dried at 60 °C to a constant weight, 2g of muscle samples were digested in Teflon tubes with concentrated nitric acid, deionised water and H_2O_2 in a microwave oven (20 min between 0 and 600 W). After cooling, samples were diluted to 50 ml with deionised water in a volumetric flask. Samples for Cd, Cu, Se, Pb, and Zn were analysed by Inductively Coupled Plasma-Mass Spectrometer (ICPMS)(Elan DRC II). Samples for Hg were analysed by Direct

Mercury Analyzer (DMA Milestones). A mean water content of 74.0 ± 4.1% was calculated in our samples. Concentrations are expressed in $\mu g.g^{-1}$ dry weight (DW). Parallel to samples, a set of certified control material samples (DOLT-3 liver and DORM-2 muscle, National Research Council Canada) went through each set of analyses to ensure the accuracy and precision of the method. Recoveries for control materials ranged from 92% to 109% for Cd, Ni, Cu, Zn, Se, Pb (Table 1). Instrumental detection limits were: Cu, 0.020 ppb; Zn, 0.042 ppb; Se, 0.166 ppb; Cd, 0.005 ppb; Pb, 0.002 ppb; Hg, 1 ppb, respectively. All samples were above the detection limit.

Table 1 : Quality control results (µg/g dry weight) acquired with certified materials

Element	Assigned value	Measured value	% Recovery
Cadmium (Cd)	19,4	17,8	92
Nickel (Ni)	2,72	2,5	93
Copper (Cu)	31,2	31,2	100
Zinc (Zn)	86,6	80,4	93
Selenium (Se)	7,06	6,19	88
Lead (Pb)	0,319	0,349	109

Statistical analysis

Statistical analysis of the data was performed using SPSS for Mac® software (SPSS Inc., version 16.0.2). The Kolmogorov–Smirnoff test was used to test for normality of the statistically treated variables, and to ensure the utilization of adapted tests. The non-parametric Mann-Whitney *U*-test were used to compare differences in organic and trace element compound concentrations among sexes and Spearman correlation test followed by Fishers Omnibus post hoc tests were used to compare differences in organic and trace element compound concentrations in relation to length and weight.

Intersite comparison of the contamination patterns were realised using discriminant analysis to assess the ability of organic and trace element compound concentrations

to discriminate among the different collection locations. Results were judged significant when $p < 0.05$.

Results

Sampling

Mean body length was 31 ± 4.6 cm and there were no significant differences in mean body length and weight among study location groups. Based upon available length and age data, all sea bass were estimated to be of 1-2 years and juvenile; gross observation of the gonads confirmed that all sea bass were immature. There was an overall sex ratio of 1:1.

All samples were collected before the spawning season (May, August in the Atlantic), as it is known that feeding intensity and consequently lipid concentrations and pollutant concentrations decrease in fish during spawning activity (Vassilopoulou and Georgakopoulosgregoriades, 1993). Thus, variability of lipid contents originated through differences in sexual maturity was minimized. The lipid proportion in the muscles of the European sea bass ranged from 0.1 to 3.9% of the wet weight with a mean value of 0.7%. The lipid content varied significantly between the sampling locations (ANOVA; $p<0.05$). Sea bass from the coastal region near the Gironde showed the highest lipid content in their muscles (mean value of 1.7) and the sea bass from the coastal region near the Charente had the lowest lipid content in their muscles (mean value of 0.4) (Table 2).

Table 2a: Lipid proportion, mean contamination levels in the white muscle of European sea bass. The concentrations are given in ng g^{-1} wet weight

Wet weight						
coastal region near	Gironde	Charente	Loire	Seine	Scheldt	ANOVA
n	8	8	34	26	11	
lipid (%)	1.7 ± 1.1 (1.4) 0.5 - 3.2	0.4 ± 0.4 (0.2) 0.1 - 1.2	0.9 ± 0.7 (0.7) 0.3 - 3.9	0.9 ± 0.3 (0.9) 0.1 - 1.4	0.6 ± 0.6 (0.4) 0.1 - 2.1	F(4,83)= 2.6 p=0.059
Σ PCB (ng g^{-1})	49.7 ± 23.4 (55.8) 15.1 - 82.3	18.1 ± 10.0 (19.8) 10.5 - 29.5	40.4 ± 19.1 (36.5) 17.2 -101.5	46.4 ± 21.6 (41.9) 15.8 - 101.8	42.5 ± 31.4 (34.5) 17.2 - 124.7	F(4,83)= 2.7 **p=0.035***
Σ ICES PCB (ng g-1)	26.8 ± 13.1 (29.9) 7.7 - 45.0	7.9 ± 4.6 (8.6) 3.7 - 13.6	14.6 ± 8.7 (11.3) 5.7 - 45.9	26.0 ± 11.6 (23.8) 8.4 - 54.7	17.4 ± 14.2 (11.8) 6.2 - 53.1	F(4,83)= 7.5 **p<0.001***
Σ *p'p*-DDT (ng g-1)	2.2 ± 0.9 (2.4) 0.8 - 3.3	0.4 ± 0.3 (0.5) 0.2 - 0.8	1.8 ± 1.5 (1.4) 0.3 - 8.0	1.2 ± 0.6 (1.2) 0.2 - 2.3	3.0 ± 2.2 (1.9) 1.3 - 7.9	F(4,83)= 5.6 **p=0.001**
Σ HCH (ng g-1)	0.1 ± 0.1 (0.1) 0.0 - 0.2	0.2 ± 0.1 (0.2) 0.0 - 0.3	0.4 ± 0.2 (0.4) 0.0 - 0.7	0.1 ± 0.2 (0.1) 0.0 - 0.8	0.1 ± 0.0 (0.1) 0.1 - 0.2	F(4,83)= 13.1 **p<0.001***
Σ aldrin-dieldrin (ng g-1)	0.3 ± 0.2 (0.4) 0.0 - 0.5	0.2 ± 0.1 (0.2) 0.1 - 0.5	0.7 ± 0.1 (0.6) 0.1 - 1.9	0.5 ± 0.3 (0.4) 0.1 - 1.2	0.3 ± 0.5 (0.1) 0.0 - 1.8	F(4,83)= 3.6 **p=0.010***

Table 2b: Lipid proportion, mean contamination levels in the white muscle of European sea bass. The concentrations are given in ng g^{-1} lipid weight

Lipid weight						
coastal region near	Gironde	Charente	Loire	Seine	Scheldt	ANOVA
n	8	8	34	26	11	
lipid (%)	- ± - (-) - - -	- ± - (-) - - -	- ± - (-) - - -	- ± - (-) - - -	- ± - (-) - - -	
Σ PCB (ng g^{-1})	1821.0 ± 2101.6 (2422.5) 295.8 - 8487.4	3756.2 ± 3076.6 (4546.1) 1550.2 - 13917.4	4473.2 ± 2783.1 (4216.9) 1696.4 - 20185.0	4716.1 ± 2880.0 (4500.0) 615.6 - 10478.2	6564.4 ± 3101.9 (6187.6) 2775.7 - 15382.9	F(4,83)= 1.1 **p=0.364**
Σ ICES PCB (ng g-1)	878.8 ± 1062.0 (1227.6) 132.9 - 5765.4	1360.9 ± 1128.6 (1420.8) 660.9 - 4381.7	1374 ± 550.1 (1411.3) 578.6 - 5228.5	2543.3 ± 1550.3 (2550.3) 615.6 - 10478.2	2627.8 ± 1239.5 (2556.5) 1406.6 -5379.4	F(4,83)= 4.4 **p=0.003***
Σ *p'p*-DDT (ng g-1)	90.5 ± 101.2 (97.4) 17.8 - 374.1	92.2 ± 122.9 (108.0) 43.7 - 561.4	143.9 ± 175.7 (127.7) 0.0 - 690.7	129.8 ± 113.4 (122.6) 27.8 - 534.1	394.9 ± 323.6 (392.4) 130.9 - 1219.1	F(4,83)= 14.0 **p<0.001***
Σ HCH (ng g-1)	3.5 ± 2.5 (4.4) 1.3 - 9.9	54.1 ± 42.7 (51.3) 14.2 -167.4	35.5 ± 29.0 (40.1) 5.8 - 183.0	8.5 ± 24.8 (6.8) 0.9 - 196.9	15.2 ± 11.7 (15.7) 2.8 - 52.1	F(4,83)= 5.4 **p=0.001***
Σ aldrin-dieldrin (ng g-1)	26.6 ± 15.7 (28.9) 9.0 - 70.9	47.5 ± 27.5 (81.5) 12.6 - 121.6	51.9 ± 67.6 (59.2) 8.7 - 438.1	46.6 ± 48.8 (44.3) 9.8 - 276.0	10.8 ± 20.2 (15.9) 0.1 - 85.0	F(4,83)= 1.9 **p=0.114**

Table 3a : Mean contamination levels of trace elements in the white muscle of European sea. The concentrations are given in µg g^{-1} wet weight.

	Wet weight					
coastal region near	Gironde	Charente	Loire	Seine	Scheldt	ANOVA
n	8	8	34	26	11	
Zn (µg g-1)	5.33 ± 0.75 (5.25) 4.45 - 6.94	3.91 ± 1.76 (3.28) 2.72 - 7.39	3.97 ± 0.76 (3.79) 2.95 - 5.78	5.18 ± 2.20 (4.71) 3.15 - 15.1	5.37 ± 0.76 (5.37) 4.83 - 5.90	F(4,83)= 3.8 **p=0.008***
Se (µg g-1)	0.47 ± 0.03 (0.46) 0.42 - 0.51	0.31 ± 0.08 (0.29) 0.24 - 0.47	0.35 ± 0.06 (0.34) 0.24 - 0.59	0.33 ± 0.08 (0.32) 0.20 - 0.57	0.79 ± 0.54 (0.79) 0.41 - 1.17	F(4,83)= 14.2 **p<0.001***
Cu (µg g-1)	0.35 ± 0.08 (0.34) 0.26 - 0.53	0.38 ± 0.30 (0.27) 0.16 - 0.72	0.28 ± 0.12 (0.26) 0.16 - 0.98	0.29 ± 0.11 (0.28) 0.13 - 0.56	0.41 ± 0.06 (0.41) 0.37 - 0.45	F(4,83)= 1.1 p=0.384
Mn (µg g^{-1})	0.28 ± 0.09 (0.27) 0.16 - 0.42	0.13 ± 0.05 (0.13) 0.07 - 0.22	0.22 ± 0.16 (0.14) 0.08 - 0.62	0.21 ± 0.11 (0.19) 0.07 - 0.49	0.22 ± 0.06 (0.22) 0.17 - 0.62	F(4,83)= 1.4 p=0.244
Hg (µg g-1)	0.09 ± 0.03 (0.09) 0.06 - 0.16	0.18 ± 0.06 (0.17) 0.11 - 0.25	0.15 ± 0.04 (0.15) 0.06 - 0.25	0.21 ± 0.08 (0.19) 0.12 - 0.44	0.13 ± 0.02 (0.13) 0.11 - 0.14	F(4,83)= 7.9 **p<0.001***
Ni (µg g-1)	0.08 ± 0.03 (0.07) 0.04 - 0.11	0.06 ± 0.03 (0.05) 0.03 - 0.12	0.10 ± 0.06 (0.08) 0.03 - 0.33	0.07 ± 0.08 (0.05) 0.03 - 0.43	0.06 ± 0.03 (0.06) 0.04 - 0.08	F(4,83)= 1.3 p= 0.273
Pb (µg g-1)	0.02 ± 0.01 (0.02) 0.01 - 0.03	0.03 ± 0.04 (0.02) 0.01 - 0.11	0.05 ± 0.02 (0.04) 0.01 - 0.12	0.04 ± 0.03 (0.03) 0.01 - 0.12	0.06 ± 0.02 (0.06) 0.04 - 0.07	F(4,83)= 2.6 **p=0.041***
Cd (µg g-1)	0.001 ± 0.004 (0.001) 0.001 - 0.010	0.008 ± 0.013 (0.001) 0.001 - 0.030	0.003 ± 0.007 (0.001) 0.001 - 0.030	0.001 ± 0.003 (0.001) 0.001 - 0.010	0.001 ± 0.001 (0.001) 0.001 - 0.002	F(4,83)= 2.5 **p=0.047***

Table 3b : Mean contamination levels of trace elements in the white muscle of European sea. The concentrations are given in µg g^{-1} dry weight.

	Dry weight					
coastal region near	**Gironde**	**Charente**	**Loire**	**Seine**	**Scheldt**	**ANOVA**
n	8	8	34	26	11	
Zn (µg g-1)	24.2 ± 3.42 (23.9) 20.2 - 31.5	17.8 ± 8.01 (14.9) 12.4 - 33.6	18.0 ± 3.46 (17.2) 13.4 - 26.3	23.5 ± 9.99 (21.4) 14.3 - 68.6	24.4 ± 3.42 (24.4) 22.0 - 26.8	F(4,83)= 3.8 **p=0.008***
Se (µg g-1)	2.13 ± 0.13 (2.10) 1.89 - 2.30	1.42 ± 0.37 (1.33) 1.11 - 2.15	1.58 ± 0.29 (1.56) 1.11 - 2.66	1.52 ± 0.37 (1.42) 0.92 - 2.60	3.59 ± 2.44 (3.59) 1.86 - 5.31	F(4,83)= 14.1 **p<0.001***
Cu (µg g-1)	1.60 ± 0.37 (1.52) 1.18 - 2.39	1.71 ± 1.37 (1.23) 0.73 - 4.45	1.29 ± 0.54 (1.19) 0.75 - 3.29	1.33 ± 0.48 (1.27) 0.60 - 2.55	1.88 ± 0.25 (1.88) 1.70 - 2.06	F(4,83)= 1.1 p=0.386
Mn (µg g^{-1})	1.26 ± 0.41 (1.19) 0.72 - 1.89	0.59 ± 0.24 (0.57) 0.33 - 1.00	0.98 ± 0.71 (0.66) 0.37 - 2.83	0.96 ± 0.50 (0.85) 0.33 - 2.21	0.99 ± 0.28 (0.99) 0.79 - 1.19	F(4,83)= 1.3 p=0.261
Hg (µg g-1)	0.42 ± 0.14 (0.41) 0.28 - 0.72	0.80 ± 0.28 (0.75) 0.52 - 1.14	0.68 ± 0.20 (0.66) 0.25 - 1.14	0.95 ± 0.35 (0.86) 0.52 - 2.00	0.57 ± 0.09 (0.57) 0.50 - 0.63	F(4,83)= 8.0 **p<0.001***
Ni (µg g-1)	0.33 ± 0.11 (0.32) 0.19 - 0.48	0.26 ± 0.14 (0.22) 0.16 - 0.52	0.44 ± 0.26 (0.37) 0.15 - 1.51	0.32 ± 0.35 (0.23) 0.14 - 1.97	0.28 ± 0.11 (0.28) 0.20 - 0.35	F(4,83)= 1.3 p= 0.262
Pb (µg g-1)	0.07 ± 0.04 (0.07) 0.03 - 0.15	0.14 ± 0.17 (0.07) 0.05 - 0.49	0.21 ± 0.10 (0.20) 0.07 - 0.54	0.18 ± 0.15 (0.12) 0.04 - 0.57	0.26 ± 0.08 (0.26) 0.20 - 0.31	F(4,83)= 2.6 **p=0.044***
Cd (µg g-1)	0.02 ± 0.01 (0.01) 0.01 - 0.04	0.04 ± 0.05 (0.01) 0.01 - 0.13	0.02 ± 0.02 (0.02) 0.01 - 0.13	0.01 ± 0.01 (0.01) 0.01 - 0.04	0.02 ± 0.01 (0.02) 0.01 - 0.02	F(4,83)= 2.3 **p=0.069**

Toxicological analysis

All individuals were regrouped for the further statistical analysis, no significant difference in contaminant concentrations could be detected between the sexes (Mann-Whitney; $p>0.05$) and no significant relationship between length and weight, and contaminant concentrations could be revealed (Spearman correlation tests followed by Fisher Omnibus post-hoc test; $p>0.05$).

All tested metals, including mercury, lead and cadmium, were found in detectable quantities in all samples (Table 3). Several trace element concentrations (Zn, Se, Hg, Pb, and Cd) varied significantly between the sampling locations (ANOVA; $p<0.05$). No clear contamination pattern could be explored by discriminant analysis, as the sampling locations reveal no clear contamination trend. Instead all individuals were regrouped together in the centre of the discriminant analysis plot (Fig. 1A).

OCs (organochlorinated compounds, on fw basis) varied significantly between sampling locations (ANOVA; $p<0.05$). The general contamination trend showed higher levels in the coastal regions near the Seine and the Gironde followed by the Scheldt and Loire while lowest levels was observed in the coastal region near the Charente (Table 2). On a lipid weight basis, Σ of PCB concentrations did not differ between the sampling areas whereas other OCs (for Σ of ICES PCBs, Σ of DDTs, Σ of Pesticides and Σ of HCHs) differed significantly (ANOVA; $p<0.05$). The data, expressed in ng g^{-1} lipid weight, shows different trends, here the coastal regions near the rivers Scheldt and Seine are the most contaminated, followed by the Loire and the Charente and finally the Gironde is the less contaminated (Table 2).

The 7 ICES (International Council for the Exploration of the Sea) PCBs (IUPAC 28, 52, 101, 118, 138, 153 and 180), the major congeners and most predominant used in the different commercial mixtures of PCBs, represent around 40-60% of all the found congeners according to the origin of the samples. They were found in sea

bass in decreasing importance: 138>153>180>101>52>118>28 with only minor changes between sampling regions.

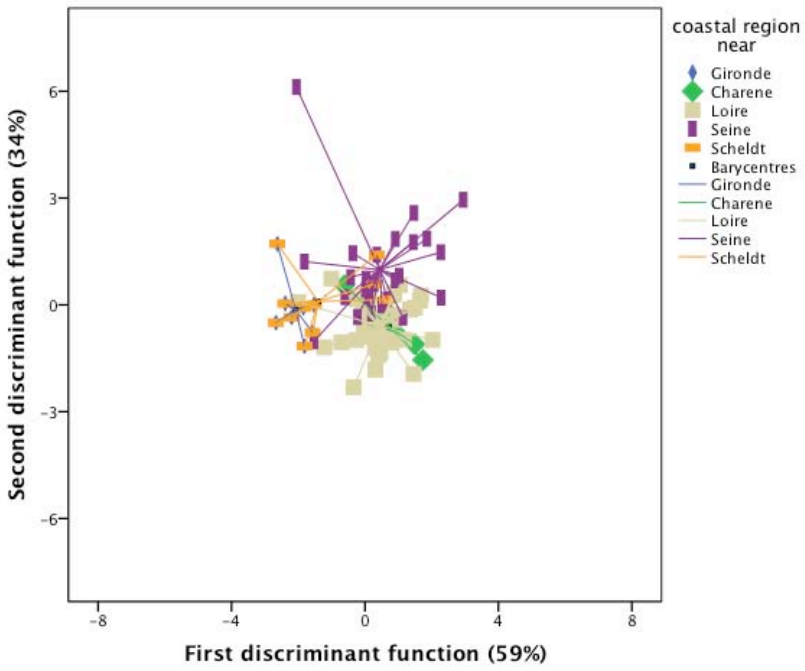

Figure 1A: Plot of the results of discriminant analysis for intersite comparison of contamination patterns on the basis of dry weight normalized trace elements concentrations

Contamination pattern differences between sampling locations were explored by discriminant analysis to show how several predefined groups of individuals (sampling location of sea bass) may be separated by given measurements of several variables. It provides linear functions of variables that best separate the cases into the predefined groups. The five sea bass groups could be discriminated by their contamination pattern (lipid weight basis), principally by the compounds β-HCH, aldrin, dieldrin, IUPAC PCB 70, 101, 87, 149, 153, 180, 170, 194, *pp'*DDT, *pp'*DDE and *pp'*DDD. The first discriminant function (root) explained 36 % of the variations between groups involving mostly βHCH, dieldrin, IUPAC PCB 70, 101, 87, 153, 194, *pp'*DDE and *pp'*DDD, and the second discriminant function explained a further 31%

of the variation between groups, involving the aldrin, IUPAC PCB 149, 180, 170 and *pp*'DDT concentrations. Together, the two discriminant functions explained 67 % of the variance. The plot of the discriminant analysis is shown on Figure 1B. Each sampling region had his well-defined contamination pattern; the 5 groups are well separated from each other.

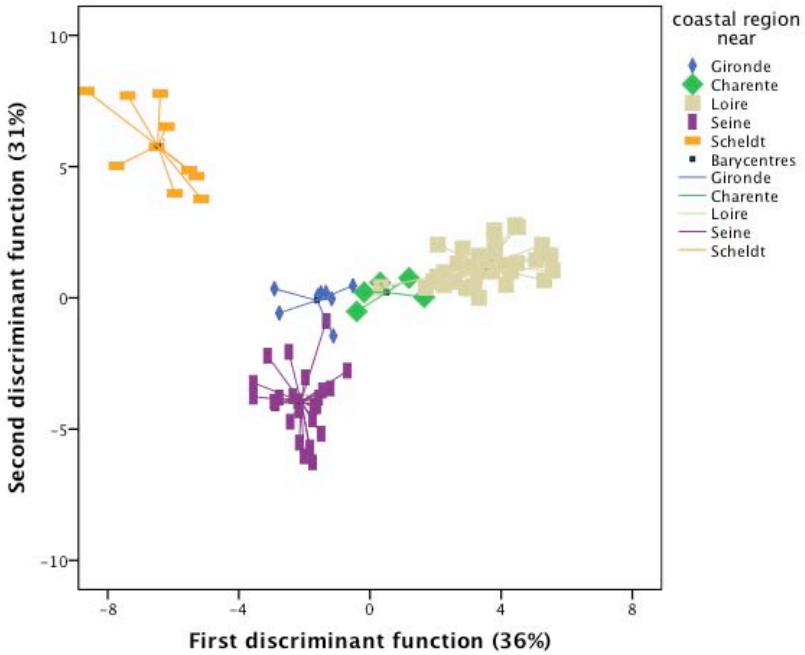

Figure 1B: Plot of the results of discriminant analysis for intersite comparison of contamination patterns on the basis of lipid weight normalized organochlorine compound concentrations

Discussion

Contamination level

The trace element concentrations measured in our sampling are quite low and are part of the background contamination level that are relatively homogeneous all along the French coast. Generally metal accumulation is highest in liver and gills, while it is low in gonad and muscle in all fish species (Dural et al., 2006). These

organs are also good indicators of chronic exposure to heavy metals because they are the sites of metal metabolism (Dugo et al., 2006). The liver is often considered a good monitor of water pollution with metals since their concentrations are proportional to those present in the environment, whereas muscle was a poor indicator (Dural et al., 2006). In sea bass, the contamination levels in liver are two to ten times higher than in muscle, although no direct relation was published.

The levels of zinc (Zn), selenium (Se), copper (Cu), manganese (Mn), mercury (Hg), nickel (Ni), lead (Pb) and cadmium (Cd) were determined in the muscle because of its importance for human consumption. Our measured concentrations are mostly in the lower range of concentrations measured in sea bass (Dugo et al., 2006; Dural et al., 2006; Durrieu et al., 2005; Miramand et al., 2001) and other fish (Bustamante et al., 2003; Canli and Atli, 2003; Cohen et al., 2001; Kalay et al., 1999; Kwon and Lee, 2001; Topcuoglu et al., 2002; Türkmen et al., 2005; Tüzen, 2003) (Table 4).

Table 4 : Comparison of mean contamination levels of cadmium (Cd), copper (Cu), lead (Pb), selenium (Se), zinc (Zn), manganese (Mn), and nickel (Ni) with values taken from literature on sea bass and other marine fish species

	Sample area	Cd	Cu	Pb	Se	Zn	Mn	Ni	References
D. labrax	Tyrrhenian Sea (Italy)	0.1	2.07	0.26	0.41	3.48			Dugo 2006
	Sea of Sicily (Italy)	0.0746	1.73	0.26	0.33	3.07			Dugo 2006
	Camlik Lagoon (Turkey)	0.06	0.34	0.82		52.22			Dural 2007
	Seine Estuary (France)	0.025	3.28	1.10		66.50			Miramand 2001
	Seine Estuary (France)	<0.02	1.12			5.20			Durrieu 2005
	Gironde Estuary (France)	<0.02	0.25			3.20			Durrieu 2005
	This study	*0.02*	*1.83*	*0.18*	*1.66*	*20.61*	*0.97*	*0.37*	
Other Fish	Middle Black Sea (Turkey)	0.09–0.48	1.28–2.93	0.22–0.85		9.5–22.9	1.06–3.76		Tüzen (2002)
	Black Sea Coast (Turkey)	<0.02–0.24	1.01–4.54	<0.05–0.06		25.7–44.2	0.69–3.56	<0.01–2.04	Topcuoğlu et al. (2002)
	Kerguelen Islands (Indian	0.01–0.1	0.5–2.5			9.2–33.2			Bustamante et al. (2003)
	Masan Bay (Korea)	0.01	0.18–0.25	0.04–0.15		6.33–12.9		0.02	Kwon and Lee (2001)
	California Lagoons (USA)	0.1–0.3	1.9–7.5	0.8–4.1		36–150		0.61–12	Tamira et al. (2001)
	Mediterranean Sea	1.07–1.43	3.40–5.88	7.33–9.11		16.1–31.4		4.25–6.07	Kalay et al. (1999)
	Mediterranean Sea	0.37–0.79	2.19–4.4	2.98–6.12		16.5–37.4			Canlı and Atlı (2003)
	İskenderun Bay	0.95	1.57	2.32		4.36	1.71	2.90	Türkmen *et al.* 2005

Various authors suggested normalizing concentrations of pollutants to the lipid contents in order to reduce intra-species variability (Pastor *et al.*, 1996). Fish lipid contends can substantially influence the bioaccumulation of organochlorinated compounds (Loizeau, 2001). The organochlorine pollution of the sampled areas is attributable to many sources of industrial, urban and agricultural contamination. The sea bass sampled near the river Scheldt showed the highest levels of total PCBs

and DDTs. These results are in good agreement with the French pollution monitoring mussel watch programme which shows that rivers are the main input of pollutants in a local coastal ecosystem, with high levels in regions near industrial or urban areas and that the Seine estuary is one of the most PCB contaminated in Europe (Abarnou, 2003). Catchment areas of the rivers is one of the driving factor explain OCs variability in sea bass. Indeed, the Scheldt catchment area (20,000 km²) shelters more than 50% of the total Belgian surface and covers a very densely populated and highly industrialised area of northern France, western Belgium and the south western Netherlands (Voorspoels et al., 2004/9). A lower total PCB and total DDT contamination has been observed in sea bass sampled in the coastal regions near two longest rivers of France, Loire and Seine. The catchment area of the Loire (117,000 km²) represents 20% of the whole French surface while the Seine catchment area (78,600 km²) shelters approximately 18 million inhabitants and encompasses 40% of the French industrial activity as well as significant agricultural activity (Abarnou, 2003; Abarnou et al., 2002; Bodin et al., 2007; Loizeau et al., 2001). The lowest levels of total PCB and total DDT were observed in the sea bass sampled in the coastal regions near the Charente and the Gironde. These rivers are much shorter and have smaller catchment areas (Durrieu et al., 2005) (Table 2).

The concentrations of ICES PCBs and DDTs measured in the muscles of sea bass are reviewed and compared with those found in similar wild species from other regions. It can be seen that the mean levels of ICES PCBs detected in specimens from the French and Belgian coast are generally higher than those reported from other Atlantic and Mediterranean areas (Figure 2A). Otherwise, the DDT levels were comparable to those from Italy and Portugal (Antunes and Gil, 2004; Carubelli et al., 2007) but substantially lower than those from Spain and Greece (Pastor et al., 1996; Schnitzler et al., 2008) (Figure 2B).

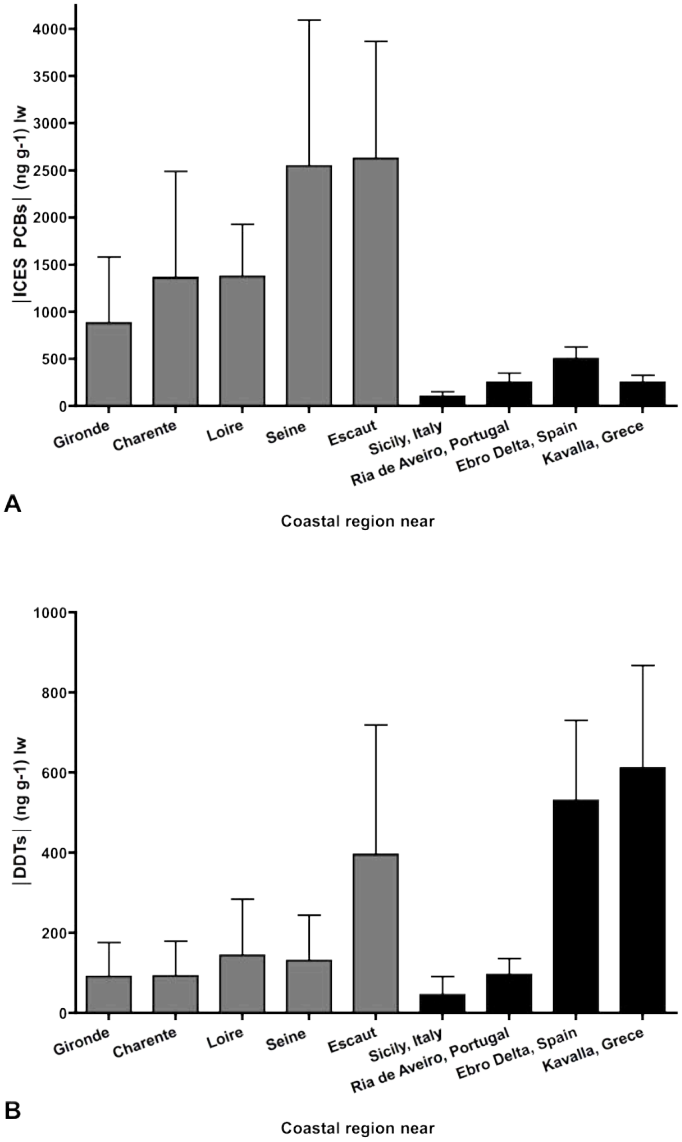

Figure 2: Organochlorine pollutant concentrations in muscles of European sea bass (ng g^{-1} lipid) Data from this study is shown in gray.
Sicily , Italy = (Lo Turco, 2007); Ria de Aveiro, Portugal = (Antunes *et al.*, 2004); Ebro delta, Spain = (Pastor *et al.*, 1996) and Kavalla, Greece = (Schnitzler *et al.*, 2008)

Contaminant profile information

Although the concentrations of several trace elements (Zn, Se, Hg, Pb and Cd) differed significantly between sampling locations, the discriminant analysis could not separate the predefined groups of individuals (sampling location of sea bass) by given measurements of trace element variables. There were no systematic differences that would have support distinct contamination patterns for the different regions. Whereas the contamination pattern differences of organochlorine pollutants between the sampling sites revealed by the discriminant analysis can be visualized by several charts showing the PCB pattern fractions, the fractioning of the different analyzed pesticides as well as the fractioning of the DDT metabolites (Figure 3).

The levels of organochlorines were as follows PCBs>DDTs> aldrin and dieldrin> HCHs. Although there are differences between sampling regions, PCB patterns were always dominated by a large contribution from the hepta-, hexa- and pentachlorinated PCBs, which collectively accounted for 81 to 86% of the PCBs. These congeners are the most abundant due to their common use in commercial mixtures such as Aroclor 1254 and 1260, but also to their high lipophilicity, stability and persistence that facilitate the adsorption to sediments and the accumulation in the aquatic system, and to their molecular structure (Naso et al., 2005). The tri-, tetra and octachlorinated PCBs were detected at noticeably lower concentrations (Figure 3A).

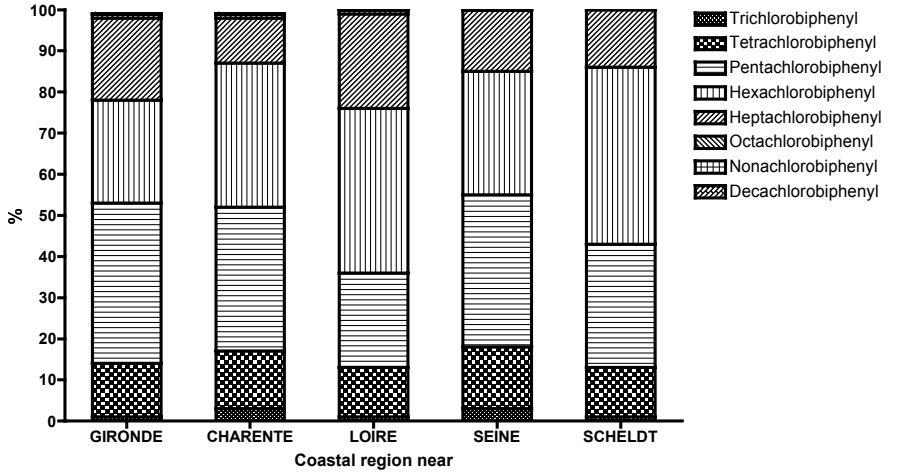

Figure 3A: Geographical differences in PCB congeners contamination patterns.

Despite the considerable length of time that has passed since 1972 when legal restrictions were introduced for the use of DDT in many European countries, DDT and his metabolites have been detected in all the samples (Figure 3B).

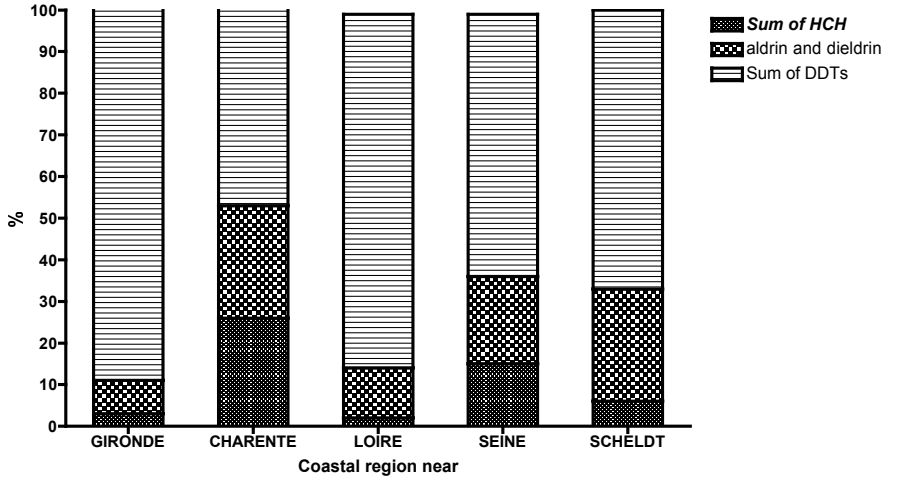

Figure 3B: Geographical differences in chlorinated pesticides contamination patterns

In most of the cases *pp'*DDD was the principal form of DDTs, constituting over 50% of the DDTs. The *pp'*DDD form was dominant in sea bass muscle from the coastal regions near the Loire, Seine and Scheldt. The *pp'*DDT was the less represented

form in all the groups constituting less than 20% of the DDTs. The *pp'*DDE constitutes around 30% of the DDT metabolites but is the dominant form in sea bass muscle from the coastal regions near the Gironde and the Charente. The DDT metabolites (*pp'*DDE and *pp'*DDD/*pp'*DDT) ratio is commonly used to asses the chronology of DDT input into the ecosystems (Bordajandi et al., 2003). The ratio greater than 1 suggests that there has been no recent input of DDT in these regions (Figure 3C).

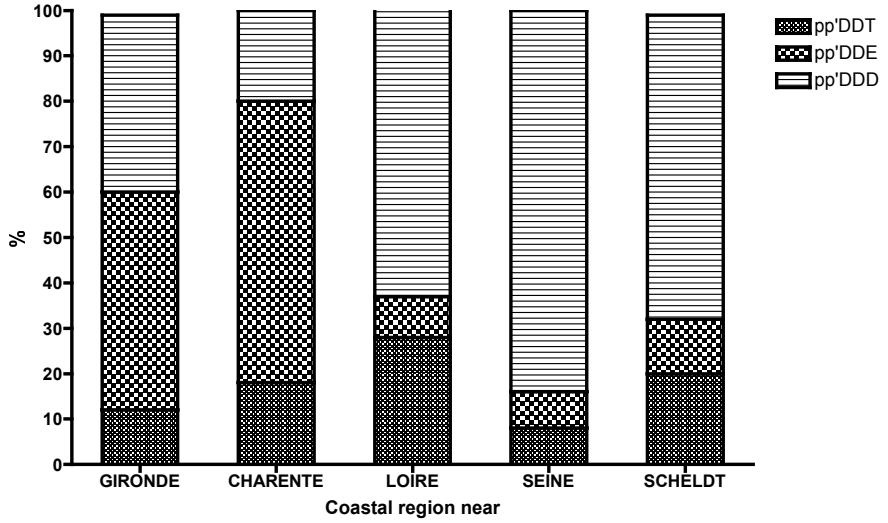

Figure 3C: Geographical differences in DDT metabolites contamination patterns

The situation for the organochlorine pesticide aldrin and his degradation product dieldrin may also give an indication of the chronology of aldrin input in the ecosystem (Gonzalez et al., 2003). Dieldrin was the dominant form measured in the muscles of sea bass sampled in the coastal regions of the Gironde, Charente, Loire, Seine and Scheldt, the dieldrin/aldrin ratio in these cases were between >1 indicating no recent input of aldrin in these regions.

The relatively low HCH concentrations might be attributed to the fact that, in contrast to PCBs and DDTs, HCHs are not magnified through the food web (Pastor et al., 1996). The observed concentrations may be ascribed to its previous use as

insecticides and fumigants on a wide range of soil-dwelling and plant-eating insects as well as in personal care products for the control of lice and mites but also the fact that it is a by-product in the manufacturing processes of various chlorine-containing chemicals and an impurity in several pesticides (Bailey, 2001; Brown et al., 2004).

Risk assessment

The highest mercury levels have been measured in sea bass from coastal regions near the river Seine, but the mean level is more than 4 times lower than the maximum authorised limit of 1 mg/kg (EC, 2005). The highest lead levels have been measured in sea bass from coastal regions near the river Loire, but the mean level is more than three times lower than the maximum authorised limit of 0.2 mg/kg (EC, 2005). Finally, the highest cadmium levels have been measured in sea bass from coastal regions near the river Charente, but the mean level is more than 6 times lower than the maximum authorised limit of 0.05 mg/kg (EC, 2005). The risk linked to metal exposure through sea bass consumption appeared therefore limited. Based on the tolerable weekly intake for Hg set by the FAO/WHO (FAO/WHO, 2003), the maximum amount of sea bass flesh that can be eaten by an average adult (60 kg) before reaching the safety limits was estimated at 1828g (for sea bass from the coastal zone near the Seine) up to 4266g (for sea bass from the coastal zone near the Gironde) per month. Combined with the average fish proportion size of 227g (Carubelli et al., 2007), this represents 8 to 18 sea bass meals per month. The safety limits for lead and cadmium (FAO/WHO, 1999, 2000) are unreachable; the amount of sea bass flesh that can be eaten by an average adult (60 kg) exceeds the 100 portions per month.

Zinc, selenium, copper and manganese are compounds that need to be present in the human diet to maintain normal physiological functions. Zinc is a component of a wide variety of enzymes, including the ribonucleic polymerases, alcohol dehydrogenase, carbonic anhydrase and alkaline phosphatase (Goldhaber, 2003). A sea bass meal provide up to 8-11% of the Recommended Dietary Allowance (RDA)

of 11mg/day for Zn. Selenium is essential due to its association with proteins, known as selenoproteins. Several selenoproteins defend against oxidative stress, others regulate thyroid hormone metabolism, and additional regulate redox status of vitamin C and other molecules (Goldhaber, 2003). A portion sea bass supply up to 128 to 194% of the RDA of 0,055 mg/day for Se. These levels are 3 to 5 times lower than the tolerable upper intake level of 0,4 mg/day. There is thus no risk of toxicity due to high selenium exposure through sea bass diet. Copper is present in important proteins and enzymes (Goldhaber, 2003). The RDA for Cu is 0,9 mg/day and a sea bass meal furnish 7-10% of it. Pyruvate carboxylase and superoxide dismutase contain Manganese (Goldhaber, 2003). A sea bass ration offer 2-4% of the RDA of 1,6 mg/day for Mn. A regular consumption of sea bass may present an interesting source for essential trace element.

Persistent organochlorinated compounds have a strong tendency to accumulate in lipids, and as a consequence, concentrations are usually normalized on lipid basis. However, if the purpose is to investigate the level of contamination in order to assess human intake, data on fresh weight basis are far more useful. Food consumption is the main exposure route for organochlorinated pollutants for the general population, and fish and fishery products seem to be the main contributors to the total dietary intake of these pollutants (Foran et al., 2005; Gochfeld and Burger, 2005; Hites et al., 2004; Marcotrigiano and Storelli, 2003; Mozaffarian and Rimm, 2006; Sidhu, 2003; Sioen et al., 2008).

The concentrations of HCHs and DDTs found in all analysed samples were well below the Maximum Residue Limits for organochlorine pesticides in some food products of animal origin (FAO, 2008). The 7 ICES PCB levels were below the limit of 75 ng g^{-1} fresh weight Maximum Residue Limits for muscle meat of fish (A.R. du 06/03/02).

But based on the tolerable daily intake for the seven indicator PCBs of 0.01 μg kg^{-1} set by the AFSSA (Afssa, 2009), the maximum amount of sea bass flesh that can be

eaten by an average adult (60 kg) before reaching the safety limits was estimated at 680g (for sea bass from the coastal zone near the Seine) up to 2250g (for sea bass from the coastal zone near the Charente) per month. Combined with the average fish proportion size of 227g (Carubelli et al., 2007) this represents 3 to 10 sea bass meals per month. These results may be an important issue for human communities who regularly consume fish, such as coastal populations. The levels in sea bass are among the highest measured in edible marine fish species, next to eel and trout (Naso et al., 2005). This probably reflects their feeding habits and the nature of the habitat of these benthic and euryhaline species. They usually inhabit shallow waters with sandy or muddy bottoms along the coast, ports and estuaries, which are generally considered to be more heavily polluted than open waters (Lewis et al., 2002; Loizeau, 2001).

Various national scientific bodies formulates the recommendations that the general population should consume fish at least twice a week, including some oily fish, and that pregnant or breast-feeding women should consume predator fish not more than once a week (Hites et al., 2004; Marcotrigiano and Storelli, 2003; Sidhu, 2003). Looking beyond these general recommendations, this study highlights the advantages of diversifying the consumed fish and seafood species in terms of proportions and provisioning origins in order to ensure a rational balance between benefits and risks compatible with nutritional and toxicological recommendations (Foran et al., 2005; Gochfeld and Burger, 2005; Mozaffarian and Rimm, 2006; Sioen et al., 2008).

Conclusions

The non-essential trace elements concentrations measured in our sampling were quite low and the risk linked to metal exposure through sea bass consumption appeared therefore limited. Meanwhile a regular consumption of sea bass may present an interesting source for essential trace element, that are well present in sea bass muscle. High contamination levels of organochlorinated compounds were

measured in the muscles of European sea bass sampled in the coastal regions near the Scheldt, Seine, Loire, Charente and Gironde. The Scheldt and the Seine are still among the most contaminated estuaries in Europe. These levels were generally higher than those reported in literature in sea bass from other regions. Each region presented their specific contamination patterns reflecting different sources due to the input of the respective rivers. As fish and fishery products are the main contributors of the total dietary intake of organochlorinated pollutants, regular consumption of sea bass with the reported contamination levels may represent an important exposure route for the general population. Especially as sea bass generally present the highest measured concentrations in edible marine fish species

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Chapter 3

Environmental factors affecting thyroid function of wild sea bass (*Dicentrarchus labrax*) from European coasts

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Abstract

Thyroid functional status of wild fish in relation with the contamination of their environment deserves further investigation. We here applied a multi-level approach of thyroid function assessment in 87 wild sea bass collected near several estuaries: namely the Scheldt, the Seine, the Loire, the Charente and the Gironde. Thyroxine (T_4) and triiodothyronine (T_3) concentrations in muscle were analyzed by radioimmunoassay. The activity of hepatic enzymes involved in extrathyroidal pathways of thyroid hormone metabolism, *viz*. deiodination, glucuronidation and sulfation were analyzed. Last, follicle diameter and epithelial cell heights were measured. We observed changes that are predicted to lead to an increased conversion of T_4 to T_3 and lowered thyroid hormone excretion. The changes in the metabolic pathways of thyroid hormones can be interpreted as a pathway to maintain thyroid hormone homeostasis. From all compounds tested, the higher chlorinated PCBs seemed to be the most implicated in this perturbation.

Keywords

Dicentrarchus labrax, persistent organic contaminants, thyroid hormone metabolism, deiodination, glucoronidation, sulfation

Introduction

Thyroid hormones are essential for normal development, and for maintenance of normal physiological functions in vertebrates (Janz, 2000; Zoeller et al., 2007). In fish, thyroid hormones are involved in the control of osmoregulation, metabolism, somatic growth and post-hatching metamorphosis (Janz, 2000; Power et al., 2001; Yamano, 2005). The regulation of thyroid hormone bioavailability in tissues and cells represents a very complex and unique web of feedback systems (Zoeller et al., 2007). In fish and other vertebrates the thyroid cascade involves two components. First, thyroxine (T_4) biosynthesis and secretion are largely under central control by the brain-pituitary-thyroid axis (Bernier et al., 2009). Second, there is the conversion of T_4 to its biologically active form 3,5,3'-triiodothyronine (T_3) and its metabolism and receptor-mediated actions that seems largely to be under peripheral control in extra-thyroidal tissues (Eales and Brown, 1993).

The regulatory mechanisms involved in thyroid hormone homeostasis are numerous and complex. As consequence, environmental chemicals can act at many levels in the thyroid system. The mechanisms involved in the endocrine disruptor mediated alteration of the thyroid function have been extensively investigated but are still not fully understood (Ishihara et al., 2003).

The thyroid system is a major target of endocrine disrupting chemicals. Today there are around 116 environmental compounds which are suspected to disrupt thyroid function (Howdeshell, 2002). Numerous environmentally relevant chemicals, including polychlorinated hydrocarbons, polycyclic aromatic hydrocarbons, organochlorine pesticides, chlorinated paraffins, organophosphorous pesticides, carbamate pesticides, cyanide compounds, methyl bromide, phenols, ammonia, metals, acid loads, sex steroids, and pharmaceuticals, exert acute or chronic effects on the thyroid cascade in the approximately 40 teleost fish species tested to date (Blanton and Specker, 2007; Brown et al., 2004; Brucker-Davis, 1998; Rolland, 2000).

The effects of PCB mixtures on the thyroid system have been examined extensively in vertebrates. Brouwer et al. have compiled an excellent review on the interactions of persistent organohalogens, including PCBs, on the thyroid status in mammals and birds. In general, PCB mixtures increase the metabolism and excretion of thyroid hormones and lower circulating T_4 levels (Brouwer et al., 1998). Research on fish reported changes in the histology of the thyroid gland and in plasma thyroid hormone levels in coho salmon (*Oncorhynchus kisutch*), chinook salmon (*Oncorhynchus tshawytscha*), rainbow trout (*Oncorhynchus mykiss*) and flounder (*Platichthys flesus*) (Besselink et al., 1996; Leatherland, 1993; Leatherland and Sonstegard, 1978, 1980). Overall, these studies indicate that PCB mixtures can alter indices of thyroid status in fish but their mode of action is not well understood.

Organochlorine pesticides including p,p′-dichlorodi-phenyltrichloroethane (DDT) and hexachlorocyclo-hexanes, can alter thyroid function. Tilapia (*Oreochromis mossambicus*) exposed to DDT showed greater thyroid epithelial cell height and nuclear diameter (Shukla and Pandey, 1986), indicative of increased thyroid gland activity. Mullet (*Liza parsia*) exposed to DDT displayed opposite symptoms such as a decrease in thyroid epithelial cell height, degeneration of epithelial cells, and depletion of colloid (Pandey et al., 1995). Exposure to lindane, the gamma isomer of hexachlorocyclohexane (γ-HCH), increased plasma T_4 levels but decreased plasma T_3 levels in *H. fossilis* (Yadav and Singh, 1987), whereas a similar treatment induced no differences in serum T_4 in rainbow trout (Aldegunde et al., 1999). The β-isomer of hexachlorocyclohexane induced thyrocyte hypertrophy and a diminished colloid content in the thyroid gland's follicles, and also an increase in the number of pituitary thyrotropes in medaka (*Oryzia latipes*) (Wester and Canton, 1988). All in all, organochlorine pesticides have multiple and species-specific effects in teleostean species, and a general picture cannot, as yet, be constructed.

Our current knowledge is derived mostly from laboratory studies on the effects of endocrine disrupting chemicals in fish (Adams et al., 2000; Boas et al., 2006; Brown

et al., 2004; Leroy et al., 2006). Only a very small number of studies have assessed the effects of the environmental pollution on wild animals, and most of them concern the influence of PCBs on the thyroid system of Great Lakes organisms (Leatherland and Down, 2001; Leatherland et al., 1989b; Raine, 2001). The interpretation of the results from field studies is proving to be more difficult because of normal variations in thyroid hormones associated with age, gender, diet, nutritional status, season and physiological condition (Rolland, 2000).

To our knowledge no comprehensive study has been carried out in European waters. Concentrations of organic contaminants in fishes may vary widely in relation to feeding habits, the nature of the habitat and environmental factors such as coastal marine pollution (Lewis et al., 2002; Loizeau, 2001; Naso et al., 2005). In European waters, PCBs concentration in tissues from juvenile sea bass varied from 10 ng g^{-1} (ww) in Gironde to 125 ng g^{-1} (ww) in Scheldt (Schnitzler et al., submitted), with unknown consequences for their thyroid function. This study therefore aimed at establishing a correlation between exposures to contaminants and effects on the thyroid endocrine system. To this end, we investigated the thyroid status of wild sea bass collected near major estuaries of European coastlines: the Scheldt, the Seine, the Loire, the Charente and the Gironde. In order to examine the status of thyroid function at multiple levels, we have studied simultaneously different endpoints. The centrally controlled thyroidal secretion of T_4 was monitored from muscular T_4 levels and thyroid gland histology. The peripherally controlled conversion of T_4 to T_3 was monitored by in-vitro deiodination activities, and muscular T_3 levels were measured to reflect peripheral thyroidal (T_3) status. We applied multivariate statistical analysis to identify associations between chronic exposition to previously described organic pollutants (Schnitzler et al., submitted) and thyroid function in wild sea bass.

Materials and Methods

Sampling

Eighty seven sea bass (*Dicentrarchus labrax*) were collected between 20th September and 1st November 2007 during different scientific missions of CEMAGREF (Institut de recherche pour l'ingénierie de l'agriculture et de l'environnement), IFREMER (Institut Français de Recherche pour l'Exploitation de la Mer) and INBO (Instituut voor Natuur- en Bosonderzoek) as previously described (Schnitzler et al., submitted). Sea bass were caught in the coastal regions of the European rivers Gironde, Charente, Loire, Seine, and Scheldt.

The caught fish were immediately dissected. The length and the weight of each fish were measured. Gonads were inspected to sex the fish and to evaluate the macroscopic characteristics of the maturity stages of the ovary and testes of sea bass. Approximately 30 g of skeletal muscle was excised from the area directly caudal to the head, dorsal to the lateral line and anterior to the dorsal fin. The muscle samples were stored at -70°C until analysis.

Thyroid parameters

Standards and Reagents

Thyroxine (T_4), uridine 5'-diphosphate glucuronic acid (UDPGA) and 3'-phospho-adenosine-5'-phosphosulfate (PAPS), were from Sigma Chemical Co. (St. Louis, MO). Sephadex LH-20 was purchased from Amersham Pharmacia Biotech Benelux (Roosendaal, The Netherlands). Outer ring labelled [^{125}I]T_4 (23.3 TBq/mmol) was obtained from Perkin-Elmer Life Science, Inc. (Boston, MA). All other chemicals were analytical grade and obtained from commercial suppliers. Radiolabeled iodothyronines were purified shortly before use by Sephadex LH-20 column chromatography. Radioactivities were measured in a 1272-Clinigamma gamma counter (LKB/Wallac Oy, Turku, Finland). Protein concentrations were determined

using a Coomassie Brilliant Blue G-250 kit (Bio-Rad, München, Germany) and bovine serum albumin as a standard.

Muscular thyroid hormone determinations

Muscular total T_3 and T_4 concentrations were measured by radio immunoassay (Siemens Coat-a-Count, Brussels, Belgium) according to the manufacturer's instructions. Details of extraction methods and the elaborated assay protocol are described elsewhere (Schnitzler et al., 2008). The accuracy of the assay was determined by blind analysis of quality control standards at high, medium and low concentrations. These samples were inserted in duplicate at the front, middle and end of the assay and mean measured concentrations were then compared to actual concentrations to determine assay reliability. The assay was accepted with reliability between 90 and 110%. To determine the efficacy of the extraction process in recovering thyroid hormones as well as transfer of samples into different types of tubes, two recovery systems were used. Unlabelled thyroid hormone was added to the minced fish muscle prior to homogenization. The samples were then subjected to the same homogenization, extraction, reconstitution and thyroid hormone assay procedures as the unknown and standard curve samples. The percentage of thyroid hormone recovered from each spiked tube was calculated and revealed quantitative recoveries of 92% T4 and 93 % T3.

Sulfotransferase activity

Sulfotransferase activities were measured in duplicate with T_4 as conjugate group acceptors. PAPS was used as the sulfate group donor. Sulfotransferase activity towards T_4 was measured by the incubation of approximately 50 µg homogenate protein at 37 °C for 120 min in 200 µl buffer composed of 100 mM Na-phosphate buffer and 2 mM EDTA (pH 7.2), 1 µM ^{125}I-labeled T_4 and 50 µM PAPS. The reaction was terminated with 800 µl ice-cold 0.1 M HCl, and the quenched incubate was applied to Sephadex LH-20 minicolumns (2 ml of a 10% w/v suspension) to

resolve liberated iodide, water-soluble conjugates and native iodothyronines, respectively, as described in detail previously (van der Heide et al., 2002). Radioiodide activities in the water-soluble fractions were interpreted to have originated from the presence of sulfated iodothyronines. Control incubations in these assays were in the absence of PAPS. Net sulfotransferase activities are expressed as a percentage of the total sum of all fractions of the Sephadex LH-20 chromatograms.

UDP glucuronyltransferase activity (UGT)

UGT activities were measured in duplicate with T_4 as conjugate group acceptors. UDPGA was used as the glucuronosyl group donor. The glucuronidation of T_4 was measured by the incubation of 50 µg homogenate protein at 37 °C for 120 min in 200 µl buffer containing 100 mM Tris/HCl (pH 7.4), 5 mM $MgCl_2$ and 0.05% Brij56, supplemented with 1 µM ^{125}I-labeled T4 and 5 mM UDPGA. The reaction was quenched with 200 µl ice-cold methanol, and the incubate was centrifuged for 10 min at 1500g. To 300 µl of the supernatant thus obtained 700 µl 0.1 M HCl was added, and the mixture was subjected to Sephadex LH-20 column chromatography as described above. Radioiodide activities in the water-soluble fractions were here interpreted to have originated from the presence of glucuronidated iodothyronines. Control incubations were in the absence of UDPGA.

Outer Ring Deiodinase activity (ORD)

5'-Deiodinase activities were measured in duplicate as described in detail elsewhere (Klaren et al., 2005). Briefly, 50 µg homogenate protein was incubated under saturating substrate conditions of 20 µM T_4 in 200 µl of 100 mM Na-phosphate buffer (pH 7.2). Outer ring labelled [^{125}I]T_4 was used as a tracer, and was purified on a 10% (w/v) Sephadex LH-20 mini-column shortly before use. The reaction was quenched by the addition of 100 µl ice-cold 5% BSA, followed by 500 µl ice-cold 10% TCA, and centrifuged at 1400g (15 min, 4 °C). To 500 µl of the deproteinized supernatant thus obtained an equal volume of 1.0 M HCl was added, and liberated

iodide was separated from the native iodothyronine using Sephadex LH-20 column chromatography. Non-enzymatic outer ring deiodination was determined in the absence of a preparation.

Thyroid histomorphometric analysis

The thyroid tissues enclosed in the subpharyngeal area were fixated in formalin. The tissue was then decalcified in 5% formic acid and 5% formaldehyde for one day and transferred to a 22.2% sodium sulphate solution for another day. The tissues were dehydrated in a graded series of ethanol before being embedded in paraffin wax. The paraffin blocks were longitudinally sectioned (8 μm) through all the thyroid tissues. The haematoxylin-eosin stain method was used for the microscopically diagnostic study of the histological samples.

Images of 50 randomly selected follicles at 100 times magnification were analysed. Thyroid histomorphometry was measured using Macnification® software (version 1.6.1 Orbicule Enhanced Labs). The different measurements in the thyroid tissue were determined by manually marking the contours of the follicles in the tissue. The follicle area, perimeter, diameter, length and width of every follicle cross section were thus measured. The shapes of the follicles were described with three dimensionless shape descriptors: roundness, form factor and aspect ratio, which were calculated as follows:

Roundness = 4 Area (μm^2) / π Diameter^2 (μm). A follicle with a maximum roundness value of 1 perfectly resembles a circle.

Form factor = 4π Area (μm^2) / Perimeter^2 (μm). The form factor expresses the evenness of the follicles outline; as its value approaches 1, the outline resembles a circle.

Aspect ratio = maximum length (μm) / maximum with (μm). The larger the aspect ratio, the more elongated the follicle is; a ratio of 1 corresponds to a perfectly circular follicle.

Chemical analysis

Extracts of 10 g of dorsal muscular tissue of each fish were analyzed for polychlorinated biphenyls (PCBs), dichloro-diphenyl-trichloroethane (DDTs), hexachlorocyclohexanes (HCHs), aldrin and dieldrin by gas chromatography using a Thermo Quest Trace 2000 gas chromatograph equipped with a 63Ni ECD (Thermo Quest, Trace 2000). Detailed method and results are presented elsewhere (Schnitzler et al., 2008; Schnitzler et al., submitted).

Calculations and statistics

Mean values ± standard deviation, (median) and min-max are presented, unless indicated otherwise. Pollutant concentrations are normalized for fresh (wet) tissue weight and the thyroid hormone concentrations and metabolic activities are also expressed on wet weight basis.

Statistical analysis of the data was performed using SPSS for Mac® software (SPSS Inc., version 16.0.2). The Kolmogorov–Smirnoff test was used to test for normality of the statistically treated variables. The non-parametric Mann-Whitney *U*-test was used to compare differences in organochlorine compound concentrations between sexes and correlation tests followed by Fishers Omnibus post hoc tests were used to compare differences in organochlorine compound concentrations in relation to length and weight.

Intersite comparison of the thyroid parameters were realised using an analysis of variance (ANOVA) to compare means between the different collection locations. The relationships between thyroid parameters (follicle histomorphometry, thyroid hormone concentrations and enzyme activities) and toxicological data were analysed in two steps. First, a correlation test was used to identify the contaminants related to the thyroid parameters. Then a correlation-based principal component analysis (PCA) was performed to reduce the 24 identified contaminant variables in order to

avoid misleading results due to correlating independent variables ('multi-collinearity') in subsequent analysis. Thereafter a correlation test, with the four factor scores revealed by the PCA as independent variables and thyroid parameters as dependent variable, was applied. Results were considered significant when $p < 0.05$.

Results

Sampling

Overall mean body length of the collected sea bass was 31 ± 4.6 cm (n=87). There were no significant differences in mean body length and weight between sampling locations. Based upon available length and age data, all sea bass were estimated to be juveniles 1-2 years of age. Inspection of the gonads confirmed that all sea bass were sexually immature. The overall sex ratio was 1:1 male:female. Neither size (length and weight) (Spearman correlation tests followed by Fisher Omnibus post-hoc test; $p>0.05$), nor sex (Mann-Whitney; $p>0.05$) had a significant interaction with any of the toxicological and endocrine parameters in this study.

Organochlorine compound analysis

The complete toxicological analysis including contamination levels, intersite comparisons, profile analysis and an estimate of risk for human consumption is published separately (Schnitzler et al., submitted). This study shows that juvenile sea bass from Scheldt and Seine had higher levels of organochlorine pesticides compared to sea bass collected near the Loire, Charente and Gironde.

Intersite differences in thyroid parameters

The mean T_4 and T_3 muscular concentration in sea bass at all sites was 10.8±5.9 $ng.g^{-1}$ and 1.4±0.5 $ng.g^{-1}$ respectively. However, thyroid hormone tissue levels varied significantly between locations (ANOVA $p<0.001$ in both cases, Table 1). The highest T_4 and T_3 concentrations were observed in sea bass from Charente and

Loire coastal regions whereas the lowest levels were measured in sea bass from the Seine and the Scheldt.

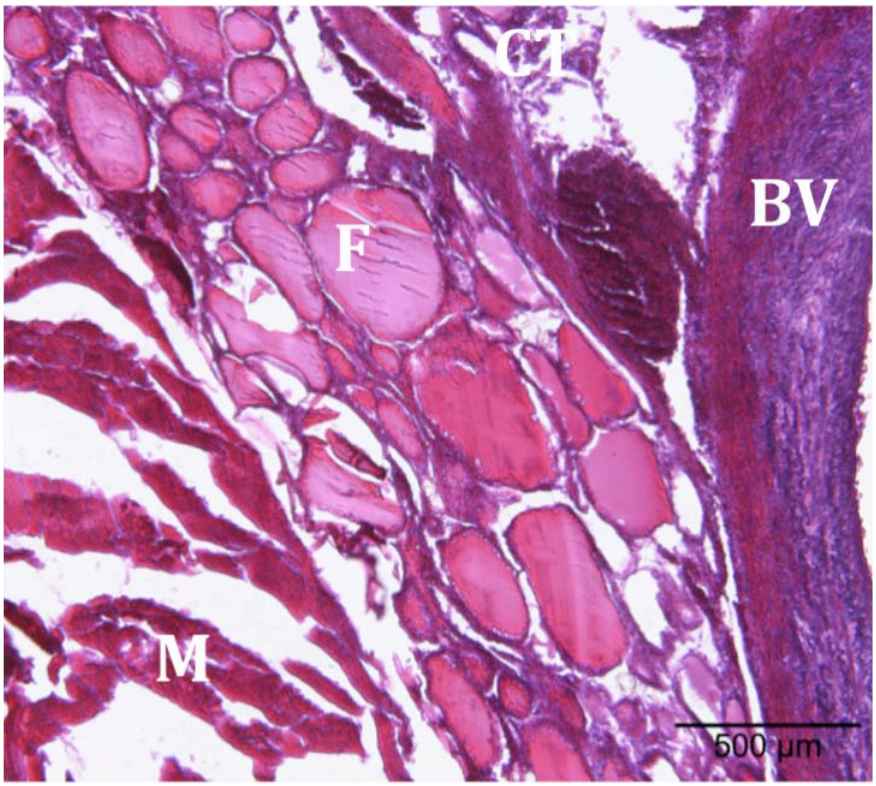

Figure 1 : Longitudinal section of European sea bass thyroid tissue in subpharyngeal area (H.E. staining ; BV= ventral aorta ; F= follicle ; M= muscle)

Analysis of thyroid gland tissue by light microscopy revealed irregular or oval follicular lumens surrounded by follicular epithelial cells. The epithelial cells were flattened, cuboid or columnar (Fig. 1).

Table 1 : Histomorphometric analysis, muscular thyroid hormone levels, mean hepatic metabolic activity and contamination levels in white muscle of European sea bass collected in several coastal regions

coastal region near	Gironde	Charente	Loire	Seine	Scheldt	ANOVA
n	8	8	34	26	11	
T4 (ng g^{-1})	12.0 ± 4.1	15.2 ± 2.5	14.0 ± 5.1	7.3 ± 5.5	5.3 ± 3.1	F(4,83)= 12.4
	(11.9)	(14.8)	(15.4)	(5.4)	(4.7)	
	3.6 - 17.1	12.7 - 18.2	2.5 - 20.3	0.6 - 18.6	1.7 - 11.4	**p<0.001***
T3 (ng g^{-1})	0.83 ± 0.54	1.52 ± 0.45	1.68 ± 0.50	1.29 ± 0.42	0.95 ± 0.10	F(4,83)= 10.1
	(0.56)	(1.51)	(1.61)	(1.23)	(0.94)	
	0.47 - 1.78	0.88 - 2.15	0.79 - 2.62	0.49 - 2.25	0.86 - 1.17	**p<0.001***
follicle diameter (µm)	74 ± 15	85 ± 24	75 ± 20	64 ± 14	58 ± 12	F(4,40)= 2.1
	(74)	(83)	(72)	(63)	(56)	
	63 - 85	64 - 111	45 - 124	43 - 84	43 - 75	*p=0.099*
cell height (µm)	17 ± 3	17 ± 4	16 ± 2	14 ± 4	13 ± 4	F(4,40)= 2.6
	(17)	(16)	(17)	(14)	(13)	
	15 - 19	14 - 23	12 - 22	8 - 19	8 - 19	**p=0.045***
roundness	0.81 ± 0.01	0.83 ± 0.02	0.79 ± 0.03	0.80 ± 0.03	0.81 ± 0.04	F(4,40)= 1.2
	(0.81)	(0.83)	(0.79)	(0.81)	(0.81)	
	0.80 - 0.82	0.81 - 0.85	0.72 - 0.84	0.74 - 0.85	0.74 - 0.85	p=0.308
form factor	0.83 ± 0.01	0.84 ± 0.02	0.81 ± 0.03	0.82 ± 0.03	0.82 ± 0.04	F(4,40)= 1.0
	(0.83)	(0.84)	(0.81)	(0.82)	(0.82)	
	0.82 - 0.84	0.82 - 0.86	0.76 - 0.86	0.75 -0.86	0.75 - 0.86	p=0.416
aspect ratio	1.20 ± 0.20	1.33 ± 0.11	1.28 ± 0.22	1.01 ± 0.19	0.91 ± 0.14	F(4,40)= 6.3
	(1.20)	(1.34)	(1.23)	(0.90)	(0.89)	
	1.06 - 1.35	1.20 - 1.46	0.95 - 1.72	0.79 - 1.35	0.79 - 1.18	**p<0.001***
deiodinase activity (fmol min^{-1} μg^{-1})	23.2 ± 10.4	5.8 ± 2.7	11.5 ± 6.3	17.6 ± 7.0	15.1 ± 3.9	F(4,83)= 9.4
	(25.9)	(5.0)	(9.7)	(15.4)	(13.3)	
	9.9 - 38.0	3.3 - 11.2	2.8 - 33.7	8.6 - 39	11.3 - 22.1	**p<0.001***
sulfatation activity (fmol min^{-1} μg^{-1})	0.3 ± 0.3	1.5 ± 1.2	1.5 ± 1.4	1.3 ± 0.6	1.0 ± 0.7	F(4,16)= 1.1
	(0.3)	(1.1)	(1.4)	(1.4)	(1.0)	
	0.01 - 0.71	0.40 - 3.16	0.04 - 3.16	0.45 - 1.76	0.46 - 1.74	p=0.392
glucuronidation activity (fmol min^{-1} μg^{-1})	6.9 ± 2.5	8.3 ± 3.8	9.9 ± 3.3	3.3 ± 2.2	6.1 ± 1.4	F(4,16)= 2.9
	(6.4)	(8.9)	(10.6)	(2.2)	(6.0)	
	3.1 - 8.3	3.6 - 11.6	6.6 - 11.6	0.2 - 8.7	4.6 - 7.9	*p=0.057*

The histomorphometrical analysis of the thyroid tissue revealed differences in epithelial cell heights (ANOVA $p<0.05$). The follicles observed in sea bass from the costal region near the rivers Charente and Loire were larger and surrounded by higher epithelial cells than those measured in sea bass from the coastal regions near the Seine and the Scheldt (Fig.2). However, follicle size, roundness, form factor and aspect ratio remained similar between locations (ANOVA $p>0.05$) (Table 1).

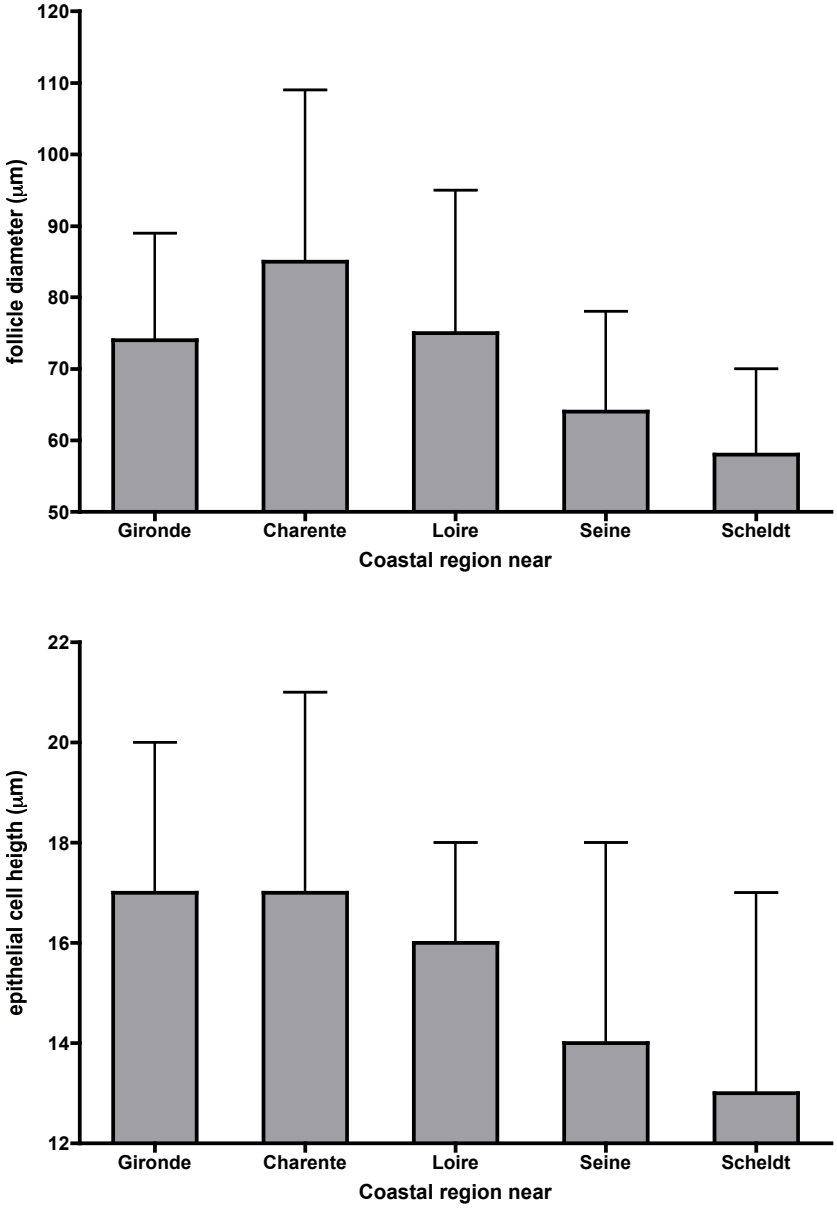

Figure 2 : Differences in thyroid histomorphometry (mean follicle diameter (µm) and mean epithelial cell height (µm)) in sea bass from coastal regions near European river mouth

Outer ring deiodination (ORD) activities were detected in sea bass livers samples and differed significantly between locations (ANOVA $p<0.001$). The activity of ORD was significantly lower in sea bass from Charente and Loire coastal regions whereas the highest activities were measured in the liver of sea bass from the Seine and the Scheldt (Table 1). Glucuronidation and sulfation were similar between locations (ANOVA $p>0.05$).

Relationships between thyroid endocrine status and contaminant exposure

Environmental levels of higher chlorinated PCB congeners (IUPAC nos. 101, 153, 170, 180, 183, 194 and 195) correlated negatively with the T_4 and T_3 concentrations in skeletal muscle. Levels of CB congeners 52 70, 87, 95, 101, 110, 118, 128, 138, 153, 156, 170, 180, 183, 187, 194 and 195 correlated positively with hepatic ORD activity. Levels of DDD metabolites correlated negatively with skeletal muscle T_3 concentrations, and positively hepatic ORD activity. HCHs concentrations correlated positively with muscular T_4 and T_3 concentrations and negatively with the hepatic ORD activity. Finally we observed a negative correlation between the muscular T_3 concentration, and a positive correlation between hepatic ORD activity and dieldrin levels, whereas aldrin has no effect on any thyroid parameter measured. With respect to the other thyroid parameters (i.e., thyroid gland morphology, glucuronidation and sulfation) no relationship with environmental contaminants could be revealed in this study.

A principal component analysis permitted us to reduce the 24 organochlorine compound variables (18 PCB congeners IUPAC nos. 52, 70, 87, 95, 101, 110, 118, 128, 138, 153, 156, 170, 180, 183, 187, 194 and 195 and the pesticides *pp'*-DDT, *pp'*-DDE, *pp'*-DDD, αHCH, βHCH, χHCH and dieldrin), identified to have an effect on the thyroid system, to four principal components. The four components explain 77.7% of the total variance. The first component represents the PCB congeners with a lower degree of chlorination (tetra-, penta- and hexachlorobiphenyls), whereas the second component represents higher chlorinated

PCB congeners (hepta- and octachlorobiphenyls) and *pp'*-DDT and *pp'*-DDE. The third component regroups the 3 HCH isomers and the fourth represents mainly the *pp'*-DDD metabolite (Table 2).

Correlation tests were used to evaluate the effects of the different components on the thyroid parameters. No significant relationship could be revealed between the first component and the thyroid parameters. The second component revealed several significant relationships, a negative correlation with the muscular T_3 concentration (R=-0.23 p=0.030), a strong positive correlation with the hepatic ORD activity (R=0.42 p<0.001) and a negative correlation with the sulfation activity (R=-0.48 p=0.045) (Fig. 3).

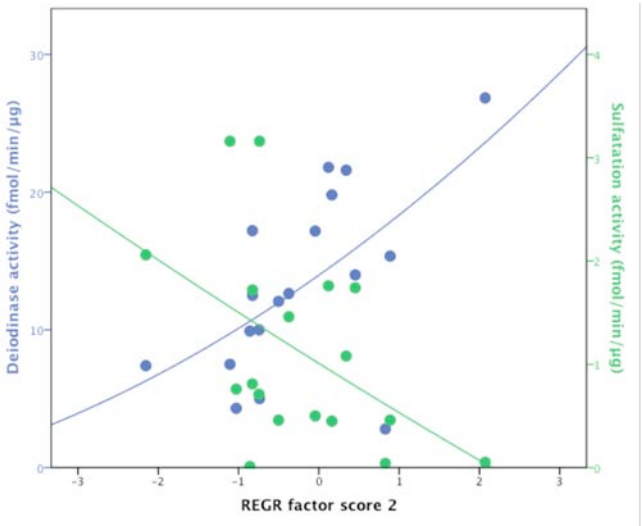

Figure 3 : Correlation between REGR factor score 2 (reflecting PCBs concentration µg.g-1 ww, (Schnitzler et al., submitted)) and the hepatic deiodinase and sulfation activity

Significant positive correlations were observed between the third component and the muscular T_4 and T_3 concentrations (R=0.39 and 0.49 respectively p<0.001) and a negative correlation with the hepatic ORD activity (R=-0.33 p=0.002). Finally the fourth component could be associated to the muscular T_3 concentration (R=-0.31

p=0.004), the epithelial cell height and follicle roundness (R=-0.30 and R=0.35 respectively p<0.05) (Table 2).

Table 2 : Principal component analysis, with the explained variance by the principal components, the classification of the different contaminants and the results of the multivariate correlation tests

	Fact. 1	Fact. 2	Fact. 3	Fact. 4
% of total variance	30.3	27.0	11.5	8.9
cumulative variance %	30.3	57.3	68.8	77.7
IUPAC#52	**0,47**	0,37	-0,20	0,37
IUPAC#70	**0,83**	0,16	0,05	0,41
IUPAC#87	**0,69**	0,17	-0,07	0,57
IUPAC#95	**0,84**	0,28	0,02	0,32
IUPAC#101	**0,83**	0,36	-0,26	0,17
IUPAC#110	**0,81**	0,30	0,00	0,00
IUPAC#118	**0,86**	0,25	-0,05	0,08
IUPAC#128	**0,79**	0,41	-0,12	-0,12
IUPAC#138	0,58	**0,74**	-0,01	-0,15
IUPAC#153	**0,56**	0,24	-0,55	-0,40
IUPAC#156	0,67	0,53	0,03	0,13
IUPAC#170	0,24	**0,88**	-0,14	0,27
IUPAC#180	0,16	**0,92**	-0,06	0,20
IUPAC#183	0,39	**0,85**	-0,05	0,04
IUPAC#187	0,49	**0,78**	0,00	-0,14
IUPAC#194	0,27	**0,83**	-0,21	0,13
IUPAC#195	0,20	**0,76**	-0,31	0,32
p'p-DDT	0,30	**0,62**	-0,18	0,48
p'p-DDE	0,16	**0,38**	0,22	0,05
p'p-DDD	0,21	0,24	-0,10	**0,78**
a-HCH	-0,16	-0,12	**0,82**	0,01
b-HCH	0,04	-0,04	**0,74**	-0,18
c-HCH	-0,09	-0,04	**0,88**	-0,05
dieldrine	**0,77**	0,24	-0,27	0,17
T4 (ng g^{-1})	R= -0.076 n= 87 p= 0.482	R= 0.055 n= 87 p= 0.613	R= 0.391 n= 87 **p< 0.001**	R= -0.026 n= 87 p= 0.811
T3 (ng g^{-1})	R= 0.059 n= 87 p= 0.588	R= -0.232 n= 87 **p= 0.030**	R= 0.490 n= 87 **p< 0.001**	R= -0.307 n= 87 **p= 0.004**
follicle diameter (µm)	R= -0.101 n= 44 p= 0.515	R= 0.027 n= 44 p= 0.861	R= 0.115 n= 44 p= 0.459	R= -0.232 n= 44 *p= 0.129*
cell height (µm)	R= -0.094 n= 44 p= 0.543	R= 0.103 n= 44 p= 0.507	R= 0.180 n= 44 p= 0.242	R= -0.300 n= 44 **p= 0.047**
roundness	R= 0.120 n= 44 p= 0.404	R= -0.116 n= 44 p= 0.454	R= -0.039 n= 44 p= 0.802	R= 0.161 n= 44 p= 0.307
form factor	R= -0.193 n= 44 p= 0.210	R= -0.027 n= 44 p= 0.863	R= 0.266 n= 44 *p= 0.081*	R= -0.088 n= 44 p= 0.578
aspect ratio	R= 0.059 n= 87 p= 0.588	R= -0.232 n= 87 **p= 0.030**	R= 0.490 n= 87 **p< 0.001**	R= -0.307 n= 87 **p= 0.004**
deiodinase activity (fmol min^{-1} μg^{-1})	R= 0.179 n= 87 *p= 0.106*	R= 0.424 n= 87 **p< 0.001**	R= -0.334 n= 87 **p= 0.002**	R= 0.046 n= 87 p= 0.682
sulfatation activity (fmol min^{-1} μg^{-1})	R= 0.435 n= 20 *p= 0.071*	R= -0.478 n= 20 **p= 0.045**	R= -0.092 n= 20 p= 0.715	R= -0.072 n= 20 p= 0.775
glucuronidation activity (fmol min^{-1} μg^{-1})	R= 0.119 n= 20 p= 0.639	R= -0.100 n= 20 p= 0.693	R= 0.401 n= 20 *p= 0.099*	R= 0.433 n= 20 *p= 0.073*

Discussion

Sea bass can accumulate various PCB congeners and other chlorinated compounds in their tissues. The levels measured in sea bass are generally the highest measured in edible marine fish species (Naso et al., 2005). This probably reflects their feeding habits and the nature of the habitat of these benthic and euryhaline species which usually inhabits shallow waters with sandy or muddy bottoms along the coast, and ports and estuaries, which are generally considered to be more heavily polluted than open waters (Lewis et al., 2002; Loizeau, 2001). Recently, we showed that pesticide levels in juvenile sea bass from Scheldt and Seine are higher than in sea bass collected near the Loire, Charente and Gironde (Schnitzler et al., submitted), and it can be concluded that sea bass tissue pesticide concentrations reflect the environmental pesticide load.

Intersite differences in thyroid parameters

The muscular thyroid hormone concentrations are in the same range as those observed in previous studies on sea bass (Schnitzler et al., 2008), and are comparable to other investigations into tissue thyroid hormone concentrations. Total T_4 and T_3 concentrations have been measured in extracts from whole eggs, yolk, larvae and fry of salmonids (de Jesus and Hirano, 1992; Leatherland et al., 1989a; Leatherland et al., 1989b; Tagawa and Hirano, 1987), flounder (Tagawa et al., 1990a), striped bass (*Morone saxatilis*, Percichthyidae) (Parker and Specker, 1990), the conger eel (*Conger conger*, Anguillidae) (Yamano et al., 1991), the tilapia (*Oreochromis mossambicus*, Cichlidae) (Weber et al., 1992) and other species (Tagawa et al., 1990b). The data are not easy to interpret because the concentrations vary between the different species, but they do support the roles of thyroid hormones in early development and during metamorphosis. Muscle contains low amounts of T_3, but owing to its large mass comprises a total tissue pool representing about 80% of all the T_3 in the rainbow trout (Fok et al., 1990). The muscular thyroid hormone concentrations should thus provide a good index of the thyroid hormone reserves in fish.

Thyroid parameters differed between sampling locations suggesting environmental influence on endocrine activities. Levels of thyroid hormones can be influenced by many other factors including age, gender, diet, nutritional status, season and physiological condition (Rolland, 2000). We therefore designed the study to control for these variables, i.e. sampling was carried out in a short period of time in autumn when the weather conditions were fairly stable. As neither body size (length and weight) nor sex had a significant interaction with any endocrine parameters in this study, endocrine disrupting chemicals are likely to be a causative independent variable. Indeed, sea bass from the more contaminated locations, showed lower muscular thyroid hormone concentrations, smaller follicles surrounded by epithelial cells with a less pronounced cell height, and with a higher hepatic ORD activity.

Relationships between thyroid endocrine status and contaminant exposure

Multivariate analysis revealed complex interactions between previously published pollutant concentrations (Schnitzler et al., submitted) and thyroid parameters. The current study demonstrates that exposure to a mixture of organochlorine compounds has effects on thyroid hormone status in juvenile sea bass. The muscular T_3 and T_4 concentration were lower in sea bass from more polluted areas (coastal regions near river Seine and Scheldt) (Figure 4). This observation correlates with the chlorination level of PCB congeners and the DDTs concentrations. In vertebrates, thyroid gland activity is homeostatically regulated by the brain (hypothalamus) – pituitary – thyroid axis. A key thyrotropic signal is thyroid stimulating hormone (TSH) from the pituitary pars distalis, which controls synthesis, storage and secretion of T_4 in a classical negative feedback system (Blanton and Specker, 2007; Brown et al., 2004; Zoeller et al., 2007). As we observed no changes in histological appearance, especially no thyroid hyperplasia, we could conclude that the HPT-axis was not affected.

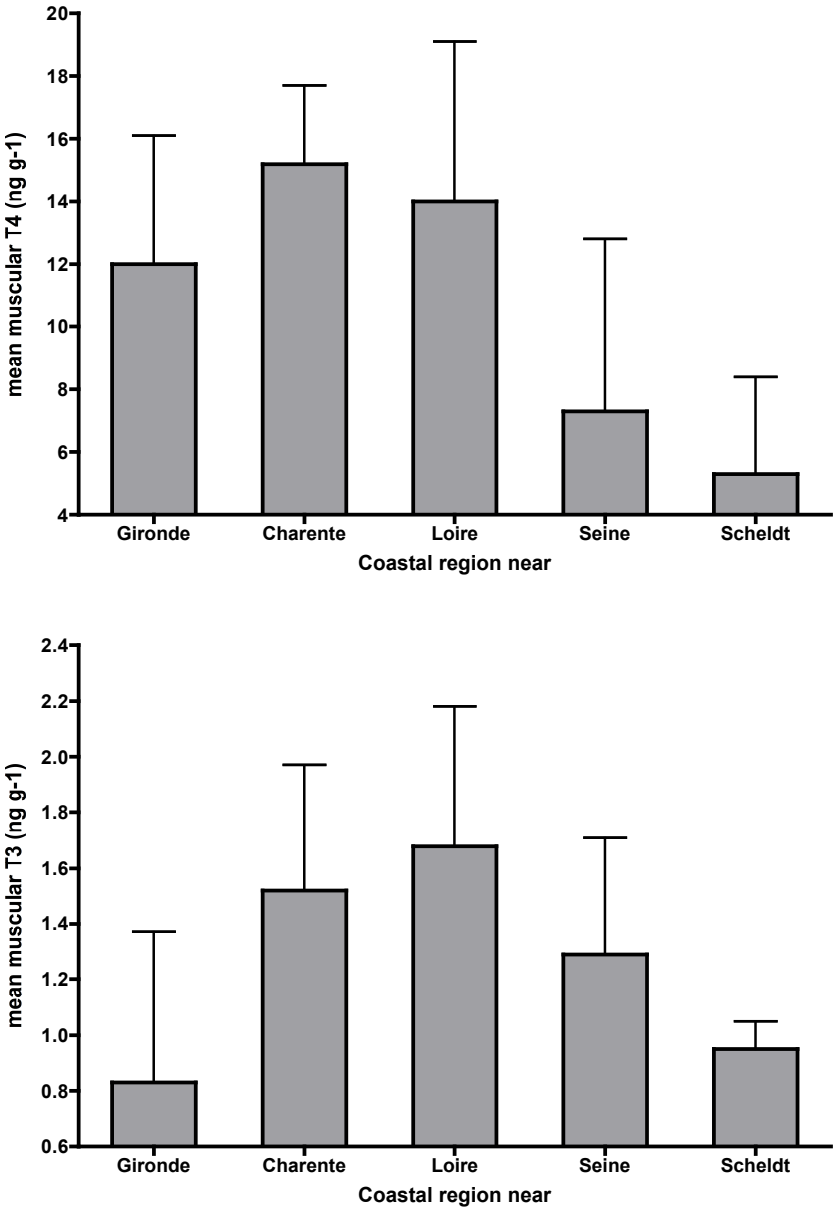

Figure 4 : Differences in muscular thyroid hormone concentration (mean T4 (ng g^{-1}) and mean T3 (ng g^{-1}) concentrations) in sea bass from coastal regions near European river mouth

Peripheral T_3 levels in teleost fish are largely controlled by enzymatic 5'-deiodination activities in extra-thyroidal tissues such as liver that control the conversion of T_4 to the biologically active form T_3, and other enzymes involved in extra-thyroidal metabolism (Brown et al., 2004). A slight depression in deiodinating activities can have severe consequences, as kinetic studies showed that about 80% of the T_3 in salmonids reside in a slowly exchanging reserve pool, mainly represented by skeletal muscle (Brown et al., 2004). Our findings support the hypothesis of the disturbance of the peripheral control of thyroid hormone homeostasis. Higher chlorinated PCB congeners and DDTs increased hepatic T_4ORD activity while decreasing the hepatic sulfation activity. The PCB induced changes in deiodinating activity likely represent compensatory responses to disrupting effects that might otherwise have depressed the T_3 levels (Adams et al., 2000).

Thyroid hormones are conjugated by sulfation and glucuronidation. These Phase II reactions are usually detoxification in nature, and involve the interactions of the polar functional groups of phase I metabolites (Schuur et al., 1999). Products of conjugation reactions have an increased water solubility and are usually inactive. Sulfotransferases inactivate thyroid hormones and facilitate their excretion in bile and urine. Furthermore, thyroid hormone sulfates do not bind to T_3 receptors and are thus unable to mimic T3 activity and are rapidly degraded by inner ring deiodinases (Brouwer et al., 1998; Schuur et al., 1999). We observed a slight decrease of SULT activity in liver of sea bass from higher contaminated regions. This is in accordance with in vitro studies using rat and human hepatoma cell lines that related a strong inhibition of thyroid hormones sulfation by hydroxylated metabolites of PCB (Brouwer et al., 1998; Schuur et al., 1999).

While the DDT metabolites exert the same effects on muscular thyroid hormone concentrations and deiodination activity, but especially the DDD metabolite seem to induce changes in thyroid histological appearance. Sea bass from higher DDD contaminated regions showed a decrease in thyroid cell height and depletion of

colloid. Similar effects are reported in mullet (*Liza parsia*) exposed to DDT (Brown et al., 2004).

The isomers of hexachlorocyclohexane (HCHs), had opposite effects on the thyroid system. Similar results are reported from catfish (*H. fossilis*), HCHs induced an increase of muscular T_4 and T_3 levels while the hepatic T_4ORD activity was reduced and no changes in histological appearance could be related (Yadav and Singh, 1987). Whereas no effect on thyroid hormone levels could be reported from rainbow trout (*Oncorhynchus mykiss*) (Aldegunde et al., 1999) and thyroid hypertrophy was reported in medaka (*Oryzia latipes*) (Wester and Canton, 1988) in association with an HCH exposure. The HCHs contribute only for less than 0.5% of the total muscular organochlorine contamination of the tested fish.

Conclusions

In this study we established correlations between contaminant concentrations and effects on the thyroid endocrine system in sea bass from the major European coastal regions. The exposure to environmental doses of pollutants alters hepatic T_4 outer ring deiodinase and T_4 sulfation whereas T_4 glucuronidation was not affected. Muscular T_4 levels were preserved despite a slight contaminant-induced diminution in T_3 concentration, which probably reflects a tight homeostatic control of T_4. These changes in dynamics lead to an increased conversion of T_4 to T_3 and a reduced excretion of thyroid hormones. The alteration of metabolic pathways of thyroid hormones can be interpreted as a pathway to homeostatically maintain thyroid hormone status. Of all tested compounds, the higher chlorinated PCBs seemed to be the most implicated in this perturbation. These metabolic pathways have a certain fitness cost and could ultimately be manifested at the population community, or ecosystem level of biological organisation. Further analysis, especially laboratory experiments, are required to evaluate the underlying mechanisms and effects of such endocrine disruption and should help to get a better understanding on these complex interactions.

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Chapter 4

Underlying mechanisms and effects of polychlorinated biphenyls on thyroid hormone physiology and metabolism

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Abstract

The current study examines the effect of subchronic exposure to a mixture of Aroclor standards on thyroid hormone physiology and metabolism in juvenile sea bass. After 120 days of exposure, histomorphometry of thyroid tissue, muscular thyroid hormone concentration and activity of enzymes involved in metabolism of thyroid hormones were assessed. The results show that the effects of PCB exposures on the thyroid system are dose-dependent. Exposure to environmentally relevant doses of PCB (0.3 to 1.0 μg Σ7PCBs per g food pellets) increases thyroid hormone synthesis and stimulates hepatic T_4 outer ring deiodinase and T_4 sulfation. Thyroid hormone tissue levels were preserved thanks to the PCB induced changes in T_4 dynamics. At 10 times higher concentrations (10 μg Σ7PCBs per g food pellets) an important depression of T_3 and T_4 levels could be observed which are apparently caused by degenerative histological changes in the thyroid tissue.

Keywords

Dicentrarchus labrax, polychlorinated biphenyls, thyroid hormones, deiodination, glucuronidation, sulfation, *in vivo* exposure

Introduction

In a recent field study we have established correlations between exposure to organochlorine contaminants and thyroid function in wild sea bass from European coasts (Schnitzler et al., submitted). Multivariate statistical analysis specifically revealed the involvement of higher chlorinated PCBs in thyroid dysfunction. Indeed, fishes with higher PCB concentrations displayed alterations in metabolic pathways, *viz*. deiodination and sulfation, that affect circulating and tissue thyroid hormone levels, (Schnitzler et al., submitted).

The mechanisms of how endocrine disruptors alter thyroid function have been extensively investigated but are still not fully understood. The regulatory pathways involved in thyroid homeostasis are numerous and complex. As a consequence environmental chemicals can act at many levels in the thyroid system (Ishihara et al., 2003). There are at least three independent, but possibly interacting, mechanisms that may explain the ability of PCB to reduce circulating and tissue levels of thyroid hormones. First, PCBs have been shown to change thyroid gland structure, possibly directly interfering with thyroid gland function (Collins and Capen, 1980b) and disrupting directly the hormone synthesis in the thyroid gland (Boas et al., 2006; Brown et al., 2004a; Ishihara et al., 2003). PCBs may directly interfere with the ability of the thyroid gland to synthesize thyroid hormones, by altering mechanisms involved in active accumulation of iodide and proteolysis of thyroglobulin. Second, PCBs can target thyroid hormone metabolism. They may affect extrathyroidal iodothyronine deiodinases, enzymes that control the conversion of thyroid hormones and are thus essential in the regulation of levels of biologically active T_3 locally and systemically (Ishihara et al., 2003; Zoeller et al., 2007). It has been shown that PCB exposure increases bile flow rate as well as the biliary excretion of T_4 (Collins and Capen, 1980a). PCB exposure also induces the expression and activity of the phase-II enzymes glucuronosyltransferase and sulfotransferase that also utilize thyroid hormones as conjugate group acceptors and increase T_4 conjugation (Klaassen and Hood, 2001; Visser et al., 1993). These actions facilitate T_4 clearance by hepatic

metabolism, reducing the biological half-life of T_4. Finally, PCBs competitively bind to thyroid hormone binding proteins in blood like transthyretin (TTR) (Boas et al., 2006; Ishihara et al., 2003; Wade et al., 2002) and can potentially displace thyroid hormones from their carrier molecules. Moreover these may interact to produce summative effects. Besides these direct effects, indirect effects via disruption of thyroid hormone receptors and accessory proteins that directly control the gene expression through thyroid hormone responsive elements can also interfere with the thyroid system (Blanton and Specker, 2007; Ishihara et al., 2003).

Disruption of thyroid function can have severe consequences as thyroid hormones play an important role in the maintenance of a normal physiological status in vertebrates. In adult fish, thyroid hormones are of primary importance in the regulation of such fundamental physiological processes as growth, nutrient utilization, and reproduction. Fish grow faster and are healthier when thyroid hormone levels are adequate (Power et al., 2001; Yamano, 2005), providing an economic rationale to study thyroid disruptors in a fishery and aquaculture context. This explains our choice of the test species, European sea bass (*Dicentrarchus labrax*), as it is an important commercial species, top predator of a simple food web, commonly found in European coastal waters, and with a well documented biology (Loizeau et al., 2001; Pickett and Pawson, 1994).

Polychlorinated biphenyls have been shown to alter thyroid hormone levels in experimental animals, including fish (Brouwer et al., 1989/7; Collins and Capen, 1980a, b; Fowles et al., 1997; Hallgren, 2001, 2002). Most studies on fish thyroidology have involved ambient concentrations of xenobiotics delivered at sublethal, concentrations that, however, are still higher than those encountered in the field (Blanton and Specker, 2007; Brown et al., 2004a). Our study aimed to gain an integrated insight into the effects of a 4-month *in vivo* exposure to various environmentally relevant doses of commercial mixtures of polychlorinated biphenyls (PCBs) on the thyroid system of *Dicentrarchus labrax*. Several endpoints were analyzed simultaneously: thyroid gland histology, hepatic 5'-deiodination (or outer ring

deiodination, ORD) activities that convert the thyroid prohormone T_4 to the bioactive hormone T_3, and muscular T_4 and T_3 levels. In addition, two biochemical pathways i.e. sulfation and glucuronidation, involved in thyroid hormone metabolism and phase-2 response to toxicants, were assayed. This approach allowed us to determine underlying mechanisms and dose dependency of the effects of these pollutants on the thyroid system of these fish, and to examine the consequences of a potential disruption of the thyroid system on growth performance and condition factor in these commercially important fish species.

Methods

Food preparation

The contaminant mixture was formulated to reflect the persistent organic pollution to which the European sea bass population could conceivably be exposed. Loizeau *et al.* 2001 caught suprabenthic species (gobies, shrimps and mysidaceans) that are potential preys of sea bass (Loizeau, 2001) using a trawl in the Seine estuary. Based on the reported the PCB concentrations (on dry weight basis with standard deviation), we calculated the Σ of the 7 marker PCB congeners (IUPAC nos. 28, 52, 101, 118, 138, 153, 180). A field study on sea bass from European coastal regions revealed PCB patterns dominated by a large contribution from the hepta-, hexa- and pentachlorinated PCBs (Schnitzler et al., submitted). These congeners are the most abundant due to their widespread use in commercial mixtures such as Aroclor 1254 and 1260. We therefore decided to work with a 1:1 mixture of Aroclor 1254 and 1260 in concentration ranges of the 7 ICES marker congeners from 300 to 1000 $ng \cdot g^{-1}$ food. These reflect the observed concentration in common sea bass prey (Table 1).

Table 1: Contamination levels for the 7 tracer PCB congeners in current prey of *D. labrax* and in artificially Aroclor 1254 and 1260 contaminated food (According to: Loizeau, V., Abarnou, A., Ménesguen, A., 2001. A Steady-State Model of PCB Bioaccumulation in the Sea Bass (*Dicentrarchus labrax*) Food Web from the Seine Estuary, France Estuaries Vil.24, No 6B, p 1074-1087.)

		28	52	101	118	153	138	180	sum 7 PCB
Preys	*N. Integer*	12.5 ± 1.4	40.2 ± 4.2	65.1 ± 6.6	53.3 ± 5.4	119.6 ± 12.0	94.9 ± 10.0	59.0 ± 6.0	444.6 ± 35.1
	P. Microps	9.3 ±1.0	36.5 ± 3.3	74.6 ± 7.2	71.5 ± 6.9	146.5 ± 15.0	121.5 ± 12.8	44.0 ± 4.6	503.9 ± 48.3
	P. Longirostris	5.6 ± 0.6	29.2 ± 3.1	23.2 ± 2.0	52.6 ± 5.4	96.4 ± 10.0	75.2 ± 8.1	51.2 ± 5.3	333.4 ± 31.3
	C. Crangon	8.4 ± 0.5	31.2 ± 3.3	26.5 ± 2.1	59.7 ± 6.1	156.4 ± 16.0	131.5 ± 13.6	81.9 ± 9.0	495.6 ± 55.8
	D. Labrax	10.3 ± 1.5	44.1 ± 4.8	126.5 ± 13.0	144.8 ± 15.0	338.8 ± 35.0	298.7 ± 27.1	131.3 ± 12.7	2397.7 ± 502,4
Food	Control	5.7 ± 2.4	10.6 ± 3.0	4.7 ± 1.2	1.4 ± 0.5	2.5 ± 0.5	1.9 ± 0.3	0.3 ± 0.1	27.1 ± 3.5
	0.3 µg.g-1	5.7 ± 0.1	29.8 ± 0.7	55.6 ± 0.8	36.2 ± 0.9	87.3 ± 3.5	73.0 ± 2.6	41.2 ± 1.6	328.8 ± 27.5
	0.6 µg.g-1	5.9 ± 0.6	52.0 ± 0.1	105.0 ± 4.0	74.9 ± 1.6	170.4 ± 4.5	139.4 ± 4.4	81.2 ± 3.3	628.7 ± 54.7
	1.0 µg.g-1	10.0 ± 2.1	79.9 ± 12.0	167.2 ± 18.9	121.5 ± 12.0	278.6 ± 30.0	226.7 ± 23.4	136.9 ± 17.4	1020.8 ± 89.5
	10 µg.g-1	82.3 ± 5.6	825.9 ± 110.7	1907.6 ± 219.1	1342.7 ± 141.2	3106.4 ± 312.3	2579.8 ± 263.4	1549.9 ± 1023.9	11394.6 ± 1023.9

Appropriate chemicals were added to 100 mL hexane and 100-g portions of commercial fish food (T-2P Classic. Trouw. France) into a 1000 ml round bottom flask. The mixture was slowly stirred by a rotary evaporator (water bath at 60 °C, refrigeration at 5 °C and pressure at 875 Pa) till dryness (ca. 1 h). The resulting food spiked with chemicals was then thoroughly dried at 40 °C overnight. The food pellets kept their initial form and consistency. Control food was prepared in the same manner aside from adding the test mixture.

The concentration for the Σ7 ICES PCB congeners in pellets designed for the five different exposure conditions was measured, and the obtained results are: (1) 27 $ng \cdot g^{-1}$ (assigned label: Control), (2) 329 $ng \cdot g^{-1}$ (assigned the nominal value of 0.3 $\mu g \cdot g^{-1}$ dw), (3) 629 $ng \cdot g^{-1}$ (assigned value 0.6 $\mu g \cdot g^{-1}$ dw), (4) 1021 $ng \cdot g^{-1}$ (assigned value 1 $\mu g \cdot g^{-1}$ dw) and (5) 11395 $ng \cdot g^{-1}$ (assigned value 10 $\mu g \cdot g^{-1}$ dw). The correlation factor between nominal and effective concentrations is 0.9999 (Table 1).

Husbandry

Experimental trials were conducted in the Biology Department of Antwerp University, Belgium. Seventy-five juvenile sea bass (*Dicentrarchus labrax*, L.) were obtained from a commercial fish farm (Ecloserie marine de Gravelines, Gravelines,

France). Their body mass ranged from 7 to 20 g (mean 13.2 ± 2.8 g). Fish were housed in 200-L tanks with a natural photoperiod. The water temperature was maintained at 15°C during the experiment. Water aeration was set to maintain 100% oxygen saturation. The water was continuously filtered through mechanical, charcoal and extensive biological filters before being recycled.

Fish were randomly assigned to a control group and four treatment groups (group size n=15 in each case) that received contaminated food at 0.3, 0.6, 1.0 and 10.0 $\mu g \cdot g^{-1}$ (Σ of [7 ICES PCBs] per g of food pellets), respectively. Fish were fed spiked food for 120 days. The daily feeding ration was 2.0% of the mean body mass of the fish, adjusted after each sampling period based on mean weight of the sub-sample fish that were sacrificed. Feed was presented by sprinkling at the surface of the water and was generally completely consumed by each group of fish within 1 minute. Five fish were sampled from each tank on days 40, 80 and 120. Fish were always sampled 24 hours after the previous feeding. Weight and length were measured and the specific growth rate (SGR=100%*(ln final weight – ln initial weight) / total days) and condition factor (CF= weight*100 / length^3) were calculated. The subpharyngeal area, gills, liver, kidney and gonads were removed and immersed in formalin fixative (VWR International BVBA). Approximately 10 g of skeletal muscle was excised caudally of the head, dorsal to the lateral line and anterior to the dorsal fin. Muscle and liver samples were frozen immediately on dry ice and stored at -80 °C until analysis.

Organic contaminant analysis

Standards and reagents

All individual PCBs and pesticides standards were obtained from Dr. Ehrenstorfer Laboratories GmbH (Augsburg, Germany). Acetone, *n*-hexane (Hex), dichloromethane, and isooctane were of pesticide-grade (Merck, Germany). Anhydrous sodium sulfate, basic aluminium oxide, and silica gel (Merck) were used after pre-washing with Hex and heating overnight at 120 °C. An accelerated Soxhlet

extractor B-811 (Buchi, Switzerland) was used for the extraction of target compounds from fish tissues and feed.

Sample preparation and analysis

A fish fillet was thawed at room temperature, and approximately 1 g was precisely weighted, ground with 20 g anhydrous sodium sulfate and placed into an extraction thimble. After addition of internal standards (PCB 46 and PCB 143), the mixture was extracted for 2.5 h by hot Soxhlet with 80 mL of hexane/acetone = 3:1 (*v/v*). The extract was subjected to cleanup on 5 g of acid silica (44% sulfuric acid, *w/w*). Hexane (20 mL) was used for the complete elution of PCBs. The final eluate was concentrated under nitrogen until 100 μL and transferred to a GC vial.

One μL was injected in pulsed splitless mode on a Hewlett-Packard 6890 GC connected via direct interface to a HP 5973 mass spectrometer. A 50 m × 0.22 mm × 0.25 mm, HT-8 capillary column (SGE, Zulte, Belgium) was used with helium as carrier gas at a constant flow of 0.7 mL/min. Injector and interface temperatures were set at 270 and 300 °C, respectively. The oven temperature program began at 90 °C, kept 1 min, and then increased with 15 °C/min to 170 °C, held for 3 min, then increased at 4 °C/min to 270 °C, held for 1 min, and was further increased at 10 °C/min to 290 °C and held for 15 min. The mass spectrometer was operated in electron impact ionization mode. Two most abundant ions were monitored for each level of chlorination for PCBs. Method limits of detection (LOD) for individual PCB congeners ranged between 0.1 and 0.5 ng/g lipid. Recoveries of target compounds ranged between 72% and 80%. We investigated 38 PCB congeners (IUPAC nos. 18, 31, 28, 52, 49, 47, 44, 74, 95, 101, 99, 87, 110, 118, 105, 151, 149, 146, 132, 153, 138, 128, 156, 187, 183, 174, 177, 171, 172, 180, 170, 199, 196/203, 195, 194, 205, 206 and 209) in all 75 muscle samples, and 21 metabolites (IUPAC nos. 4-HO-CB119, 4-HO-CB120, 3HO-CB118, 4HO-CB109, 3HO-CB153, 4HO-CB146, 4HO-CB127, 3HO-CB138, 4HO-CB130, 4HO-CB163, 4HO-CB187, 4-HO-CB162, 4-HO-CB202, 4-HO-CB177, 3HO-CB180, 4HO-CB172, 4HO-CB193, 4-

HO-CB198, 4-HO-CB-199, 4-diHO-CB202 and 4-HO-CB208) in five muscle and liver samples.

The method was submitted to regular quality assurance and control procedures. Retention times, ion chromatograms, and intensity ratios of the monitored ions were used as identification criteria. A deviation of the ion intensity ratios within 20% of the mean values of the calibration standards was considered acceptable. The method performance was assessed through rigorous internal quality control, which included a daily check of calibration curves and regular analysis of procedural blanks and certified material CRM 350 (PCBs in mackerel oil). The method was tested by regular participation to interlaboratory tests organized by the US National Institute of Standards and Technology (NIST) for the determination of PCBs in biological samples. The results of the individual PCB congeners deviated less than 20% from the target values.

Thyroid parameters

The detailed materials and methods used in the study of the thyroid parameters, including *Standards and reagents*, *Muscular thyroid hormone determinations*, the activity measurements of *sulfotransferase*, *UDP glucuronyltransferase (UGT)* and *outer ring deiodinase (ORD)* and *histomorphometric analysis of the thyroids* are described in Chapter 3 on pages 68 to 71.

Ultrastructural analysis

A selection of thyroid tissue was glutaraldehyde-fixed and then embedded in epoxy resin. Ultra-thin sections were obtained using a diamond knife on a Reichert–Jung ultra-microtome (Ultracut E), contrasted with uranyl acetate (alcoholic solution) and lead citrate, and observed in a Jeol JEM 100-SX electron microscope at 80 kV of accelerating voltage.

Calculations and statistics

Mean values ± standard deviation, (median) and min-max are presented, unless indicated otherwise. Statistical analysis of the data was performed using SPSS for Mac® software (SPSS Inc., version 16.0.2). The Kolmogorov–Smirnoff test was used to test for normality of the statistically treated variables. Treatment group comparisons of the thyroid parameters were done by analysis of variance (ANOVA) to compare means. The relationships between thyroid parameters (follicle histomorphometry, thyroid hormone concentrations and metabolic pathways) and toxicological data were analysed by correlation tests. Growth curves were compared by a distribution-free statistical methodology. Results were considered significant when $p < 0.05$.

Results

No differences in length, weight and condition factor were found between PCB exposed and control sea bass. However the growth curve analyses showed that the distribution of weight are not identical between groups (χ^2= 30.5, dl= 12, p= 0.002) (Figure 1). This observation is also illustrated by the specific growth rate, which is lower in higher exposed fish [1.0 and 10 $\mu g.g^{-1}$ dw] (mean SGR <0.45±0.09) compared to control (mean SGR >0.60 ± 0.13) (p=0.044). Otherwise no other external sign of adverse effects of OCB exposure could be observed. All fishes were in excellent condition with a condition factor around 1.18 ± 0.06.

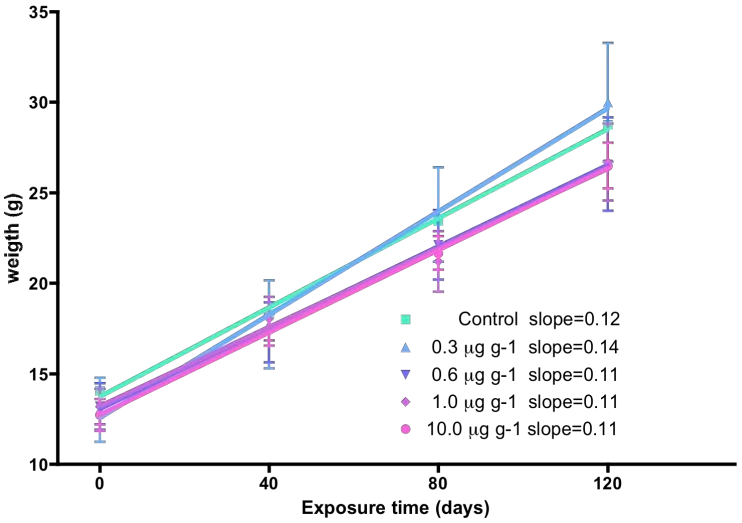


Figure 1. Growth curves of fish from different treatment groups

The initial average concentrations of PCBs measured in sea bass muscle used in accumulation experiments were close to or lower than the method LOQ (mean concentration of 10 ng·g^{-1} ww Σ 7 marker PCB congeners). This initial average concentration of PCBs did not change significantly in the muscles of sea bass from the control tank during the experimental period. On the contrary, muscular PCBs concentrations gradually increased with exposure time in all accumulation experiments and no steady state was observed up to the end of the experiment (Figure 3). The penta-, hexa- and hepta-CBs represented 88% of the PCB congeners and these top ten congeners were found in decreasing importance: CB 153 > CB 180 > CB 138 > CB 149 > CB 101 > CB 110 > CB 95 > CB 118 > CB 187 (Figure 2). The muscular concentrations of the different congeners correlated strongly and with the ΣPCBs ($R=0.65$-0.99, $p<0.001$), as the contamination mixture was formulated from the same stock solution. As a consequence, congener-specific analysis of effects on thyroid system came out with identical results. We therefore present in the following effect analysis, the correlation between effects and the ΣPCBs.

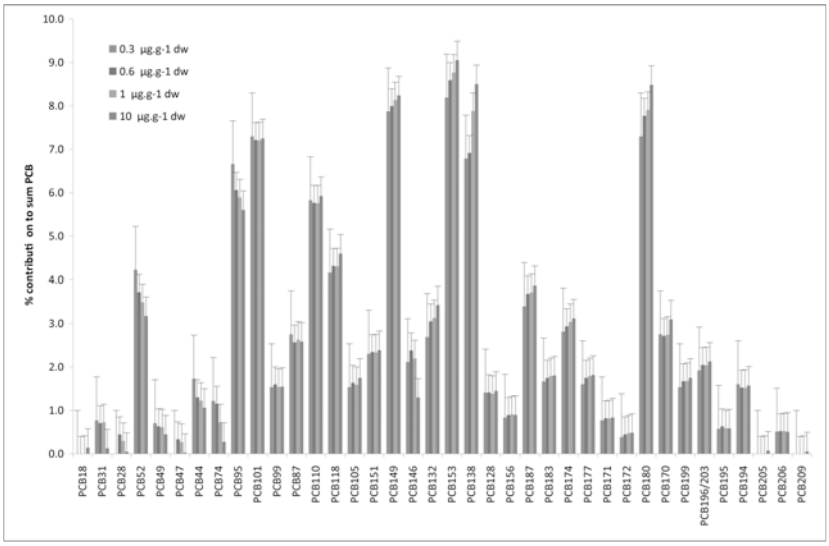


Figure 2. Distribution of individual PCB congeners in the muscle of fish from different treatment groups

Mean concentrations of 220, 360, 530 and 5530 ng g^{-1} ww (Σ all 38 PCB congeners) were determined after 120 days exposure (Figure 3). Although PCBs are not readily metabolized by fish (Letcher et al., 2000), we investigated the presence of hydroxylated PCB metabolites in both muscle and liver fish samples collected from highest treatment group. Only few HO-PCB congeners were identified in liver, but none could be detected in muscle samples. With a mean ± standard deviation (based on 5 samples analyzed) value of 6.4 ± 3 ng/g of liver, HO-PCBs consisted of approximately 0.4% from the sum PCBs measured in the same tissues. According to their rank order of concentration, the most important metabolites (mainly high-chlorinated ones) were: 4HO-CB187 > 4HO-CB163 > 4-HO-CB-199 > 4HO-CB172 > 4HO-CB193 > 4HO-CB146 > 4-HO-CB177. Lower chlorinated PCBs (penta-hexa), despite being dominant in the diet, did not form HO-PCB metabolites at detectable levels in skeletal muscle tissue. However, these results should be interpreted with caution, due to the low number of samples (5) from high-contaminated fish (10 µg.g^{-1} dw [7 ICES PCB] in food pellets).

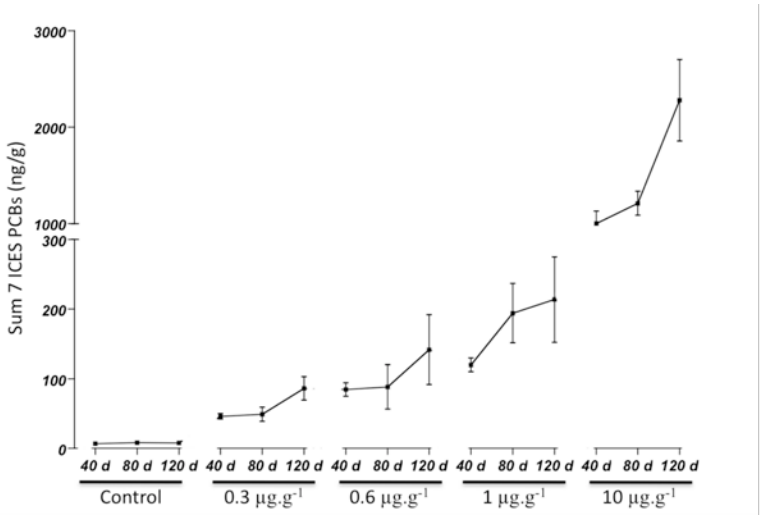


Figure 3 : Development of the muscular [7 ICES PCB] concentration as a function of time in the different exposure tanks

Muscular T_4 concentrations did not change significantly following exposure to contaminated food containing 0.3 up to 1.0 µg·g^{-1}dw [7 ICES PCB]. Muscular T_3 levels had increased 1.5 fold from 0.52 ± 0.17 to 0.76 ± 0.22 ng·g^{-1} ($F_{3,56}$=3.68, p=0.017, n=60) in fish fed control and contaminated food (1.0 ng·g^{-1} dw [7 ICES PCB]), respectively (t=-3.26, p=0.003).

Table 2 : Histomorphometric analysis, muscular thyroid hormone levels, mean hepatic metabolic activity and contamination levels in white muscle of European sea bass

Food [7 ICES PCB]	Control	0.3 µg.g-1 dw	0.6 µg.g-1 dw	1 µg.g-1 dw	10 µg.g-1 dw	ANOVA
n	15	15	15	15	15	
T4 (ng g^{-1})	9.3 ± 3.6	5.5 ± 2.7	10.0 ± 2.2	9.2 ± 3.1	2.9 ± 1.1	F(4,71)= 19.7
	(9.9)	(5.4)	(10.5)	(10.3)	(3.1)	
	1.1 - 13.9	1.6 - 11.3	4.8 - 13.0	1.3 - 13.7	0.8 - 4.6	**p<0.001***
T3 (ng g^{-1})	0.52 ± 0.17	0.56 ± 0.26	0.66 ± 0.19	0.76 ± 0.23	0.40 ± 0.14	F(4,71)= 6.7
	(0.51)	(0.54)	(0.71)	(0.83)	(0.37)	
	0.20 - 0.80	0.10 - 0.90	0.30 - 1.01	0.30 - 1.10	0.20 - 0.70	**p<0.001***
follicle diameter (µm)	92 ± 5	110 ± 14	116 ± 14	121 ± 21	116 ± 29	F(4,21)= 0.3
	(92)	(108)	(117)	(120)	(110)	
	88 - 94	98 - 126	103 - 118	92 - 169	80 - 180	p=0.897
cell height (µm)	21 ± 5	21 ± 4	22 ± 3	29 ± 3	22 ± 4	F(4,21)= 1.9
	(21)	(19)	(22)	(30)	(23)	
	18 - 25	19 - 26	20 - 25	25 - 31	18 - 25	p=0.157
roundness	0.81 ± 0.03	0.73 ± 0.01	0.77 ± 0.05	0.74 ± 0.02	0.75 ± 0.02	F(4,21)= 2.4
	(0.81)	(0.73)	(0.76)	(0.74)	(0.74)	
	0.79 - 0.83	0.73 - 0.75	0.73 - 0.83	0.72 - 0.75	0.74 - 0.77	*p=0.081*
form factor	0.83 ± 0.02	0.75 ± 0.02	0.79 ± 0.04	0.77 ± 0.01	0.76 ± 0.03	F(4,21)= 3.2
	(0.83)	(0.75)	(0.78)	(0.78)	(0.76)	
	0.82 - 0.85	0.72 - 0.76	0.76 - 0.84	0.76 -0.78	0.74 - 0.79	**p=0.034***
aspect ratio	0.98 ± 0.07	0.94 ± 0.18	1.01 ± 0.05	0.99 ± 0.07	1.23 ± 0.35	F(4,21)= 1.0
	(0.98)	(1.03)	(1.00)	(0.97)	(1.06)	
	0.93 - 1.03	0.74 - 1.06	0.98 - 1.06	0.94 - 1.08	1.00 - 1.65	p=0.433
deiodinase activity (fmol min^{-1} μg^{-1})	3.8 ± 2.8	2.8 ± 2.0	5.4 ± 3.8	6.4 ± 2.5	8.2 ± 1.3	F(4,71)= 7.0
	(2.8)	(2.2)	(4.4)	(5.3)	(7.8)	
	0.3 - 9.3	0.3 - 7.0	0.6 - 13.0	3.1 - 11.5	0.8 - 17.1	**p<0.001***
sulfatation activity (fmol min^{-1} μg^{-1})	3.2 ± 0.4	3.2 ± 1.9	2.4 ± 1.3	1.7 ± 1.0	1.5 ± 1.3	F(4,21)= 1.9
	(3.2)	(3.5)	(2.7)	(1.7)	(2.1)	
	2.5 - 3.5	0.5 - 5.7	0.6 - 3.9	0.7 - 2.8	0.1 - 2.5	p=0.154
glucuronidation activity (fmol min^{-1} μg^{-1})	7.7 ± 5.2	7.2 ± 5.7	7.1 ± 3.1	6.6 ± 3.2	5.8 ± 3.2	F(4,21)= 1.5
	(7.4)	(6.2)	(7.9)	(6.8)	(6.3)	
	1.2 - 15.0	1.3 - 15.1	3.4 - 10.0	1.4 - 9.6	1.4 - 9.1	p=0.960
Sum ICESPCB (ng g-1)	9.9 ± 10.2	60.4 ± 30.5	104.8 ± 76.8	175.9 ± 99.5	1497.0 ± 797.3	F(4,71)= 46.5
	(8.3)	(51.4)	(75.2)	(137.2)	(1234.5)	
	3.8 - 46.9	23.4 -118.6	46.2 - 338.5	79.3 - 443.1	652.6 - 3222.3	**p<0.001***
SumPCB (ng g-1)	31.3 ± 23.3	156.2 ± 76.8	268.9 ± 187.1	441.4 ± 240.5	3641.1 ± 1924.4	F(4,71)= 46.1
	(27.1)	(133.4)	(194.3)	(348.4)	(3003.7)	
	17.9 - 117.0	63.4 - 301.7	130.0 - 835.9	211.0 - 1084.1	1615.2 - 7840.7	**p<0.001***

In the case of exposure exceeding the environmental relevant range (10 ng·g^{-1} dw [7 ICES PCB]), drastically lower muscular thyroid hormone concentrations could be observed ($t=6.58$, $p<0.001$ and $t=2.03$, $p=0.045$ for T_4 and T_3 respectively) compared to control. The T_4 and T_3 concentration were reduced to 30% and 75% compared to control levels (Figure. 4 and Table 2).

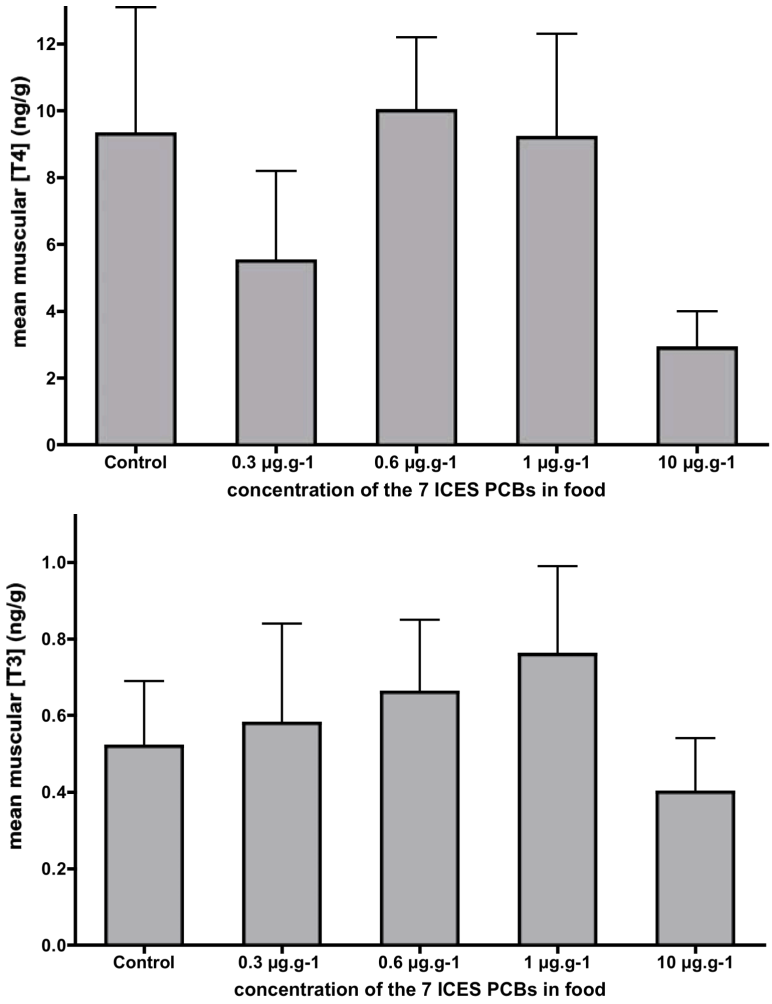


Figure 4 : Mean muscular T_4 and T_3 concentrations in muscle as a function of PCB concentration in food

A 1.9-fold increase in ORD activity could be observed in fishes with higher contamination levels (Figure 5 and Table 2). The activities of conjugating enzymes in liver responded differentially to the PCB exposure. Whereas the glucuronyltransferase (UGT) activity remained unchanged (R=-0.026, p=0.906, n=25), the hepatic sulfotransferase activity had maximally reduced to 47% of its control value (Figure 5 and Table 2).

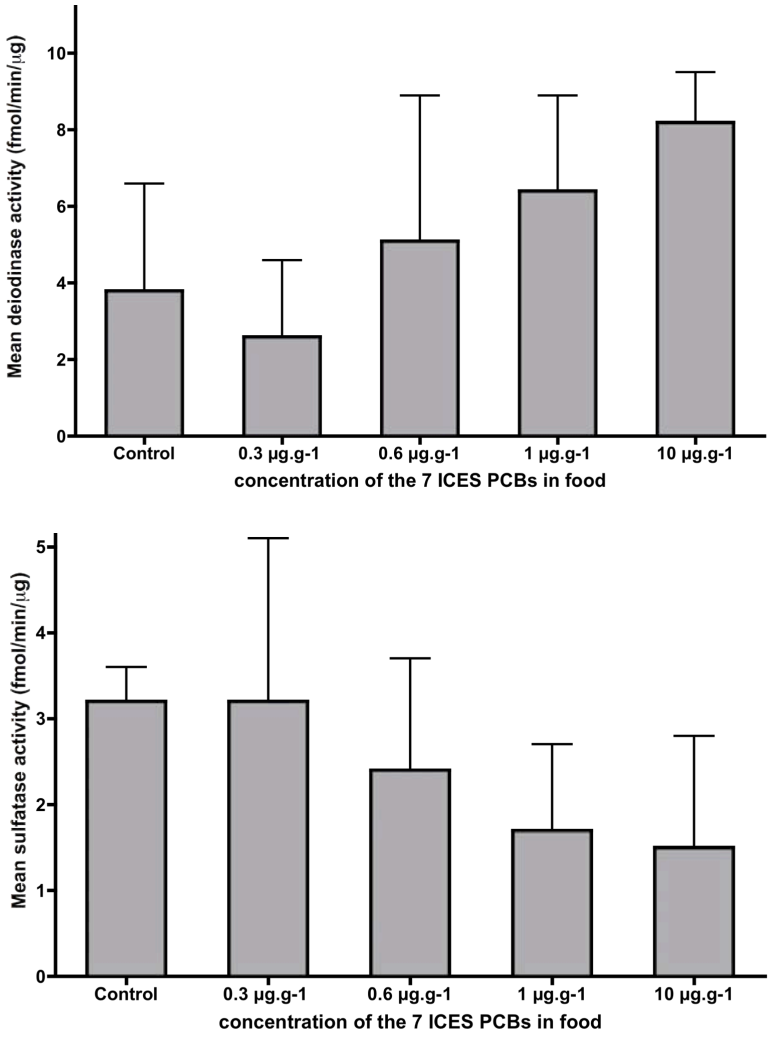


Figure 5 : Mean deiodinase and sulfation activity in sea bass liver as a function of PCB concentration in food

This observation is supported by the positive correlation between the hepatic ORD activity and the effective PCB concentration measured in the muscle of these fish ($R=0.414$, $p<0.001$, $n=74$) and by a negative correlation between the hepatic sulfotransferase activity and the effective PCB concentration measured in the muscle of these fish ($R=-0.512$, $p=0.009$, $n=25$) (Figure 6).

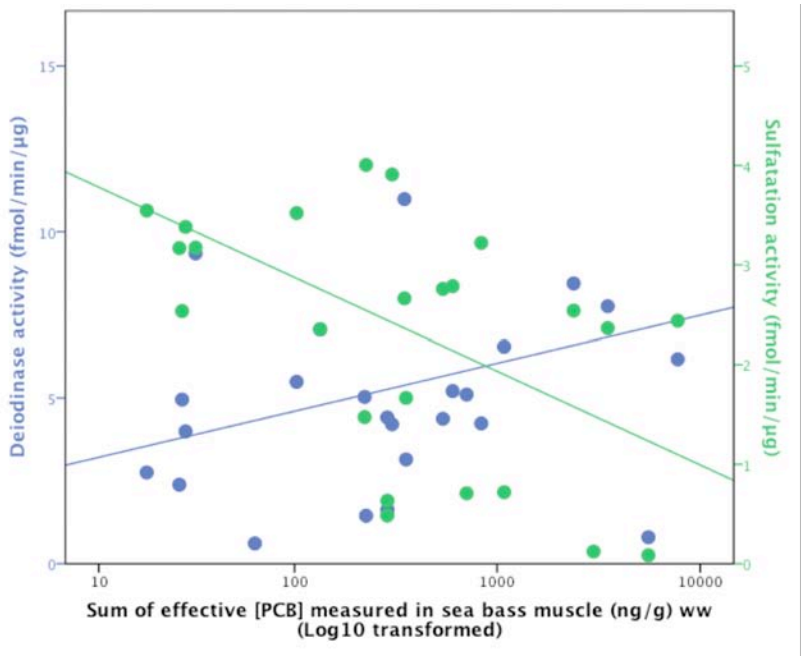
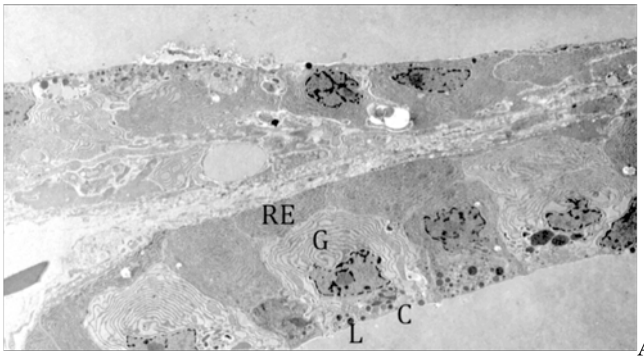


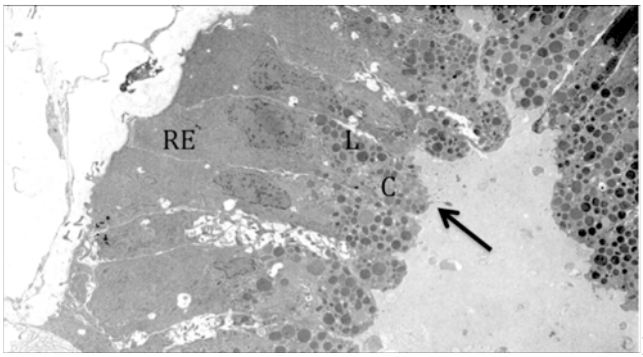
Figure 6: Correlation between the PCB concentration measured in sea bass muscle and the hepatic deiodinase and sulfation activity

Histological examination revealed changes in the thyroid follicles of fishes exposed to 0.3 to 10 µg·g^{-1} dw [7 ICES PCB]). The epithelial cell height ranged from 18 to 31 µm and did not vary significantly between the exposure groups ($F_{4,21}$=1.94, p=0.157). The mean follicle diameter in the exposure groups ranged between 92 to 121 µm and no significant difference could be identified ($F_{4,21}$=0.34, p=0.897). A larger heterogeneity of follicle size was observed in thyroids of the 1.0 and 10 µg·g^{-1}dw [7 ICES PCB] groups, the standard deviation was 4-6 times higher than in control group and twice as high than in the other exposure groups (0.3 and 0.6 µg·g^{-1} dw [7 ICES PCB]). Thyroids of highly exposed fish (1.0 and 10 µg·g^{-1} dw [7 ICES PCB]) contained small follicles (∅≈80 µm) and very big follicles (∅≈180 µm). No differences in roundness, form factor and aspect ratio could be identified among the different treatment groups (Table 1). Electron microscopy revealed that cells forming the larger follicles observed in the thyroids of high exposed fish (1.0 µg·g^{-1} dw [7 ICES PCB]), contained extensive lamellar arrays of rough endoplasmic reticulum.

There were numerous large electron dense colloid droplets similar to that of luminal colloid within follicular cells. Large lysosomal bodies with a heterogeneous internal structure were present in larger numbers than in smaller follicles. The Golgi apparatus and mitochondria are well developed and compressed by the numerous colloid droplets. Long projections of follicular cell cytoplasm often extended from the apical surface into the colloidal lumen (Figure 7).



A



B

Figure 7 : Thyroid follicular cells of sea bass exposed to 1 µg g^{-1} Σ [7 PCBs] in food (x2000): A between two smaller follicles, we can see few apical cytoplasmic processes extending into follicular lumen, well developed rough endoplasmic reticulum (RE) and large Golgi apparatuses (G) and few colloid droplets (C) and lysosomal bodies (L) B. of large follicle, we can see apical cytoplasmic processes extending into follicular lumen (Arrow), dilated profiles of rough endoplasmic reticulum (RE) and compressed Golgi apparatuses (G) and numerous large colloid droplets (C) and lysosomal bodies (L)

In the case of exposure exceeding the environmental relevant range (10 µg·g-1 dw [7 ICES PCB]), enlargement of interstitial tissue between follicles and degenerated colloid were observed (Figure 8).

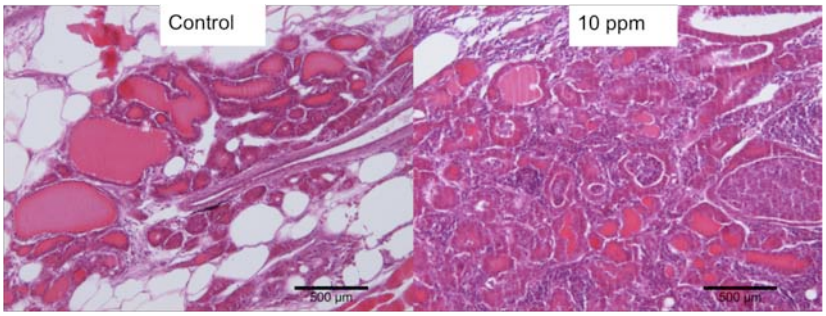


Figure 8 : Thyroid histological section of a controlled and an exposed sea bass (10 µg.g-1 dw)

Discussion

Our study demonstrates that subchronic exposure to a mixture of PCB affects thyroid hormone physiology in juvenile sea bass. Clearly, effects of exposure to environmentally relevant concentrations differed in effects and pathways from those observed at higher dose. At lower exposure doses (0.3 to 0.6 µg g^{-1} dw [7 ICES PCB] in food pellets), thyroid hormone homeostasis appeared unaffected while drastic diminution of thyroid hormone levels was observed at higher concentrations.

Environmentally relevant exposures to PCBs affected thyroid hormone metabolism and thyroid hormone synthesis. The histomorphometrical analysis showed a larger variability of the follicle diameter and especially increased epithelial cell heights with higher PCB exposure. The fine structure of fish thyroid gland is more heterogeneous than its mammalian counterpart, containing follicles and cells of different sizes and functional states (Eales, 1979). The size of the follicles and the form of the follicular cells gives an indication of the secretory activity of the gland. Thyroids dominated by small follicles lined by cuboid and columnar cells can be classified as highly active. Relatively inactive thyroids show large follicles lined by low or flattened epithelial cells (Hallgren, 2002). Our observations support the hypothesis that the contamination of PCB mixtures induces a hyperactivity of the thyroid tissue indicated by the hypertrophy of follicular epithelial cells. These observations are in

accordance with previous reported changes in thyroid histological appearance (Leatherland, 1993; Leatherland and Sonstegard, 1978, 1980; Schnitzler et al., 2008).

The simultaneous presence of small and big follicles in highly exposed fish (1.0 and 10 μg·g^{-1} dw [7 ICES PCB]) indicated an asynchrony of cellular activity in the thyroid gland. The histological ultrastructure of epithelial cells surrounding these bigger follicles was different compared to the smaller follicles, indicating different secretory activities. The organelles in the cytoplasm of these cells were more developed, especially the rough endoplasmic reticulum, probably related to an increased synthesis activity. With regard to the projections of follicular cell cytoplasm from the apical surface into the colloidal lumen, the follicular cells take up colloid droplets by endocytosis. A high number of colloid droplets accumulated noticeable in the cytoplasm of follicular cells. Comparable observations have been made in PCB fed rats (Capen et al., 1977; Collins and Capen, 1980a, b). Collins and Capen suggested that the lysosomal bodies were unable to interact with colloid droplets in a normal manner, leading to the inhibition of thyroglobulin proteolysis and thyroid hormone release (Collins and Capen, 1980b).

The main metabolic pathways for thyroid hormones are deiodination, glucuronidation and sulfation (Brouwer et al., 1998; Eales and Brown, 1993). Enzymatic outer ring deiodination activities, that convert the prohormone T_4 to the bioactive hormone T_3 were significantly higher in animals exposed to 1 and 10 μg·g^{-1} dw [7 ICES PCB] in food pellets. This increase was accompanied by an increase in muscular T_3 in fish exposed to environmental relevant doses of PCB. Adams et al. (2000) examined thyroid hormone deiodination in plaice following short-term exposure to PCB 77 and 126 (Adams et al., 2000). They found that PCB 77 increases the deiodination enzyme activity whereas PCB 126 did not alter T_4ORD in plaice. These differences in effect may be due to the greater potential for PCB 77 to be hydroxylated (Brouwer et al., 1998). In mammals, co-planar PCBs generally depressed hepatic T_4ORD (Brouwer et al., 1989/7). Unfortunately, due to the cross-

correlation of the different PCBs, it was not possible to differentiate congener-specific effects.

Thyroid hormone conjugation to glucuronic acid inactivates the thyroid hormones, increases their solubility and facilitates their excretion in bile and urine (Brouwer et al., 1998). In our experiment no effect of PCB exposure on T_4UGT could be observed, whereas other studies reported marked induction in rats following exposure to individual PCB congeners (Adams et al., 2000; Brown et al., 2004b; Morse et al., 1993; Spear et al., 1990; Visser et al., 1993) and mixtures (Hood, 1999; Klaassen and Hood, 2001; Morse et al., 1996). In most of these reports reduced T_4 levels in the same animal accompanied an increased T_4UGT activity and negative correlations between those two parameters suggested a causal relationship. Generally, phenolic PCBs undergo detoxification by glucuronidation and induce hepatic UGTs to facilitate excretion of PCBs (Klaassen and Hood, 2001; Visser et al., 1993). Although there was no increase in hepatic T_4-UGT activity in our experiment. This may be because the T_4-glucuronidation enzyme assay does not evaluate activity for specific UGT isoforms. UGT activity induction can be dependent on aryl hydrocarbon receptor (AhR) activation (Richardson et al., 2008). Antagonistic interactions between AhR agonists such as PCB 126 and other AhR-inactive PCBs have been demonstrated (Safe et al., 1998).

Another essential step in metabolism of iodothyronines is the sulfation by sulfotransferases (Schuur et al., 1999). They also inactivate the thyroid hormones, increase their solubility and facilitate their excretion in bile and urine like UGT. Furthermore TH sulfates do not bind to T_3 receptors and are thus unable to mimic thyroid hormone activity and are rapidly degraded by inner ring deiodinases (Brouwer et al., 1998; Schuur et al., 1999). In our experiment we observed a general decrease of SULT activity. This is in accordance with *in vitro* studies using rat and human hepatoma cell lines that related a strong inhibition of thyroid hormones sulfation by hydroxylated metabolites of PCB (Brouwer et al., 1998; Schuur et al.,

1999). This reduced T_4SULT activity is accompanied by an increase of muscular T_4 levels in animals exposed to environmental relevant doses of PCB.

The heterogeneity of fish thyroid systems and their resilience to perturbations make it difficult to interpret these changes in activity. It is well accepted that some PCB congeners, due to their structural similarity, are able to serve as binding ligands for T_4-binding proteins (Darnerud et al., 2001). By this means they reduce thyroid hormone levels by displacing them from transport proteins and increasing their excretion. Both T_4 and T_3 have a negative feedback effect on TSH secretion by the pituitary in teleost fish species (Yoshiura et al., 1999). That may have stimulated the production of T_4, revealed by thyroid histomorphometry. The deiodinase activity was increased, thus more conversion of T_4 to T_3, and there is less excretion of thyroid hormones through the hepatic pathway as the sulfation activity decreased with raising PCB exposure. These modifications in thyroid hormone dynamics contribute to maintain thyroid hormone levels in an acceptable range. These observations are consistent with those made in our field study. The PCB induced disruption of thyroid system is countered by an extensive self-regulatory feedback control.

Nevertheless, we observe an important lowering of muscular T_3 and T_4 levels in animals exposed to 10 $\mu g.g^{-1}$ dw [7 ICES PCBs] in food pellets. This observation indicates that at those exposure levels other causes than the metabolic pathways are involved. In these fishes the histological examination revealed lymphoid cell infiltration and enlargement of the interstitial tissue between follicles and degenerated colloid. The follicles appeared in lower number and the tissue seems disorganized. These degenerative changes might have caused the observed hypothyroidism in these fish. Probably, the pollutants at this dose interfere with the synthesis and secretion of thyroid hormones.

The thyroid status has pronounced effects on growth and development in fish (Blanton and Specker, 2007; Inui et al., 1995; Klaren et al., 2008; Power et al., 2001;

Shiao and Hwang, 2006; Yamano, 2005). Depending on the dosage used, T_3 supplementation has anabolic and catabolic effects whereas hypothyroidism always results in growth retardation (Theodorakis et al., 2006; Van der Geyten et al., 2001). In this study, neither size nor weight differences could be found between the treatment groups, which can be explained by the high feeding rate of these fishes. Thyroid hormone reserves have not been determined fully in any fish species but based on human values it would seem advisable to continue studies for several months to determine a true measure of effect on the thyroidal status (Brown *et al.* 2004).

Conclusions

The presented results show clearly that the effects of PCB exposures on the thyroid system are dose dependent. Exposure to environmental relevant doses of PCB modifies hepatic T_4 outer ring deiodinase and T_4 sulfation and increases the hormone synthesis and secretion. Ultrastructural histological investigations showed a potential inhibition of thyroglobulin proteolysis and thyroid hormone release in the thyroids of high exposed fish (1.0 $\mu g.g^{-1}$ dw [7 ICES PCB]). Meanwhile, the thyroid hormone levels were preserved. The presented mechanisms are part of the extensive autoregulatory feedback control at both central and peripheral levels, and the induced changes in thyroid hormone dynamics keep the levels in an acceptable range. At 10 times higher concentrations, an important depression of muscular T_3 and T_4 levels could be observed which are apparently caused by other mechanisms than metabolic pathways. Here we observed degenerative histological changes in the thyroid tissue that might have caused the hypothyroidism in these fish.

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Chapter 5

Summary and general discussion

In this thesis, several components of the endocrine disruption of the thyroid system in European sea bass are described, ranging from the contamination levels of potential endocrine disrupting chemicals in European coastal waters, the effects of those environmental factors on the thyroid system of sea bass, to the underlying mechanisms and effects of such an endocrine disruption. This section includes a summary of the main findings presented in the chapters 2 to 4. Afterwards, we will entirely dicuss these results and propose accurate interpretations for them.

Summary of the main findings

Several potential endocrine disrupting chemicals (including Polychlorinated biphenyls (PCBs) and organochlorine pesticides like Dichloro-Diphenyl-Trichloroethane (DDTs), Hexachlorocyclohexanes (HCHs), aldrin, dieldrin and trace elements (Cd, Cu, Se, Pb, Zn and Hg)) are well present in coastal regions near several important European river mouths (Gironde, Charente, Loire, Seine and Scheldt) (chapter 2). Each region presented their specific contamination patterns reflecting different sources due to the input of the respective rivers. High contamination levels of organochlorinated compounds were measured in the muscles of European sea bass sampled in the coastal regions near the Scheldt, Seine, Loire, Charente and Gironde. Even if their concentrations were below the Maximum Residue Limits set by the European governments (EC, 2005; FAO, 2008), they might induce alterations of the endocrine system. Indeed, the fact that these so-called xenobiotics may generate adverse low dose effects is becoming more widely accepted (Tabb and Blumberg, 2006).

The thyroid systems of fish and mammals are similar in many aspects, with one major difference. The mammalian system is driven primarily through the central brain-pituitary-thyroid axis that regulates thyroid secretion of both T_4 and T_3. In fish instead, the central brain-pituitary-thyroid axis has the primary role of ensuring T_4 homeostasis. T_3 production and homeostasis is regulated in the peripheral tissue by conversion of T_4 to T_3 by deiodination. This implies that no single biomarker

examines all facets of fish thyroid function. A series of recommended measurement endpoints have been put forward in recent reviews to assess thyroid function at different levels of the fish thyroid cascade (Brown et al., 2004).

Table 1: Summary of the results concerning the different studied points of thyroid disruption in fish

Endpoint	Field study	Experimental study	
	European coastal regions (Gironde, Charente, Loire, Seine and Scheldt)	**Environmental relevant concentrations** (0.3 to 1.0 µg Σ7 tracer PCBs per g food pellets)	**Exceeding environmental concentrations** (10 µg Σ7 tracer PCBs per g food pellets)
Mean muscular concentration of Σ7 tracer PCBs	8 - 27 ng/g ww	10 - 176 ng/g ww	1497 ng/g ww
muscular T4 concentration	no significant multivariate relationship with contaminant exposure, correlated negatively with levels of following PCB congeners (101, 153, 170, 180, 183, 194 and 195)	no significant change following exposure to contaminated food	reduced to 30% of its control value
muscular T3 concentration	significant multivariate relationship with higher chlorinated PCB exposure, correlated negatively with levels of following PCB congeners (101, 153, 170, 180, 183, 194 and 195) and DDD	increased 1.5 fold compared to control group	reduced to 75% of its control value
histological examination	unaffected, no significant relationship with contaminant exposure	no significant change following exposure to contaminated food, but bigger heterogeneity in follicle size and ultrastructural alterations	enlargement of interstitial tissue and degenerated colloid
deiodination activity	significant positive multivariate relationship with higher chlorinated PCB exposure, correlated positively with levels of following PCB congeners (52,70, 87, 95, 101, 110, 118, 128, 138, 153, 156, 170, 180, 183, 194 and 195) and DDD	increased 1.9 fold compared to control group	
sulfation activity	significant negative multivariate relationship with higher chlorinated PCB exposure	reduced to 47% of its control value	
glucuronidation activity	unaffected, no significant relationship with contaminant exposure	unaffected, no significant relationship with contaminant exposure	

These endpoints were applied in a large field study investigating the thyroid functional status of wild sea bass sampled in coastal regions near several important

European river mouths in relation with the contaminants of their environment (chapter 3). And in a 120-day experimental model that was especially designed after this field study to reflect the persistent organic pollution to which the European sea bass population could conceivably be exposed (chapter 4). By this approach we could determine a dose dependency of the effects of these pollutants on the thyroid system and allowed us to describe underlying mechanisms and effects of such an endocrine disruption.

Observations made in the experimental exposure to environmental relevant doses of PCB were consistent with those made in our field study (Table 1). The centrally controlled thyroidal secretion of T_4 was monitored adequately from the muscular T_4 levels and from thyroid histological appearance. In both studies the muscular T_4 levels were preserved and no multivariate relationship with contaminant exposure could be revealed. The thyroid gland activity is homeostatically regulated by the thyroid-stimulating-hormone (TSH) which controls synthesis, storage and secretion of T_4 in a classical negative feedback system. Measurements of follicular diameter and epithelial cell heights showed no significant differences. In the following discussion we present the difficulties to determine any minor proliferation of thyroid tissue and we interpret the results of ultrastructural histologial investigations suggesting a potential inhibition of thyroglobulin proteolysis and thyroid hormone release in the thyroids.

Peripheral T_3 levels in teleost fish are largely controlled by enzymatic deiodinase activities in extra-thyroidal tissues. Our findings support the hypothesis of the disturbance of the peripheral control by higher chlorinated PCB congeners and DDTs that increased the hepatic T_4ORD activity. These changes likely represent compensatory responses to disrupting effects that might otherwise have depressed T_3 levels. The muscular T_3 levels were well preserved in both studies. We tested the thyroid hormone conjugation by glucuronyl transferases and sulfatases that increases their solubility and facilitates their excretion. In both studies, no effect of contaminant exposure on T_4UGT could be observed, whereas literature reported

marked inductions of this pathway. In both studies, we observed a general decrease of SULT activity. In the following discussion we propose some interpretations of these results.

The observed effects were different depending if the exposure was environmental relevant or exceeding the environmental concentrations. In fish exposed to exceeding environmental concentrations, a depression of muscular T_3 and T_4 levels could be observed which are apparently caused by other mechanisms than metabolic pathways. Here we observed degenerative histological changes in the thyroid tissue that might have caused the hypothyroidism in these fish. In the general discussion we propose possible mechanisms for this alteration.

The experimental exposure permitted us to examine the consequences of a potential disruption of the thyroid system on growth performance and condition factor. Neither size nor weight differences could be found between the treatment groups, tough slight differences in growth curves and specific growth rates could be observed. At the end of the discussion we point out the fact that xenobiotic-induced changes in thyroid hormone function have yet to be conclusively causally linked to decreased fitness or survival.

General discussion

To interpret the different observations we reviewed the stages in teleost thyroid function and its regulation, from the initial biosynthesis of the thyroid hormones to their eventual interaction with putative receptors (Figure 1). The fish thyroid cascade can be broken down into the following three elements. First is the centrally controlled brain-pituitary-thyroid axis, which is primarily responsible for synthesis, storage and secretion of T_4 and maintenance of T_4 levels for a given physiological state. The second element is the peripherally controlled availability of the active hormone T_3. The primary production of the biologically active form of the thyroid hormone T_3 via outer-ring deiodination of T_4 occurs in peripheral organs or tissues

such as the liver. The third phase is the receptor-mediated effects of T_3 on target cells to regulate development, growth and aspects of reproduction.

Disruption mechanisms of thyroxin production and liberation

Thyroid hormone biosynthesis depends on an adequate plasma iodide level, determined partly by dietary iodide and partly by active branchial iodide uptake from the water. For normal thyroid function the presence of iodide is essential. However, marine fish in natural and most artificial conditions do not suffer from having iodide deficiency (Eales and Brown, 1993) and is thus not an issue in this study.

The thyroid gland can concentrate iodine 20-40 fold over blood levels under normal physiological conditions. The sodium-iodide symporter (NIS) mediates the initial step in thyroid hormone synthesis, the uptake of iodide into the cell. NIS can be blocked by the anions thiocyanate and perchlorate (Crane et al., 2005). Iodide, the form of iodine that enters the cell, must be oxidized by the enzyme thyroperoxidase (TPO). The thioamide drugs, such as propylthiouracil and methimazole (Bradford et al., 2005), inhibit TPO. The thyroid cell synthesizes a large glycoprotein called thyroglobulin, which is secreted into the follicle and forms the colloid. The tyrosyl residues within the thyroglobulin are iodinated at the cell/colloid interface.

Finally the apical cell membrane engulfs colloid by endocytosis and the resulting cytoplasmic colloid droplets fuse with lysosome in which proteolysis of thyroglobulin releases T_4, that then diffuses to the blood. The metabolic pathway required to liberate T_4 and T_3 from the thyroglobulin molecule is an important physiological event and its potential disruption by environmental chemicals could be an important mechanism by which adverse effects of specific toxicants could occur. However little is known about the potential vulnerability of this site of action in thyroid toxicity.

The thyroid follicular cells of PCB exposed sea bass accumulate large numbers of colloid droplets and large lysosomes, indicating such a disorder (please see colour figures 1 and 2 on page 180-181). Similar observations have been made in PCB administered rats. Ultrastructurally, PCB-induced changes in thyroid gland include an increased development of endoplasmic reticulum, vacuolization of mitochondria and a decrease in the colloid droplet-lysosome interaction necessary for the secretion of thyroid hormones (Capen et al., 1977; Collins and Capen, 1980a, b). These studies show evidences of possible direct effects of PCB on the thyroid gland. The lysosomal bodies appeared unable to interact with colloid droplets in a normal manner and hydrolyse the cleavage of thyroid hormone from the molecular structure of thyroglobulin in colloid droplets.

Measurements of follicular diameter and epithelial cell heights showed that higher contamination levels were capable of temporarily inducing a mild hypertrophy, indicating an increase of synthesis and secretion activity of the gland. There was a remarkable heterogeneity in how individual follicles responded to contaminant exposure (please see colour figures 3 and 4 on page 182-183). The mechanisms that generate the marked heterogeneity in follicle architecture in response to contaminants are unknown. Eales (1979) recognized the importance of this heterogeneity of fish thyroid containing both follicles and cells of different sizes and functional states that are hypothesized to go through a histophysiological cycle of generation, maturation and decay (Eales, 1979). Therefore, any minor proliferation of thyroid tissue is fairly difficult to determine.

The dynamic relationship among hormones secreted by the HPT axis has the effect of maintaining thyroid levels within a narrow range. Both, T_4 and T_3 have a negative feedback effect on TSH secretion by the pituitary in teleost fish species (Yoshiura et al., 1999). Plasma levels of TSH are difficult to measure and rarely reported in fish studies (Kumar et al., 2000). Because of the species differences in the primary structure of TSH and the degree of glycosylation, mammalian antibodies typically do not recognize non-mammalian TSH and homologous antisera are not

available for the majority of teleost species. In our field study as well as in our experimental exposure to environmentally relevant doses of PCBs a certain thyroid homeostasis could be maintained, even when a slight decrease of T_4 could be observed in higher contaminated wild sea bass. Serum half-life of thyroxin (T_4) is controlled in part by serum binding proteins like Thyroxin-Binding-Globulin (TBG), Transthyretin (TTR) and albumin. PCBs and related compounds are structurally similar to thyroid hormones and are well known to displace T_4 from serum binding proteins. This may cause a decline in serum thyroid hormone concentration by biliary clearance, inducing a release of TSH provoking the observed increase of synthesis and secretion activity of the gland. This observation has led to the concept that the negative feedback loop of the HPT axis can compensate for dysfunction of the thyroid gland (Zoeller et al. 2007).

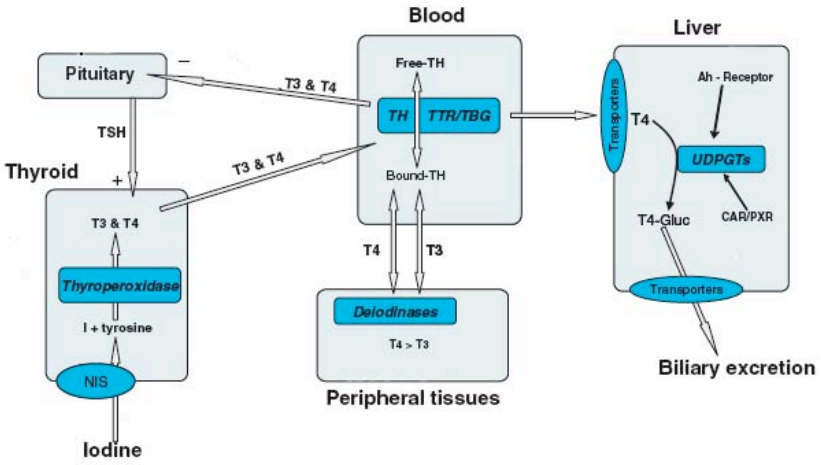

Figure 1: TH control pathways and sites of disruption by xenobiotic chemicals. Sites or processes where xenobiotics are known or hypothesized to act as TDCs are indicated in the boxes and ovals. Abbreviations: NIS, sodium/iodide symporter; T4-Gluc, T4-glucuronide; TBG, thyroid-binding globulin; TSH, thyroid-stimulating hormone; TTR, transthyretin; UDPGT, uridine diphosphate glucuronyltransferase Modified from Crofton *et al.* (2008) International Journal of Andrology

The literature suggests that prolonged stimulation of thyroid by TSH promotes initially hypertrophy, followed by follicular cell proliferation and thyroid gland enlargement (Capen, 1997). Histological examinations of thyroids from animals exposed to 10 $\mu g.g^{-1}$ dw [7 ICES PCBs] in food pellets revealed an enlargement of

the interstitial tissue between follicles and degenerated colloid (please see colour figures 5 and 6 on page 184-185). The follicles appeared in lower number and the tissue seems disorganized. These degenerative changes might be a consequence of the mitogenic activity of TSH. The conclusion whether changes represent a compensatory or adverse effect rests questionable. For example, the responsive increase in TSH following thyroid hormone deficiency are compensatory as they maintain the T_4 levels in an acceptable range, but could become adverse within the context of increased risk of thyroid cancer (Capen et al., 1977).

Thyroid hormone deiodination and metabolism

Iodothyronine deiodinase are enzymes involved likewise in the activation of thyroid hormone. In fish, apparently more than in other vertebrates, these important thyroid hormone transformations are controlled outside the thyroid and occur mainly in peripheral tissues (liver, brain, kidney, gill). Fish liver expresses the highest T_4ORD activity, which support the notion that liver could play a dual role, contributing to both the local and systemic supply of T_3 (Blanton and Specker, 2007). Exposure of rats to PCBs resulted in an inhibition of hepatic deiodinase activity (Brouwer et al., 1998b; Morse et al., 1996; Visser et al., 1993). In this thesis we observed in both field and experimental study an increase of T_4ORD related to contaminant exposure. Similar observations have been made in American plaice (*Hippoglossoides platessoides*) (Adams et al., 2000). It was concluded that the PCB-induced changes in deiodinating activity likely represents compensatory responses to disrupting effects that might otherwise have depressed the plasma T_3 levels. The different responses between mammalian and fish species rests on the fact that in fish the T_3 levels are primarily under peripheral control.

Additional pathways are important in metabolizing iodothyronines. These conjugation pathways include glucuronidation and sulfation of the phenolic hydroxy group. Conjugation changes the solubility of iodothyronines, allowing their concentration in bile acids and excretion through the hepatic pathway.

In general, there is a very large literature about the role of pollutants inducing glucuronidation, changing circulating levels of thyroid hormones (Hood, 1999; Klaassen and Hood, 2001; Morse et al., 1993; Spear et al., 1990; Visser et al., 1993; Zhou et al., 2000). Generally, phenolic PCBs undergo detoxification by glucuronidation and induce hepatic UGTs to facilitate excretion of PCBs (Klaassen and Hood, 2001; Visser et al., 1993). Although there was no increase in hepatic T_4-UGT activity whether in the field study nor in our experimental exposure. This may be because the T_4-glucuronidation enzyme assay does not evaluate activity for specific UGT forms. There are several isoenzymes of UDP-glucuronyltransferase involved in T_4 glucuronidation, which can be measured using specific substrates (phenol, bilirubin, or sex steroids) or reaction conditions (addition of brij 56) (Visser et al., 1993). Moreover, UGT activity induction can be dependent on aryl hydrocarbon receptor (AhR) activation (Richardson et al., 2008). Antagonistic interactions between AhR agonists such as PCB 126 and other AhR-inactive PCBs have been demonstrated (Safe et al., 1998).

Another essential step in metabolism of iodothyronines is the sulfation by sulfotransferases (Schuur et al., 1999). They also inactivate the thyroid hormones, increase their solubility and facilitate their excretion in bile and urine like UGT (Brouwer et al., 1998b; Schuur et al., 1999). In our experiment we observed a general decrease of SULT activity. This is in accordance with *in vitro* studies using rat and human hepatoma cell lines that related a strong inhibition of thyroid hormones sulfation by hydroxylated metabolites of PCB (Brouwer et al., 1998b; Schuur et al., 1999). On basis of the close structural resemblance between PCB-OHs and iodothyronines, it is not unexpected to observe competitive inhibition. Other thyroid hormone binding proteins such as deiodinases and transthyretin are also competitively inhibited by hydroxylated PCBs (Brouwer et al., 1998a; Lans et al., 1994; Rickenbacher et al., 1989; Schuur et al., 1998).

PCBs are not readily metabolized by fish (Letcher et al., 2000). Only few PCB-OH congeners were identified in sea bass liver, but none could be detected in muscle samples. PCB-OHs consisted of approximately 0.4% from the sum PCBs measured in the same tissues. However, these results should be interpreted with caution, due to the low number of samples (5) from high contaminated fish (10 μg.g-1 dw [7 ICES PCB] in food pellets).

The role of conjugated hormones is still poorly known. But a number of observations reflect their physiological importance. Estrogen sulfotransferase deficient mice exhibited spontaneous fetal loss (Tong et al., 2005). When the testis from the elasmobranch *Squalus acanthias* was perfused through the genital artery, androgens and estrogen were extensively metabolised and appeared as sulfates and glucuronides in the perfusate (Cuevas et al., 1992). Rat cardiac fibroblasts synthesise and secrete glucuronidated iodothyronine conjugates in vitro, and cells from the embryonic rat heart ventricle cell line H9c2(2-1) preferentially take up glucuronidated T_4 and T_3 over the native, unconjugated hormones (van der Heide et al., 2007). These findings support the role of conjugating and deconjugating enzymes in the modulation of hormone bioactivities, however it remains to be investigated if conjugating enzyme activities similarly affect hormone bioactivity in sea bass.

Thyroid hormone receptors

Thyroid hormone exerts its effects on development and physiology primarily by interacting with specific nuclear proteins, the thyroid hormone receptors (TRs). TR binds to DNA sequences known as thyroid hormone response elements (TREs) found in the regulatory regions of target genes, and according to the nature of the TREs, gene expression may be enhanced or inhibited (Wu and Koenig, 2000). In the absence of ligand, the bound receptor generally acts as a repressor, the binding of ligands however, causes a change in the conformation of TR that results in the replacement of the corepressor complex, thus activating transcription of the target

gene (Yamano, 2005). PCB exposure up-regulated the expression of genes that are positively regulated by TH (Gauger et al., 2004). It seems that PCBs can activate TRs, perhaps directly, and the implication is important because inappropriate activation of TRs may produce adverse consequences on brain development (Zoeller et al., 2002). This issue has not been treated in this thesis; nonetheless it may be an important mechanism in thyroid endocrine disruption. For fish there are to date only thyroid hormone receptor assays described that employs the principle of saturation. This method used the classical reversible biomolecular binding and could be used as a screen to identify chemicals that have the potential to interact with thyroid hormone receptors. The disadvantage are that the saturation kinetics need to be determined for the used species and tissue and that the assay is technically challenging.

Thyroid hormone effects

The thyroid status has pronounced effects on growth and development in fish (Blanton and Specker, 2007; Inui et al., 1995; Klaren et al., 2008; Power et al., 2001; Shiao and Hwang, 2006; Yamano, 2005). Depending on the dosage used, T_3 supplementation has anabolic and catabolic effects whereas hypothyroidism always results in growth retardation (Theodorakis et al., 2006; Van der Geyten et al., 2001). In this study, neither size nor weight differences could be found between the treatment groups, tough slight differences in growth curves and specific growth rates could be observed. Thyroid hormone reserves have not been determined fully in any fish species but based on human values it would seem advisable to continue studies for several months to determine a true measure of effect on the thyroidal status (Brown et al., 2004). Xenobiotic-induced changes in thyroid hormone function have yet to be conclusively causally linked to decreased fitness or survival (Blanton and Specker, 2007; Brown et al., 2004). The attribution of xenobiotic effects to the thyroid function is extremely complex. Numerous variables must be taken into account to distinguish indirect and direct actions on the thyroid cascade from chemical exposure (Brown et al., 2004) (Figure 2). Assays for post-receptor biologic

actions of T_3 are difficult to develop in fish. However, biological responses that are unique to thyroid function such as parr-smolt transformation, flounder metamorphosis, and young fish early development could become effective thyroid hormone tests.

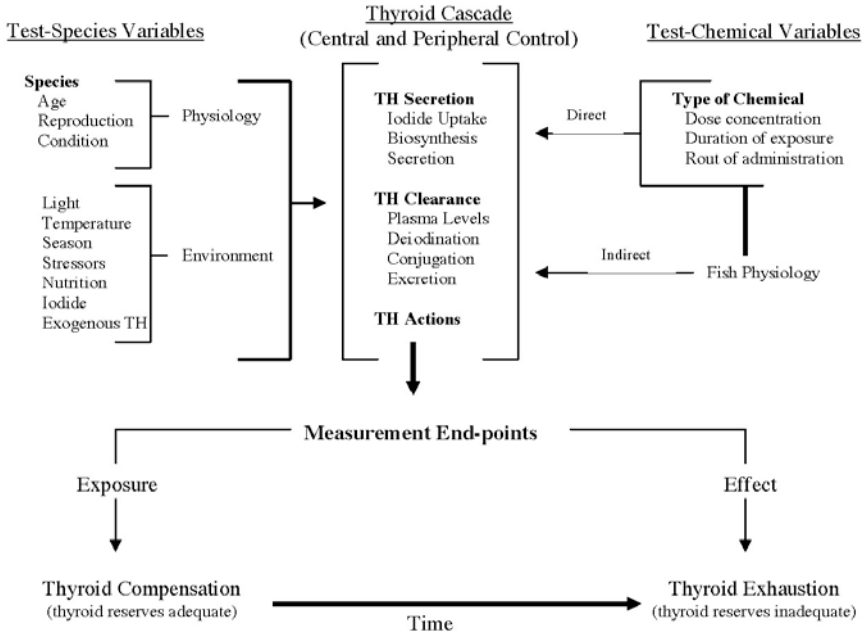

Fig 2: Factors involved in the interpretation of responses of the fish thyroid system to xenobiotics (TH=thyroid hormones) Adapted from Brown *et al.*, (2004) Environmental Toxicology & Chemistry

Perspectives

Thyroid toxicants are generally defined as toxicants that alter circulating levels of thyroid hormone (Brucker-Davis, 1998). Today there are around 116 environmental compounds that are suspected to disrupt thyroid function (Howdeshell, 2002). However, studies of thyroid disruption often incorporate an incomplete picture of the dynamic relationship within the HPT axis. These relationships are quite complex, and measurements of some of these parameters can be very difficult to obtain. Thus, it is important to capture endpoints that are more indicative of thyroid disruption as well as reflective of adverse effects.

Based on the literature review of this thesis, there are currently no in vitro or in vivo assay that are sufficiently developed to warrant recommendation for use to efficiently screen chemicals for thyroid disruption. Methods are available that can be used to measure thyroid hormones, measure their metabolisation and assess the thyroid histological appearance. Although our ability to interpret the causes and implications of potential alterations in T_4 and T_3 levels in fishes is nonetheless limited without further research. No standardized protocol/method had been validated to assess thyroid disruption in fish.

A recent review (Blanton and Specker, 2007) failed to find a satisfactory assay for evaluation of biological responses that are unique to thyroid function. Consideration should be given to early development of fishes that could become an interesting thyroid hormone effect screen. The early life stage may prove to be very susceptible to thyroid disruption. This needs necessarily a refinement of the presented assays to apply the thyroid analysis to very small fish.

Most potential thyroidal endocrine disruptors have been studied in isolation, but it is likely that individuals experience multiple exposures that could have additive or synergistic effects. Additional studies are needed to further elucidate the risk posed by potentially thyroid-disrupting compounds alone and in combination.

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Abstract

Thyroid functional status of wild fish in relation with the contamination of their environment deserves further investigation. Polychlorinated biphenyls (PCBs) and organochlorine pesticides like Dichloro-Diphenyl-Trichloroethane (DDTs), Hexachlorocyclohexanes (HCHs), aldrin, dieldrin and trace elements (Cd, Cu, Se, Pb, Zn and Hg) were analysed in the muscle of sea bass (*Dicentrarchus labrax*) sampled in coastal regions near several important European river mouths (Gironde, Charente, Loire, Seine and Scheldt). We applied a multi-level approach of thyroid function assessment. Thyroxine (T_4) and triiodothyronine (T_3) concentrations in muscle were analyzed by radioimmunoassay. The activity of hepatic enzymes involved in extrathyroidal pathways of thyroid hormone metabolism, *viz*. deiodination, glucuronidation and sulfation were analyzed. Last, follicle diameter and epithelial cell heights were measured. We observed changes that lead to an increased conversion of T_4 to T_3 and lowered thyroid hormone excretion. The changes in the metabolic pathways of thyroid hormones can be interpreted as a pathway to maintain thyroid hormone homeostasis. The higher chlorinated PCBs seemed to be the most implicated in this perturbation. To gain a more integrated insight, we examined the effect of subchronic exposure to a mixture of Aroclor standards on thyroid hormone physiology and metabolism in juvenile sea bass. After 120 days of exposure, histomorphometry of thyroid tissue, muscular thyroid hormone concentration and activity of enzymes involved in metabolism of thyroid hormones were assessed. The results show that the effects of PCB exposures on the thyroid system are dose-dependent. Exposure to environmentally relevant doses of PCB (0.3 to 1.0 µg Σ7PCBs per g food pellets) increases thyroid hormone synthesis and stimulates hepatic T_4 outer ring deiodinase and T_4 sulfation. Thyroid hormone tissue levels were preserved thanks to the PCB induced changes in T_4 dynamics. At 10 times higher concentrations (10 µg Σ7PCBs per g food pellets) an important depression of T_3 and T_4 levels could be observed which are apparently caused by degenerative histological changes in the thyroid tissue. We propose accurate interpretations, by reviewing the different stages of teleost thyroid function and regulation.

Résumé

L'objectif de cette thèse est d'étudier la fonction thyroïdienne des poissons sauvages en relation avec la contamination de leur environnement. Les polychlorobiphényles (PCB) et les pesticides organochlorés tels que le dichloro-diphényl-trichloréthane (DDT), les hexachlorocyclohexanes (HCH), l'aldrine, la dieldrine et éléments traces (Cd, Cu, Se, Pb, Zn et Hg) ont été analysés dans le muscle de bars (*Dicentrarchus labrax*) échantillonnés dans les régions côtières à proximité de plusieurs importants estuaires européens (Gironde, Charente, Loire, Seine et l'Escaut). Nous avons évalué la fonction thyroïdienne à plusieurs niveaux. Les concentrations musculaires de thyroxine (T_4) et triiodothyronine (T_3) ont été analysés par dosage radio-immuno-assay. L'activité des enzymes hépatiques impliqués dans la métabolisation des hormones thyroïdiennes, à savoir déiodation, la glucuronidation et la sulfation ont été analysés. Enfin, le diamètre des follicules et la hauteur des cellules épithéliales ont été mesurés. Nous avons observé des changements indiquant une conversion accrue de T_4 en T_3 et une diminution de l'excrétion des hormones thyroïdiennes. Ces modifications des voies métaboliques des hormones thyroïdiennes peuvent être interprété comme une voie pour maintenir l'homéostasie des hormones thyroïdiennes. De tous les composés testés, les PCB fortement chlorés semblent être le plus impliqués dans cette perturbation. Afin d'acquérir une vision plus intégrée, nous avons examiné les effets d'une exposition à un mélange d' Aroclor sur la physiologie et le métabolisme des hormones thyroïdiennes chez les bars juvéniles. Après 120 jours d'exposition, l'histomorphométrie du tissu thyroïdien, les concentrations musculaires en hormones thyroïdiennes et l'activité des enzymes impliquées dans le métabolisme des hormones thyroïdiennes ont été évalués. Les résultats montrent que les effets de l'exposition aux PCB sur le système thyroïdien sont dosé dépendants. L'exposition à des doses environnementales de PCB (0.3 à 1.0 μg Σ7PCBs par g de nourriture) stimulent la synthèse des hormones thyroïdiennes et la deiodinase et inhibent la sulfation. Les niveaux d'hormones thyroïdiennes ont été conservés grâce aux changements dans la dynamique induite par les PCB. À des concentrations 10 fois plus élevées (10 μg Σ7PCBs par g de nourriture) une diminution des taux d'hormones thyroïdiennes peut être observée, qui est apparemment causés par des changements histologiques dégénératifs du tissu thyroïdien. Nous proposons une discussion de ces observations, en passant en revue les différentes étapes de la fonction et de la réglementation thyroïdienne chez les téléostéens.

Appendix

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HARBOR PORPOISE THYROIDS: HISTOLOGIC INVESTIGATIONS AND POTENTIAL INTERACTIONS WITH ENVIRONMENTAL FACTORS

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ABSTRACT: The thyroid plays an important role in development and is of primary importance in metabolism and heat loss for cetaceans, including the harbor porpoise (*Phocoena phocoena*). Several studies have demonstrated that environmental contaminants can alter various aspects of thyroid function in mammals and may contribute to various histologic changes. The present study completes the data set of a 2006 study by Das et al., by performing histological and immunohistologic investigations on thyroids of 36 harbor porpoises from Belgian and United Kingdom waters. The number and mean diameter of follicles (µm) and the relative proportion of follicular, connective, and vascular tissue (%) were quantified in the thyroid gland of each individual. Interfollicular fibrosis has been observed in these thyroid glands, and the collective findings support the hypothesis of an endocrine disruption of thyroid function through organochlorinated compounds. Our study aimed also to reveal potential relationships between thyroid morphometric data and metal levels (Cd, Fe, Zn, Cu, Se, and Hg) using multivariate statistical analysis. The multiple regressions revealed statistically significant relationships between trace elements (cadmium, selenium, and copper) and thyroid fibrosis. The largely negative relationships are interesting findings but do not support the hypothesis that these elements have an adverse effect on thyroid morphometry. Further research is needed to understand the nature of any relationship between organochlorine and trace element exposure and thyroid gland morphology and function in harbor porpoises.

Key words: Endocrine disruption, harbor porpoise, metals, organochlorine, *Phocoena phocoena*, thryroid.

INTRODUCTION

The harbor porpoise (*Phocoena phocoena*) is a native cetacean species in the North Sea (Benke et al., 1998). Along with the impacts of by-catch and reduced prey by overfishing, concern is growing about the adverse effects of environmental pollution on this marine mammal species (Reijnders, 1994; Siebert et al., 1999). The thyroid of marine mammals is a bilobated gland located on both sides of the larynx (Slijper, 1973). As with other vertebrates, the thyroid gland is composed of thyroid follicles that synthesize and store thyroid hormones (Bloom and Fawcett, 1975; Jubb et al., 1993; Junqueira et al., 1995; Feldman and Nelson, 1998). Several studies have demonstrated that environmental contaminants can alter various aspects of thyroid function (Hutchinson et al., 2000). The thyroid hormones contribute to the regulation of metabolism and growth, cell differentiation, and the development and function of the immune system (Woldstad and Jenssen, 1999). In cetaceans they are also believed to play an important role in controlling heat loss (Gregory and Cyr, 2003). Small cetaceans such as the harbor porpoise are obliged to remain active to maintain body temperature (Worthy and Edwards, 1990).

During recent years it has become evident that many xenobiotic chemicals may act as endocrine disrupters (Hutchinson et al., 2000). Endocrine-disrupting chemicals consist of synthetic and naturally occurring chemicals that affect the balance of normal hormonal functions in animals (Keith, 1997). Endocrine disrupt-

ers interfere with the functioning of the endocrine system in at least three possible ways (Damstra et al., 2002), including mimicking the action of a naturally produced hormone, such as oestrogen, testosterone, or thyroid hormones, and thereby setting off similar chemical reactions in the body (Hutchinson et al., 2000; Baker, 2001); blocking the receptors in cells receiving the hormones (hormone receptors), thereby preventing the action of normal hormones (Hutchinson et al., 2000; Gelbke et al., 2004); or by affecting the synthesis, transport, metabolism, and excretion of hormones, thus altering the concentrations of natural hormones (Zhou et al., 2000; Ishihara et al., 2003).

The thyroid gland represents one of the major target organs of endocrine disruptors (Brouwer et al., 1999). Synthetic chemicals and trace elements can disrupt nearly every step in the production and metabolism of thyroid hormones. They can interfere with uptake of iodine and cause inhibition of the peroxidase enzymes, displacements of the hormones from the transport proteins, and disruption of the hormone metabolism by influencing deiodinase, glucoronidase, and sulfatase activity (Howdeshell, 2002).

Organohalogens such as polychlorinated biphenyls (PCBs), pesticides (e.g., DDT, DDE), polybrominated diphenyl ethers (PBDEs), and chlorinated paraffins (CPs) are well-described endocrine disruptors (Damstra et al., 2002). Numerous studies have reported the presence of pesticide residues and metabolites, organochlorinated compounds, and other environmental compounds in a variety of tissues and species of marine mammals (Gregory and Cyr, 2003). Relationships between thyroid function and the concentration of organochlorine compounds are reported in wildlife animals. Significant decreases of T_3 and T_4 were found in sea lions in relation with PCBs and DDTs (Debier et al., 2005), in polar bears in relation with PCBs (Skaare et al., 2002), and in seals in relation with PCBs (Brouwer et al., 1989) and PBDEs (Hall et al., 2003).

Beside these results, several field studies reported alterations in thyroid gland morphology probably accompanied with impairment of thyroid function in marine mammals associated to exposure to persistent organic pollutants. Histologic examinations of 40 thyroid glands from harbor seals that died during the epizootic of phocine distemper infection in the North Sea (1988–89) exhibited colloid depletion and fibrosis that have been associated with chronic PCB exposure (Schumacher et al., 1993). Morphologic changes in thyroid gland have also been reported in beluga (*Delphinapterus leucas*) inhabiting the St. Lawrence estuary that have very high levels of organochlorine pollutants (De Guise et al., 1995). These results were similar to those of experiments with rats (Byrne et al., 1987) and seals (Brouwer et al., 1989) fed directly with PCBs. The effects of PBDEs, PCBs, and CPs on the thyroid are well documented in rats. Histopathological changes were reported to be associated the decrease of circulating thyroid hormones, especially T_4 (Hallgren and Darnerud, 2002). A relationship between PCBs, PBDE, DDE, and DDT compounds and interfollicular fibrosis has also been reported in the thyroids of harbor porpoises (Das et al., 2006b).

In contrast, fewer studies have focused on potential relationships between essential (zinc [Zn], copper [Cu], iron [Fe], selenium [Se]), and nonessential metals (cadmium [Cd] and mercury [Hg]) and thyroid histology. Essential and nonessential metals may also interact with the thyroid (Rolland, 2000). The cellular mechanisms involved in thyroid pathology are poorly understood. Generally the trace elements act at multiple sites via multiple mechanisms of action. These elements play a physiologic role in the metabolic regulation(s) of a thyroid disorder and can intervene in the secretion and distribution of thyroid hormones (Tsou et al., 1993;

Gupta et al., 1997b). They can stimulate or inhibit the secretion via the pituitary by inhibiting other hormones to connect with the corresponding receptors on the pituitary cell membranes (Oliver, 1975; Esipenko and Marsakova, 1990; Bedwal and Bhuguna, 1994; Goel et al., 1994; Kralik et al., 1996). Trace elements can also affect the hepatic iodothyronine deiodinase activity preventing the conversion of T_4 to T_3 (Arthur et al., 1991; Kralik et al., 1996; Gupta et al., 1997a; Gupta and Kar, 1998) or may accelerate the iodine depletion of thyroid (Wu et al., 1995). The adverse effect of these trace elements can be observed at several endpoints, such as a decreased thyroid hormone concentration in the plasma and peripheral tissues (Oliver, 1975; Kawada et al., 1980; Nishida et al., 1986; Esipenko and Marsakova, 1990; Ghosh and Bhattacharya, 1992; Goel et al., 1994; Nishijo et al., 1994; Pavia J'unior et al., 1997; Gupta and Kar, 1999; Zimmermann et al., 2000), reduced thyroid gland volume and weight, or the thyroid may show changes of atrophy and degeneration in the follicles (Oliver, 1975; Zimmermann et al., 2000).

There are indications that zinc is important for normal thyroid homeostasis. Its roles are complex and may include effects on both the synthesis and mode of action of the hormones. Thyroid hormone binding transcription factors, which are essential for modulation of gene expression, contain zinc bound to cysteine residues (Ruz et al., 1999). In the thyroid gland itself, transcription factor 2 (TF-2), which interacts with the promoters for the thyroglobulin and thyroperoxidase genes, is a zinc-containing protein (Tsou et al., 1993; Gupta et al., 1997b). Iron and copper status have also been linked to decreased plasma T_3 concentrations in animals and humans. It remains to be determined whether the changes in thyroid metabolism are a direct result of the iron and copper deficiencies or a nonspecific response to poor health (Oliver, 1975; Esipenko and Marsakova, 1990; Bedwal and Bhuguna, 1994; Goel et al., 1994; Kralik et al., 1996; Zimmermann et al., 2000). Selenium is a component of iodothyronine deiodinases, which transforms T_4 to T_3 in liver, kidney, muscle, and thyroid. It also plays a role in oxidative stress control at the thyroid as a component of the enzyme glutathione peroxidase (Arthur et al., 1991; Wu et al., 1995; Ruz et al., 1999). Cadmium alters the thyroid function at glandular as well as peripheral levels by preventing the conversion of T_4 to T_3 by inhibiting the iodothyronine deiodinase activity (Ghosh and Bhattacharya, 1992; Nishijo et al., 1994; Pavia J'unior et al., 1997; Gupta et al., 1997a; Gupta and Kar, 1998, 1999). Mercury is a toxic element with significant effects on many tissues, including the thyroid. It has been shown that moderate occupational exposure affects the enzyme deiodinase responsible for the deiodination of T_4 toT_3 (Kawada et al., 1980; Nishida et al., 1986; Ghosh and Bhattacharya, 1992).

Unlike their exposure to modern synthetic organic chemicals, the exposure of marine mammals to metals has occurred throughout history, during which they may have developed mechanisms either to control their concentration or to mitigate their toxic effects, such as the metallothioneins, which play an important role in the transport storage and detoxification of metals in vertebrates (Das et al., 2000, 2006a).

Recently Zn and Hg were found in high concentration in the livers of southern North Sea harbor porpoises; these high concentrations were linked to degrading body condition (Siebert et al., 1999; Bennett et al., 2001; Das et al., 2004). Questions arise about potential relationship between essential and nonessential metals and the thyroid histomorphometry.

The aims of the present study are 1) to evaluate the proportion of follicular, connective and vascular tissues in the thyroid of harbor porpoises collected around UK and Belgian waters by histomorphometry using the image acquisition software DP-

Soft; 2) to compare the observed histologic lesions with those previously observed in harbor porpoises from Germany, Norway, and Iceland (Das et al., 2006b); and 3) to use a multivariate analysis to investigate the potential relationships between thyroid histomorphometric parameters and previously described trace metal concentration in the liver (Zn, Fe, Cu, Se, Cd, and Hg) (Jepson, 2003; Das et al., 2004).

MATERIALS AND METHODS

Tissue sampling

Between 1998 and 2001 tissue samples (thyroid, liver) were collected from 113 porpoises from Belgian (n=46) and UK waters (n=67). Post mortem examinations were performed according to standard protocols (Law, 1994). For each histologic section of the thyroid the state of preservation, the presence of artifacts, and the presence of lesions such as congestion, cystic lesions, hyperplasia, and interfollicular fibrosis was assessed. The sections presenting signs of autolysis were discarded from this study. Of the 36 best-preserved animals included in this study, 22 were male and 14 were female (comprising 13 adults, 16 juveniles, and seven neonates). Thirteen harbor porpoises were by-caught and 23 animals stranded (Table 1). The age was determined for 24 porpoises by counting the dental growth layers (Lockyer, 1995) or were classified in age classes (neonate, juvenile, and adult) according to their size and development of the gonads.

Histology and immunohistochemistry

Samples of the thyroid glands were fixed in 10% formalin, processed by conventional techniques, then embedded in paraffin wax at 60 C for histologic and immunohistohistochemical investigations. Paraffin wax-embedded tissue sections (5 µm) were stained with haematoxylin and eosin (HE) and by elastic van Gieson for the detection of collagen (Siebert et al., 2002).

For immunohistochemistry a polyclonal rabbit antihuman thyroglobulin antibody (Code No. A 0251, DAKO Corporation Hamburg, Germany) and the Avidin-Biotin-Peroxidase complex method were used as described previously (Baumgärtner et al., 1989). The blocking serum used was from a goat (PAA Laboratories GmbH, Pasching, Austria). The polyclonal antibody against thyroglobulin was used in a solution of 1:2,600 in TBSc (900ml 0.85% NaCl, 100ml 0.05M Tris-Buffer, 37ml 1N HCL, 2.5ml Triton x-405, Aquadest, pH 7.6). A biotinylated anti-rabbit-immunoglobulin (Vector Laboratories Inc., BA 1000, Peterborough, UK) was used as a secondary antibody. The sections were then treated with avidin-biotin-peroxidase complex (Vector Laboratories Inc., PK 4000). As a negative control, thyroid gland sections were treated with a monoclonal antibody against the T-cell surface antigen of chicken lymphocytes (T1), which was used as control antibody. Previously positively stained sections were used as a control.

Scoring of the thyroid gland

For the histomorphologic analysis, images of 10 randomly selected visual fields in the microscope with a magnification of 200 of each section were observed. Thyroid histomorphology was measured using DP-Soft® software (version 3.2, Soft Imaging Systems GMBH) with a digital camera (Olympus C-4040 Olympus, Hamburg, Germany) connected to a light microscope (Olympus Statif CX 41 Olympus). The images showed a visual field of 633 µm in width and 475 µm in height. The proportion of different tissue types in the thyroid gland was determined by circumscribing the perimeter of the different tissue types (connective, follicular, and vascular tissue) present in the thyroids. The surface occupied by the follicular, vascular, and connective tissue was thus interactively measured, and the diameter and number of follicles present in each vision field were determined, and the mean value of these parameters from the 10 scored fields was used for statistical analyses.

Integration of previously published data

Thyroid histomorphometric measurements collected previously on porpoises from German (n=31; 24 from the Baltic Sea and seven from the North Sea), Norwegian (n=14), and Icelandic (n=11) waters and presented in Das et al. (2006b) were integrated in the study after intercalibration. This increased the sample size and statistical power for the analysis investigating potential relationships between thyroid parameters and trace element concentrations.

Trace metal results were extracted from larger studies presented previously (Das et al., 2003; Jepson, 2003; Das et al., 2004). Briefly, atomic absorption spectrophotometry (ARL 3510, Thermo Scientific, Breda, The Netherlands) was used to determine Cu, Zn, Fe, and Cd concentrations. Mercury was analyzed by flameless atomic absorption spectrophotometry (Perkin-Elmer MAS-50A Perkin-Elmer

Massachusetts, USA). Selenium was analyzed by fluorimetry. Concentrations are expressed as $\mu g\ g^{-1}$ dry weight.

Statistical analyses

Statistical analysis of the data was performed using Statistica® software (Statsoft Inc., version 7.1 Statsoft, Maison-Alfort, France). The Kolmogorov-Smirnoff test was used to test for normality of the statistically treated variables of thyroid morphology (the surface area occupied by follicular, vascular, and connective tissue and the diameter of follicles) and the hepatic trace metal concentrations (Zn, Cu, Fe, Se, Cd, and Hg). The trace metal concentrations had been log-transformed to normalize their distribution. The nonparametric Mann-Whitney *U*-test followed by Fisher's Omnibus post hoc tests were used to compare differences among sexes, age categories (neonate, juvenile, and adult), geographic origin (Belgian and UK waters), and cause of death (by-catch and stranding).

Sources of the potential differences between histologic quantification methods of thyroid tissues were analyzed by Wilcoxon test, which permitted us to evaluate systematic differences. The effect size, which in statistics is a measure of the strength of a relationship between two variables, has been extracted from the ANOVA method. It allowed us to know along with the statistically significance of the differences in evaluation methods the size of any observed effects. Thus we evaluated the size of the variance due to the quantification method, in other words, the size of the effect due to the differences in evaluation methods. A Spearman correlation permitted to evaluate the relation of the tissue definition concepts between the two evaluation methods.

Intersite comparison was realized using discriminant analysis to asses the ability of thyroid parameters and trace metals to discriminate among the different collection locations (Iceland, Norway, Germany [North Sea and Baltic Sea], UK, and Belgium). Multiple regressions were performed to examine the relationship between the hepatic trace metal concentrations and the thyroid parameters (connective tissue proportion and mean follicle size). Results were judged significant when $P<0.05$.

RESULTS

Histology

Using light microscopy, irregular or oval follicular lumens were seen in the parenchyma of the thyroid, surrounded by follicular epithelial cells. Follicular epithelial cells were often invaginated into the follicular lumen (Fig. 1). The follicular cells varied in height and shape and were commonly low cuboidal to flattened. Nuclei were spherical, central, poor in chromatin, and contained one or more nucleoli (Fig. 2). The follicles were surrounded by a variable layer of connective tissue and blood vessels. Three tissue compartments were distinguished in the thyroid gland: the follicular tissue (comprising follicular epithelial cells and colloid), the vascular tissue, and the connective tissue.

Rabbit polyclonal antibody human thyroglobulin, cross-reacted with the thyroid of harbor porpoises (Fig. 3). Thyroglobulin was detected in the lumen of the follicles and in the follicular epithelial cells. In some histologic sections the color was also disseminated in the parenchyma, probably a consequence of autolysis.

Tissue proportions

The follicular tissue occupied a mean surface of 70% of the total thyroid surface (ranging from 54% to 84%), whereas the vascular tissue occupied a surface of 20% (ranging from 2% to 33%) and the connective tissue a surface of 10% (ranging from 1% to 24%). The follicular lumens were larger in the central than in the peripheral regions of the gland. The diameter of follicular lumens ranged from 40 to 192 μm (Table 2). No differences in tissue proportions were observed between the harbor porpoises from Belgian and the UK waters.

Thyroid histomorphometric measurements collected previously on porpoises from German, Norwegian, and Icelandic waters (Das et al., 2006b) were integrated into this study to increase the sample sizes for the statistical analysis investigating potential relationships between these parameters and trace metal concentrations. To intercalibrate these two studies, we quantified and compared the three different tissues (follicular, connective, and

TABLE 1. General data of the sampled harbor porpoises (*Phocoena phocoena*) from Belgian and UK waters.

Collection country	Found date	Sex	Age	Age category
Belgium	23/06/2000	M	Unknown	Adult
Belgium	29/11/1999	M	8	Adult
Belgium	13/03/2000	F	1	Juvenile
Belgium	8/12/2000	F	Unknown	Juvenile
Belgium	24/03/2001	M	Unknown	Juvenile
Belgium	26/06/2002	F	Unknown	Neonate
Belgium	19/03/2002	F	Unknown	Juvenile
Belgium	2/09/2002	M	Unknown	Adult
Belgium	3/05/1999	M	Unknown	Juvenile
Belgium	30/06/1999	M	Unknown	Neonate
Belgium	15/02/1999	M	1	Juvenile
United Kingdom	29/01/1997	F	7	Adult
United Kingdom	13/02/1999	M	0	Juvenile
United Kingdom	04/03/1999	F	4	Adult
United Kingdom	05/11/2000	M	1	Juvenile
United Kingdom	21/11/2000	M	Unknown	Adult
United Kingdom	29/02/2000	F	1	Juvenile
United Kingdom	13/03/1998	F	0	Juvenile
United Kingdom	14/03/2000	F	Unknown	Adult
United Kingdom	13/04/1999	M	2	Juvenile
United Kingdom	09/04/1998	M	2	Adult
United Kingdom	01/06/1992	M	0	Neonate
United Kingdom	18/04/1997	F	0	Juvenile
United Kingdom	04/03/1993	M	1	Juvenile
United Kingdom	09/03/1993	M	1	Juvenile
United Kingdom	24/06/1992	M	0	Neonate
United Kingdom	06/06/1997	M	0	Neonate
United Kingdom	23/09/1992	M	15	Adult
United Kingdom	21/06/2001	M	Unknown	Adult
United Kingdom	07/07/1998	F	Unknown	Adult
United Kingdom	11/07/2000	M	0	Neonate
United Kingdom	23/07/1998	M	0	Neonate
United Kingdom	12/07/2001	F	Unknown	Adult
United Kingdom	05/12/1997	F	Unknown	Adult
United Kingdom	18/12/1995	M	1	Juvenile
United Kingdom	25/04/1991	F	1	Juvenile

vascular tissue) by DP-Soft in 10 thyroid sections that have been analyzed by the previous method (Table 3). Because vascular tissue was previously integrated in follicular tissue, only connective tissue proportions were comparable between the studies (Table 4). The approximate mean value from the follicle size was used to compare the mean follicle diameter of the harbor porpoises collected on Belgian, UK, German, Norwegian, and Icelandic waters.

Collection location differences were explored by discriminant analysis to simultaneously evaluate similarities in the thyroid parameters (connective tissue proportion and mean follicle size) and hepatic element concentrations (Zn, Cu, Fe, Se, Cd, and Hg) among the porpoises sampled at the six locations. Three porpoise groups could be distinguished by their connective tissue proportion, and the Cd, Zn, and Fe values (given in order of decreasing importance). The first discriminant function (root) explained 69% of the variations between groups involving mostly the connective tissue proportion, and the second discriminant function explained a further 17% of the variation between groups, involving the

TABLE 1. Extended.

Weight	Length	Blubber thickness	Cause of death	Place found	Location
58	160	5	Unknown	Nieuwpoort	Belgium
43.4	144	14	Unknown	Koksijde	Belgium
21.5	114	20	Unknown	Westende	Belgium
29	114	20	Unknown	Koksijde	Belgium
17	110	6	Unknown	Wenduine	Belgium
7	77	8	No bycatch	Knokke-Heist	Belgium
32	130	15	Infection	Oostende	Belgium
37	137	8	Infection	Oostende	Belgium
15	108	8	Unknown	Blankenberge	Belgium
7.4	80	6	Unknown	Middelkerke	Belgium
25.5	101	25	Unknown	Nieuwpoort	Belgium
50.5	154	14	Bycatch*	Bridlington Bay	Humberside
30	124	17	Bycatch	West Looe	Cornwall
50.8	143	21	Pneumonia, parasitic, and bacterial	Westminster Bridge	Greater London
24	109	22	Bycatch*	BYC off Bridlington	Humberside
38	148	7	Pneumonia, parasitic, and bacterial	Woolacombe Bay	Devon
18.2	109	9	Pneumonia, parasitic	Blyth	Northumberland
36	131	22	Live stranding	Westward Ho	Devon
48.5	148	22	Pneumonia, unknown aetiology	Battersea Bridge	Greater London
36.8	122	23	Bycatch*	BYC Minsmere Sluice	Suffolk
35.5	138	18	Bycatch*	Scarborough	North Yorkshire
8	81	12	Physical trauma	Withernsea Beach	Humberside
26	110	26	Bycatch*	Cromer Point	North Yorkshire
28	119	23	Bycatch	Sunderland	Tyne and Wear
24	113	16	Generalized bacterial infection	Bognor Regis	West Sussex
7.5	78	12	Starvation (neonate)	Isle of Sheppey	Kent
8	90	9	Starvation (neonate)	Gt. Yarmouth	Norfolk
40.5	136	19	Bycatch	Tresaith	Dyfed
43.8	136	19	Bycatch*	BYC off Bridlington	Humberside
45	154	10	Generalized bacterial infection	Sea Palling	Norfolk
9.5	88	12	Physical trauma	Rhos-on-Sea	Conwy
9	84	13	Starvation (neonate)	Snettisham	Norfolk
37	136	12	Bycatch*	BYC off Bridlington	Humberside
51	156	12	Starvation	Whitley Bay	Tyne and Wear
26.2	127	16	Bycatch	South Shields	Tyne and Wear
25	120	17	(Meningo) encephalitis	Mablethorpe	Lincolnshire

Cd, Zn, and Fe concentrations. Together, the two discriminant functions explained 86% of the variance (Table 5). As previously described, porpoises from Iceland are clearly separated from the other locations by the decreased proportion of connective tissue in their thyroids and the high Cd values (Das et al., 2004, 2006b). Porpoises from Norwegian and German North and Baltic Seas are situated close together in the diagram and could not be discriminated based on thyroid parameters and metal values. Porpoises from UK and Belgian waters were clearly discriminated from the other porpoises by their small connective tissue proportion and their high Zn values.

Relationships between histologic thyroid parameters and environmental factors

We evaluated different factors that could be implicated in the etiology of alterations of the thyroid histology. No significant relationship was observed between sex, age category, origin, cause of death, and the thyroid morphology (Mann-Whitney followed by Fisher's Omnibus test, $P>0.05$). No significant relationship was observed between sex, origin, cause of death, and trace element con-

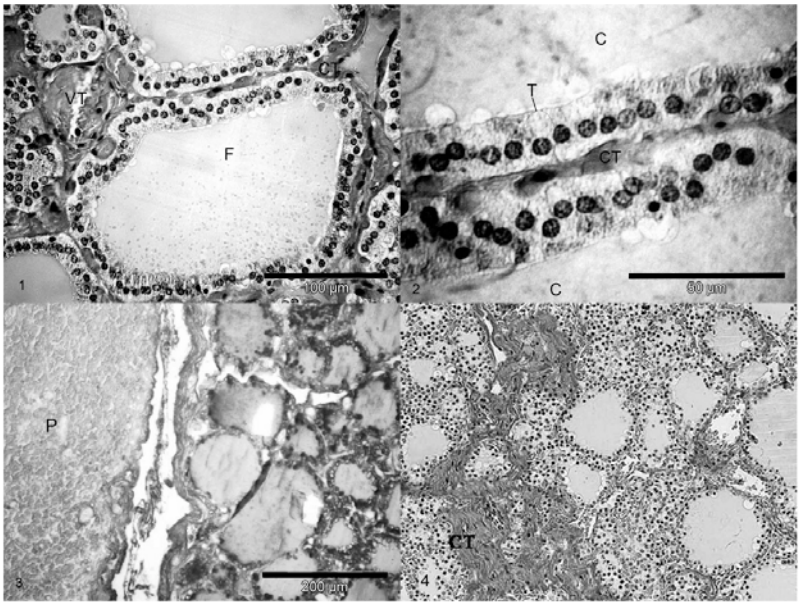

FIGURE 1. Thyroid gland of a harbor porpoise from Belgian waters. **F**: follicle is surrounded by connective tissue (**CT**) and some vascular tissue (**VT**) (Van Giesson staining, magnification 400×, scale bar = 100 µm). FIGURE 2. Follicular epithelium in a thyroid gland of a harbor porpoise from Belgian waters. C: colloid, CT: connective tissue, T: follicular epithelium (Van Giesson staining, magnification 1,000×, scale bar = 50 µm). FIGURE 3. Thyroglobulin specific reaction in the thyroid of harbor porpoise. Right: positive coloration of the thyroid tissue and left no coloration of the parathyroid tissues (immunohistochemical staining, magnification 200×, scale bar = 200 µm). FIGURE 4. Thyroid gland of a harbor porpoise showing a severe fibrosis (Van Giesson staining, magnification 200×, scale bar = 200 µm).

centration (Mann-Whitney followed by Fisher's Omnibus test, $P>0.05$). We observed a significant relationship between age category and trace element concentration (Mann-Whitney followed by Fisher's Omnibus test, $P<0.05$). The Se, Hg, and Cd concentrations increase with the age of the animals. A slight seasonal effect was observed with the thyroid parameters (Mann-Whitney followed by

TABLE 2. Morphometry of the thyroid gland in harbor porpoises from Belgian and UK waters.[a]

	n	Mean	Min	Max	SD
Follicular tissue (%)	36	71.6	53.9	83.1	7.4
Connective tissue (%)	36	10.2	0.5	23.5	4.8
Vascular tissue (%)	36	18.2	1.5	32.8	7.5
No. of follicles	36	15.7	4.7	34	7.7
Mean diameter of follicles (µm)	36	90.3	40.2	191.6	37.5

[a] Data are given as number of samples (*n*), mean, range (minimum [Min] and maximum [Max]), and standard deviation (SD).

TABLE 3. Intercalibration between the quantification method used by Das et al. (2006b) and the method used in this study.

		Quantification without DP-Soft (Das et al., 2006)			Quantification with DP-Soft (this study)		
Animal	Origin	Follicular tissue %	Connective tissue %	Solid tissue %	Follicular tissue %	Connective tissue %	Vascular tissue %
1318	Baltic Sea	19	33	48	64	27	9
1348	Baltic Sea	16	16	68	74	16	9
1423	Baltic Sea	13	13	74	72	10	18
1493	Baltic Sea	17	42	42	47	47	6
1638	Baltic Sea	2	61	37	34	62	4
1662	Baltic Sea	3	48	48	64	27	9
1666	Baltic Sea	3	48	48	58	37	8
1670	Baltic Sea	42	17	42	68	27	5
1681	Baltic Sea	3	48	48	43	51	6
1715	Baltic Sea	56	22	22	67	18	15

Fisher's Omnibus test, $P<0.05$). The porpoises that stranded in winter had a higher proportion of connective tissue.

Relationships between organohalogenated pollutants and thyroid histomorphometry have been observed in the 36 harbor porpoises collected along the Belgian and British coasts (Schnitzler, 2005). These results support the findings of Das et al. (2006b) and the hypothesis of a contaminant-induced thyroid fibrosis in harbor porpoises. In this study we examined the relationship between selected trace metals on thyroid histologic parameters, such as the proportion of connective tissue and mean follicle size by multiple regressions. This analysis revealed a negative correlation between the connective tissue proportion and the Cd, Cu, and Se concentrations in the livers of harbor porpoises from UK, Belgian, German, Norwegian, and Icelandic waters ($P=0.0004$, $R^2=0.348$; Table 6).

DISCUSSION

The morphology of the harbor porpoise thyroid was similar to thyroid glands of other mammal species (Bloom and Fawcett, 1975; Jubb et al., 1993; Junqueira et al., 1995; Cowan and Tajima, 2006). Although the follicular lumina were shrunken in size because of fixation, on average they were larger than that of goat but seem to be smaller in comparison to larger ruminants (Shimokawa et al., 2002; Table 7). The follicular tissue occupied around 70% of the thyroid surface on section, the vascular tissue 20%, and the connective tissue 10%.

TABLE 4. Analysis of the sources of differences between the tissue quantification methods used in these two studies.

		Follicular tissue	Connective tissue	Solid tissue
Wilcoxon	V	32	34	30
	P-value	0.693	0.557	0.846
	N	10	10	10
Effect size	SSeffect (=SCTr)	573.306	30.836	311.008
	SSerror (=SCE)	3,592.172	343.013	4,545.377
	Effect size	0.138	0.082	0.064
Spearman correlation	r_s	0.443	0.841	0.138
	P-value$_{app}$ 10 cases; 10,000 permutations	0.203	0.009	0.705
	Determination coefficient	0.196	0.707	0.019

TABLE 5. Summary of the discriminant analysis results for the two first principal components (PCs).[a]

	Component 1	Component 2
Cadmium	0.14	**−0.85**
Iron	−0.31	**−0.33**
Zinc	−0.37	**−0.54**
Copper	0.06	**0.27**
Selenium	0.47	**0.32**
Mercury	−0.23	**0.49**
Connective tissue	**−1.1**	−0.15
Follicle diameter	**−0.43**	0.1
Cumulative variance	0.69	0.86

[a] Boldfaced values refer to the main correlation between the PCs and the variables.

TABLE 6. Results of the multiple regression between the hepatic trace elements concentration and the proportion of connective tissue in the thyroid.[a]

	t-Value	*P*-Value	
OrdOrig.	1.3	0.2	*N*
Cadmium	−4.6	**0.01**	73
Iron	−0.3	0.74	75
Zinc	0.3	0.75	80
Copper	−2.1	**0.04**	78
Selenium	2	**0.05**	69
Mercury	−1.6	0.11	77

[a] $R^2 = 0.35$; $F(6.56) = 4.98$; p-value = 0.0004.

Generally low variability in the concentration of the serum thyroid hormones is observed across age season and sex (Rosa et al., 2007). In our study the thyroid histomorphology was consistent among age classes (neonate, juvenile, and adult) and sex. This supports the existence of strong homeostatic mechanisms for maintaining thyroid hormone concentrations in healthy animals (Rosa et al., 2007).

A slight seasonal effect has been observed on the proportion of the connective tissue, with winter-stranded porpoises having a higher proportion of connective tissue in the thyroid. This can be related to the fact that older animals were more represented in winter-stranded porpoises and had a better nutritional status than porpoises found dead in summer. St. Aubin and Geraci (1989) observed marked seasonal differences in thyroid histology of beluga and suggested that these differences related to increased water temperatures of seasonal occupied area. Such changes may be species specific, because such effects were not observed in bottlenose dolphins (St. Aubin et al., 1996) and could be related to the fact that the belugas undergo a seasonal migration (Richard et al., 2001). Most of the studied porpoises were juveniles, which maintain an active thyroid appearance during the different seasons (Rosa et al., 2007).

Compared to the results of our former study (Das et al., 2006b), we observe several geographic differences. Icelandic porpoises were characterized by a very small proportion of connective tissue (3%) and small follicle size, whereas the German and Norwegian porpoises displayed a high proportion of connective tissue (35%; Fig. 4) and larger follicles. Based on these differences, the porpoises could be separated into three groups (Fig. 5); Belgian and UK porpoises take an intermediary position between those two extremes. The size of the follicles and the shape of the follicular cells give an indication of the secretary activity of the gland. Thyroid glands dominated by small follicles lined by cuboidal or columnar cells can be classified as highly active, whereas low active glands show large follicles lined by low or flattened epithelial cells (Hallgren and Darnerud, 2002). Thyroids of the porpoises from the Icelandic coast could have a higher secretory activity than those from the German and Norwegian coasts.

The follicles are normally separated from each other by a fine irregular layer of connective tissue, mainly formed by reticular fibers. Also, some connective tissue can be associated to the vascular tissue (Junqueira et al., 1995; Cowan and Tajima, 2006). In our study the connective tissue proportion varied widely (1–23%) in the thyroids of the harbor porpoises collected along the Belgian and British coasts and occupies an intermediate mean position of 10%. This accumulation of

TABLE 7. Diameter of thyroid follicles in different mammals.

Species	Follicle diameter (μm)	Source
Mouse	41.8–52.6	Shimokawa et al. (2002)
Cat	56.0–66.4	Shimokawa et al. (2002)
Goat	89.7–102.8	Shimokawa et al. (2002)
Harbor porpoise	48.3–127.4	This work
Risso's dolphin	98.1–120.3	Shimokawa et al. (2002)
Cattle	169.1–192.0	Shimokawa et al. (2002)
Camel	155.3–240.7	Shimokawa et al. (2002)

connective tissue could be of pathologic origin. The collagen deposition and reduction of the initial vascularization in the newly formed scar tissue is a long process that takes weeks to months (Schumacher et al., 1993). Schumacher et al. (1993) showed that no morphologic signs of an increase in the collagen content occurred in his autolysis experiments, so that artificial swelling of the connective tissue due to autolysis can be excluded (Schumacher et al., 1993).

Schumacher et al. (1993) and Das et al. (2006b) related an observed colloid depletion and interfollicular fibrosis in seals and harbor porpoises, respectively, to elevated concentrations of organic contaminants (mainly PCBs, DDTs, and PBDEs). This relationship between organohalogenated pollutants and thyroid histomorphometry has also been observed in the 36 harbor porpoises collected along the Belgian and British coasts (Schnitzler, 2005). The Belgian and British harbor porpoises presented a lower concentration of organochlorinated pollutants (Mean sum of seven ICES PCBs±SD) in their blubber (1,780±1,370 and 1,990±1,553 ng g^{-1} lipid weight, respectively; Covaci et al., 2002; Jepson, 2003) compared to those from the German North and Baltic Sea coasts (7,664±5,075 and 8,247±7,949 ng g^{-1} lipid weight, respectively; Siebert et al., 2002) but still higher than those from Icelandic coasts (1,550±1,517 ng g^{-1} lipid weight; Siebert et al., 2002). These findings were therefore considered to support the hypothesis of an endocrine disruption of thyroid function mediated through chronic exposure to organochlorinated compounds (Covaci et al., 2002; Siebert et al., 2002; Jepson, 2003).

In contrast, the results of this study also demonstrated that hepatic Cd, Se, and Cu concentrations were negatively correlated with the proportion of connective tissue, thus failing to support the hypothesis that these metals may influence histologic changes in porpoise thyroid in a dose-dependent manner.

Our results also suggest that interfollicular fibrosis of the thyroid affect not only porpoises from the highly polluted Baltic Sea but also porpoises from other locations, including the Belgian and UK coasts. We have to emphasize that the high proportion of connective tissue measured in thyroid glands of the harbor porpoises from the German and Norwegian coasts indicates a severe pathologic dysfunction that, in other animals, results in a reduction of the thyroid function (Jubb et al., 1993; Schumacher et al., 1993). The effect of such dysfunction in the harbor porpoise remains poorly understood.

Thirty of the 36 analyzed Belgian and UK harbor porpoise thyroids had an interfollicular fibrosis. When these data were combined with earlier studies on thyroids of harbor porpoises from the German, Norwegian, and Icelandic coasts, the collective findings support the hypothesis of an endocrine disruption of thyroid function through organochlorinated compounds. The largely negative relationships found in this study between trace element (Cd, Se, and Cu) concentrations and histologic thyroid gland parameters (especially fibrosis) are interesting findings but do not support the hypothesis that these metals have an adverse affect on thyroid morphometry. Further research is needed to better understand the nature of any relationships between organochlorine and metals exposure and thyroid gland morphology and function in harbor porpoises.

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Effects of persistent organic pollutants on the thyroid function of the European sea bass (*Dicentrarchus labrax*) from the Aegean sea, is it an endocrine disruption?

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A B S T R A C T

We evaluated the alterations of organochlorinated compounds such as polychlorobiphenyls (PCB), dichloro-diphenyl-dichloroethylene (DDE) and dichloro-diphenyl-trichloroethane (DDT) on the thyroid in wild and cultured sea bass (*Dicentrarchus labrax*) at environmental concentrations. These compounds influence the endocrine system of many fish species and are qualified as endocrine disruptors. The thyroid seems to be a target organ. Two alteration endpoints: the thyroid histology and the muscular thyroid hormone concentrations, were used simultaneously.

High concentrations in PCBs and DDT were detected in muscles, supporting the idea that the Mediterranean fauna could be more polluted than the Atlantic fauna. The high abundance of DDE indicates a progressive degradation of remnant DDT load and the absence of new inputs in this area. Aquaculture sea bass shows a significant higher amount of pollutants on fresh weight basis (especially PCBs) in their muscles compared to the wild sea bass. Those differences may be related mainly to the contaminations of diet.

Thyroid parameters vary between wild and aquaculture sea bass, wild sea bass were characterized by higher follicle diameters, epithelial cell heights and muscular T_4 concentrations. A significant relationship between persistent organic pollutants (muscular PCBs and DDT concentration) and the different thyroid parameters (diameters of follicles, epithelial cell heights and muscular T_4 levels) could be observed, which support the hypothesis that these compounds have an adverse impact on thyroid morphometry and function.

1. Introduction

A number of naturally occurring and man-made chemicals are able to interact with the endocrine system of humans and wildlife, which can lead to a disturbance of hormone metabolism or hormone-regulated cellular and physiological processes (Damstra et al., 2002). The thyroid system is a major target of the so called endocrine disrupting chemicals. Today there are around 116 environmental compounds which are suspected to disrupt thyroid function (Howdeshell, 2002). The mechanisms involved in the endocrine disruptor mediated alteration of the thyroid function have been extensively investigated but are still not fully understood. The regulation mechanisms involved in thyroid homeostasis are numerous and complex. As consequence environmental chemicals can act at many levels in the thyroid system (Ishihara et al., 2003).

Endocrine disrupting chemicals interfere directly with hormone synthesis in the thyroid gland (Ishihara et al., 2003; Brown et al., 2004; Boas et al., 2006). They competitively bound to thyroid hormone binding proteins in blood like transthyretin (TTR) (Wade et al., 2002; Ishihara et al., 2003; Boas et al., 2006), to membrane bound transporters of target cells (Ishihara et al., 2003; Gauger et al., 2004) or to intracellular cytosolic thyroid hormone binding proteins which are thought to act as modulators of nuclear-receptor-mediated transcription (Ishihara et al., 2003; Blanton and Specker, 2007). The endocrine disruptors can also act on metabolic enzymes which activate or inactivate thyroid hormones (Ishihara et al., 2003; Zoeller et al., 2007). Peripheral iodothyronine deiodinases control the conversion of thyroid hormones in different organs and are thus essential in the regulation of levels of biologically active T_3 (Boas et al., 2006; Zoeller et al., 2007). Finally those pollutants can disrupt thyroid hormone receptors and accessory proteins which directly control the gene expression through thyroid hormone responsive elements (Ishihara et al., 2003; Blanton

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and Specker, 2007). Beside the direct effects via these sites, indirect effects via the hypothalamus and anterior pituitary gland were also possible (Ishihara et al., 2003; Zoeller et al., 2007).

An endocrine disruption of thyroid function may have severe consequences as thyroid hormones play an important role in the maintenance of a normal physiological status in vertebrates. Thyroid hormones assist in control of different physiological functions according to the development state of embryo, larva, juvenile and adult fish (Janz, 2000). In fish, thyroid status is positively correlated to reproductive status, whereas a seasonal rise in thyroid hormones coincident with gonadal maturation and reproduction has generally been observed. During ovarian maturation, thyroid hormones accumulates in the developing oocytes where they play a significant role in fish embryogenesis (Power et al., 2001; Yamano, 2005). Small thyroid follicles appear first at early stages of the larval period, then the follicles increases gradually in number and size. These small follicles are able to produce thyroid hormones which are detectable in larval fish in low concentrations (Yamano, 2005). During this phase the thyroid hormones intervene in the transformation of larvae into juveniles (Power et al., 2001).

In adult fish, thyroid hormones are of primary importance in the regulation of such fundamental physiological processes as growth, nutrient utilization, and reproduction, but the exact mode of action is unknown. Fish grow faster and are healthier when the thyroid hormone levels are high (Power et al., 2001; Yamano, 2005), which point out an economic outlet of this problematic in fishery and aquaculture.

Polychlorinated biphenyls (PCBs) and insecticides (like DDT and its metabolites), have been shown to alter thyroid hormone levels in experimental animals (Collins and Capen, 1980a, 1980b; Brouwer et al., 1989; Fowles et al., 1997; Hallgren and Darnerud, 2002). The aim of this paper is to evaluate if these alterations of the thyroid function are also observable in wild and cultured fish at environmental conditions. The European sea bass (*Dicentrarchus labrax*), is the top predator of a simple food web, it is commonly found in European coastal waters, its biology is well documented (Pickett and Pawson, 1994; Loizeau et al., 2001) and it is an important commercial species, also important from a human health view point.

To achieve this goal the concentrations of organochlorinated compounds were measured in wild and cultured specimens of *D. labrax*, and their effects on the thyroid function using simultaneously two thyroid endpoints were evaluated. The first is the thyroid histomorphometry which consist in a microscopically quantification of the diameter of follicles and the epithelial cell heights and the second is an analysis of muscular thyroid hormone concentrations. The combined analysis of histological and hormonal biomarkers allows a better estimation of the thyroid function status. The possible impact of the levels in PCBs and DDTs on the thyroid gland will be studied via a multivariate analysis.

2. Methods

2.1. Study site

Forty-six *D. labrax* specimens have been obtained during a sampling mission in the North Aegean (Eastern Mediterranean, Greece) which took place in February 2006. The fish used in this study were caught in the northern part of the Aegean Sea, fifteen of which were caught in the Stymonikos Gulf and the other thirty-one came from an cage fish farm situated in the isle of Thassos.

Strymonikos Gulf, occupies an area of 540 km^2 and its coast has a total length of 70 km. The Strymon River, found at the north of the Gulf, coming from Bulgaria with a catchment area of 17.130 km^2, and the Richios River at the west, constitute the main sources of fresh water, nutrients and pollution (domestic and agricultural pollutants) in this area (Stamatis et al., 2002; Kallianiotis et al., 2004). The Gulf is one of the most important nursery and fishing grounds for pelagic species of the North Aegean Sea (Sylaios et al., 1999). This area includes the most variable and spectacular insular, coastal and marine landscapes, as well as flora and fauna (Koutrakis et al., 2000). The gulf has a heavy pollution impact from land based sources, which includes fertilisers and pesticides from agriculture, untreated waste water from cities, and industrial waste from chemical plants are some of the localised threats, while intense oil tanker traffic and high fishing effort are widespread in this area (Dassenakis, 2000).

The isle of Thassos is located in the northern Aegean sea approximately 7 km from the mainland and 20 km south-east of Kavala. It has a total coastal length of 115 km and its total land surface is 378,84 km^2. The cage fish farm is situated at the north part of the island and is the only cage farm in the Kavala Prefecture. It has a total yearly capacity of about 70 t and its main production consists of Gild-head sea bream and European sea bass. The unit is positioned in a closed and windless bay with a depth of 12 m. In addition to its other production processes there is also a small hatchery for sea species (mainly European sea bass), witch covers its needs. The surrounding waters of both the unit and the island in general are relatively unpolluted (Kallianiotis et al., 2000).

2.2. Sampling of the different tissues

The fish were killed in ice water and stored on ice till their arrival at the Institute where they were immediately dissected. The length and the weight of each fish were measured. The lower jaw was removed from each animal and immersed in Bouin's fixative. Approximately 30 g skeletal muscle was excised from the area behind the head, dorsal to the lateral line and anterior to the dorsal fin. The muscle samples were stored at −70 °C until analysis.

2.3. Thyroid histomorphometric analysis

The thyroid tissues enclosed in the lower jaw parts were stored in Bouin's fixative. The tissue was then decalcified in 5% formic acid and 5% formaldehyde for a day and transferred into a sodium sulphate solution for one day. The tissues were dehydrated in a graded series of ethanol before being embedded in paraffin wax. The paraffin blocks were longitudinally sectioned (8 μm) through all the thyroid tissue (Zhou et al., 2000).

The hematoxylin-eosin stain and the Van Gieson stain method were used for the microscopically diagnostic study of the histological samples (Zhou et al., 2000). These staining methods were perfectly suitable for this kind of study, since the follicles and the epithelial cells were clearly designable and different measurements could be taken.

Thyroid histomorphometry was measured using DP-Soft® software (version 3.2 Soft Imaging Systems Gmbh) with a digital camera (Olympus C-4040) connected to a light microscope (Olympus Statif CX 41). DP-Soft is a basic image software for microscopy analysis, it permits the acquisition, the archivation and different measurements of the images. For the histomorphological analysis, images of ten randomly selected visual fields in the microscope, with a magnification 200 of each section, were observed.

The images showed a vision field of 633.1 μm large and 474.8 μm height. The different measurements in the thyroid tissue were determined by surrounding the contours of the follicles in the tissue. The follicle area and size and the epithelial cell height was thus interactively measured in each vision field. The calculation of the mean value used for statistical analyses was carried out by using 10 values per individual.

2.4. Organochlorine compound analysis

All solvents were of pesticide grade; *n*-hexane and acetone (Burdick & Jackson brand) were purchased from Fluka (Buchs, Switzerland). The Mirex (Dodecachloropentacyclo-[5.3.0.0.0.0]decane) used as internal standard, the pure PCB congeners (IUPAC Nos. 28, 44, 52, 66, 70, 87, 95, 101, 105, 110, 118, 128, 138, 149, 153, 156, 170, 180, 183, 187, 194, 195, 206, and 209) and the congener used as surrogate (IUPAC No. 112), were obtained from Ultra Scientific® and Dr. Ehrenstorfer®. All other chemicals used were of analytical grade.

Muscle samples were lyophilized over 20 h and dry matter was determined gravimetrically. The lipids were extracted using an accelerated solvent extractor (ASE) (Dionex ASE 2000, Dionex Corporation). A 650–750 mg sample of lyophilized muscle with 0.5 g of anhydrous sodium sulphate was extracted 3 times with a mixture of hexane, dichloromethane and methanol (5:2:1, v:v:v) at 80 °C and under a pressure of 1500 Psi. The solvent with the extracted fat was collected in pre-weighed vials and was evaporated at 40 °C under nitrogen flow (Turbovap LV Zymark). The fat content of muscle samples ('hexane-extracted fat') was determined gravimetrically. Lipids were then dissolved into 3 ml of hexane and collected into a test tube. The mixture was homogenized by vortexing during 1 min.

All prepared samples were then purified by acid and Florisil clean-ups. A 2 ml volume of sulphuric acid mixture (fuming sulphuric acid 30% and concentrated sulphuric acid 95%, 1:3, v:v) was added to the sample and the mixture was homogenized by vortexing before being centrifuged for 3 min at 1810g at 10 °C (Jouan). The organic phase was transferred to another tube and the acidic phase was extracted with 3 ml of hexane, vortexed and centrifuged for another 3 min. The organic phases were pooled and reduced to 1 ml under a nitrogen flow. The second clean-up was performed with Florisil® solid phase cartridges (Supelco, Envi-Florisil). The cartridges were first conditioned with 5 ml of acetone, 5 ml of an acetone-hexane mixture (50:50, v:v) and 12 ml of hexane, successively. The sample was then added at the top of the column. Polar molecules were retained on the Florisil® (magnesiumsilicate mixture). The test tubes containing the sample were rinsed with 3 ml of hexane and added to the cartridge. Another 3 ml of hexane were finally directly added to the column. The eluate was then evaporated just to dryness under a gentle nitrogen flow.

The dried residue of muscle samples was reconstituted with 125 μl of hexane and 125 μl of Mirex (100 pg μl^{-1}). The purified extracts were then analysed by gas chromatography using a Thermo Quest Trace 2000 gas chromatograph equipped with a 63Ni ECD detector (Thermo Quest, Trace 2000) and an automatic injector. From 1 to 5 μl of each purified extract was injected by means of a cold 'on column' injector. PCB congeners were separated on a 30 m × 0.25 mm (0.25 μm film) RESTEK RXI-5 ms capillary column (Bellefonte, USA). The temperature program was as follows: 2 min at 60 °C, gradual heating from 60 to 140 °C at the rate of 20 °C min^{-1}, 3 min at 140 °C, gradual heating from 140 to 270 °C at the rate of 25 °C min^{-1} and 12 min at 270 °C. The carrier gas was hydrogen with a flow rate of 4 ml min^{-1} and a pressure of 130 kPa, and the make-up gas was Ar:CH4 (95:5) at a flow rate of 30 ml min^{-1}. The injector was at ambient temperature and the detector was kept at 300 °C. PCBs were identified according to their retention times. Twenty-seven congeners, mostly present in Aroclor 1242, 1254 and 1260 mixtures, were measured (IUPAC 28, 44, 52, 66 + 95, 70, 87, 101, 105, 110 + 77, 118, 126, 128, 138, 149, 153, 156, 169, 170, 180, 183, 187, 194, 195, 206, and 209) and the *p,p*-DDT and *p,p*-DDE. The + sign indicates that these two congeners are not separated. Data were recorded using Chrom-Card 1.19 software. Quantification was performed by comparison with external standards of the 27 pure PCB components in a certified calibration mixture (Ultra Scientific and Dr. Ehrenstorfer®), using a linear calibration curve for each PCB congener whose concentration ranged from 2 to 100 pg μl^{-1}. PCB concentrations are expressed as the sum of the congeners measured.

Blanks were run with each sample series to control the clean-up procedures. Blanks were also used to control lyophilization and ASE steps. For each sample, a quality control (QC) was also analysed in parallel. Milk cream enriched with a defined concentration of PCBs was used as a QC for analysis. The PCB recovery was calculated on the basis of the concentration of the surrogate standard (IUPAC 112, Dr. Ehrenstorfer®) (50 pg μl^{-1}). It was added to the sample at the beginning of the clean-up for muscle samples. All results were corrected to obtain 100% recovery. However, the results of the PCB analyses were accepted only if the recoveries were between 70% and 130%.

2.5. Radio-immuno-assay

The iodothyronines were extracted from the muscle sample using procedures adapted from Parker (1988). The muscle was rinsed with NaCl solution (0.87%), minced with scissors and homogenized using a tissue grinder (60 s, 22,000 rpm) with 3 ml ice cold 100% ethanol containing 1 mM 6-*n*-Propyl-2-thiouracil. All the above procedures were performed on ice. After 15 min, the mixture was centrifuged at 1500g for 10 min at 4 °C and the supernatant was transferred to evaporations. The pellets were washed twice with 2 ml 100% ethanol by resuspension followed by centrifugation. The pooled supernatant was evaporated at 40 °C to dryness. The dried sample was stored at 0 °C until thyroid hormone content was assayed.

For the radioimmunoassay (RIA), the samples were re-suspended in 250 μl 95% ethanol and 250 μl 0.11 M sodium barbital (pH 8.6). Each 500 μl sample was vortexed for 15 s after each solution was added. The sample was transferred into an eppendorf snap cap vial and centrifuged at 3000 rpm for 10 min at 4 °C. Each sample was assayed in duplicate for T_4 content and for T_3 content. In this procedure 25 μl of the sample and 1 ml of radiolabelled thyroid hormone were added to antibody coated test tubes. The thyroid hormones compete for a fixed time (1 h at 37 °C) for sites on the specific antibodies. Since the antibody is immobilized on the wall of the test tubes, simply decanting the supernatant suffices to terminate the competition and to isolate the antibody-bound fraction of the radiolabelled thyroid hormones. Counting the tube in a gamma counter then yields a number, which converts by way of calibration curve to a measure of the thyroid hormones present in the fish sample. The kit was used according to manufactures instruction and chosen for well described affinities for fish thyroid hormones (Scholtz et al., 1992; Raine, 1998; Plate et al., 2002; Jenssen et al., 2004).

A computer program, contained in the counter, automatically determined the relative percent bound to each standard concentration and plotted a log-logit graph of the standard curve (% bound vs standard concentrations). The program then examined the percent for each unknown sample and interpolated the concentration of the sample from the standard curve graph. Total thyroid hormone content of each individual fish sample was determined by multiplying the hormone content of 25 μl subsample by 20 (500 μl total sample volume/25 μl subsample tested for TH content) and converting this concentration to total ng/sample. This number was divided by the weight of the extracted muscle to provide the hormone concentration of each fish in ng g^{-1} body weight (Scholtz et al., 1992).

To determine the efficiency of the extraction process in recovering thyroid hormones as well as transfer of samples into different types of tubes, a recovery analysis was employed. Unlabelled

thyroid hormone was added to the minced muscles prior to homogenization. Replicates tubes each were spiked with 0, 1, 4, 10, 16, and 24 μg dL^{-1} unlabelled T_4 and 0, 20, 50, 100, 200, and 600 ng dL^{-1} unlabelled T_3. The samples were then subjected to the same homogenization, extraction, reconstitution and RIA assay procedures as the unknown and standard curve samples. The percentage of thyroid hormone recovered from each spiked tube was calculated. These quality assurance procedures and recovery determinations were also performed in the study of Scholtz et al. (1992) on the Kokanee salmon (*Oncorhynchus nerka*) (Scholtz et al., 1992).

2.6. Statistical analysis

The relationships between thyroid parameters (follicle histomorphometry and thyroid hormone concentrations) and toxicological data were analysed in two steps. First, a correlation-based principal component analysis (PCA) was performed to reduce the 16 original PCB congener variables in order to avoid misleading results due to correlating independent variables ('multi-collinearity') in subsequent analysis. Thereafter a Spearman correlation test, with the three factor scores revealed by the PCA as independent variables and thyroid parameters as dependent variable, was applied. Results were considered significant when $p < 0.05$. Contaminant values were log-transformed to achieve homogeneity of variances and normal distribution. The non-parametric Mann-Whitney *U*-test was used to compare differences among sexes and the sample origin (wild or aquaculture). Statistical analysis of the data was performed using Statistica® software (Statsoft Inc., version 7.1).

3. Results

3.1. Thyroid histology of the European sea bass

The structure of the teleost thyroid, although similar to that of higher vertebrates, differs in its lack of a discrete organization. The thyroid tissue of the European sea bass consists of glandular follicles scattered around the ventral aorta and bronchial arteries that supply the gills (Fig. 1A). The follicle consists of an outer layer of thyroid epithelium that surrounds an inner lumen filled with colloid. Colloid contains a reserve of the protein-bound form of the thyroid hormones (Fig. 1B). The surrounding epithelial cells are either flattened, cuboidal, or columnar, depending on their activity. In light microscopy irregular or oval follicular lumen were seen surrounded by follicular epithelial cells (Fig. 1C). Stratified follicular epithelial cells were often invaginated into the follicular lumen. The nucleus of the gland cells were spheroidal, centrally situated, poor in chromatin and contain one or more nucleoli. The use of the *Van Gieson* method which permits to differentiate the connective tissue brought no further information of organisation (Fig. 1D). The thyroid tissue is composed of isolated thyroid follicles intermixed with the well vascularized connective tissue of the lower jaw.

3.2. Thyroid histomorphometric analysis

Wild caught specimens were found to have larger follicles than those from the aquaculture unit as their follicles have significant higher surface and diameter (Mann-Whitney; $p < 0.05$; Table 1). Beside this wild sea bass showed thicker epithelium compared to those coming from aquaculture (Mann-Whitney; $p < 0.05$; Table 1). No differences in histological thyroid parameters could be revealed by Mann-Whitney test between sexes ($p > 0.05$). No significant relationship of length and weight on the histological thyroid parameters could be showed in our sampling (Spearman correlation, $p > 0.05$). All individuals were regrouped for the further statistical analysis.

3.3. Dosage of thyroid hormones in muscle using radio-immuno-assay

Results of the recovery data from thyroid hormone spiked muscle indicate that percent recovery was 92.6% for T_3 and 92.4% for T_4.

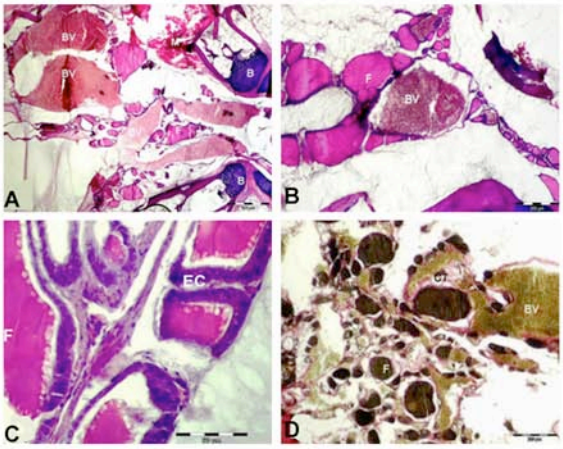

Fig. 1. (A) Longitudinal section of European sea bass thyroid tissue from the Aegean Sea (D.I. G/O/105, HE coloration, Scale bar = 500 μm) (BV = blood vessel; F = follicle; B = bone; M = muscle). (B) Longitudinal section of European sea bass thyroid tissue (D.I. G/O/106, HE coloration, Scale bar = 200 μm) (BV = blood vessel; F = follicle; B = bone). (C) Longitudinal section of European sea bass thyroid tissue (D.I. G/O/112, HE coloration, Scale bar = 50 μm) (F = follicle; EC = epithelial cells). (D) Longitudinal section of European sea bass thyroid tissue (D.I. G/A/217, Van Gieson method, Scale bar = 200 μm) (BV = blood vessel; F = follicle; CT = connective tissue).

Table 1
Histomorphometric analysis, thyroid hormone levels, lipid proportion, mean contamination levels of the 7 ICES PCBs, the 27 measured PCBs and of the pesticides (*pp'*-DDT, *pp'*-DDE, *pp'*-DDD) in the white muscle of European sea bass collected in the Aegean Sea from wild and from aquaculture and their food pellets

	Wild	Aquaculture	*p*-value
n	13	28	
Follicle area (μm²)	3319 ± 1609 3140 1533–8243	2452 ± 1540 1973 1045–8147	0.006
Follicle diameter (μm)	66 ± 12 63 48–99	56 ± 15 52 39–108	**0.002**
Cell height (μm)	7 ± 1 7 4–9	4 ± 1 4 3–6	**<0.001**
T_3 (ng g^{-1})	*n* = 13 0.21 ± 0.11 0.18 0.12–0.51	*n* = 17 0.26 ± 0.17 0.23 0.06–0.75	0.509
T_4 (ng g^{-1})	*n* = 3 11.44 ± 3.17 11.55 8.22–14.56	*n* = 19 8.07 ± 4.04 6.54 3.99–18.39	0.132
T_3/T_4 (ng g^{-1})	*n* = 2 0.012 ± 0.005 0.012 0.009–0.016	*n* = 11 0.033 ± 0.018 0.03 0.009–0.070	0.103
Lipid proportion (%)	*n* = 13 0.8 ± 0.29 0.8 0.39–1.27	*n* = 17 1.9 ± 0.65 1.8 0.95–3.02	**<0.001**

	Fresh weight	Lipid weight	Fresh weight	Lipid weight	Fresh weight	Lipid weight
Sum 7 ICES	*n* = 12 2.02 ± 0.95 1.67 1.04–3.73	*n* = 12 251.2 ± 165.8 225.9 62.3–717.5	*n* = 16 5.62 ± 1.82 4.59 3.64–8.66	*n* = 16 298.2 ± 90.1 261.4 205.7–535.7	**<0.001**	0.09
Sum 27 PCB	*n* = 12 5.53 ± 1.40 5.25 3.43–7.92	*n* = 12 746.5 ± 309.1 723.1 262.9–1369.0	*n* = 16 8.91 ± 2.53 8.04 6.21–12.77	*n* = 16 514.0 ± 142.2 467.0 364.7–759.9	**<0.001**	**0.018**
pp'-DDT	*n* = 12 0.30 ± 0.22 0.22 0.04–0.71	*n* = 12 34.3 ± 28.8 23.22 6.57–98.4	*n* = 16 0.38 ± 0.14 0.33 0.17–0.63	*n* = 16 30.0 ± 40.0 19.3 13.7–183.0	0.273	0.414
pp'-DDE	*n* = 12 4.53 ± 2.57 4.31 1.80–11.24	*n* = 12 521.6 ± 271.2 486.2 111.2–1003.9	*n* = 16 2.01 ± 0.81 1.66 1.01–3.89	*n* = 16 109.5 ± 42.2 97.6 75.5–222.6	**0.001**	**<0.001**
pp'-DDD	*n* = 12 0.52 ± 0.45 0.35 0.12–1.72	*n* = 12 59.0 ± 47.8 39.1 10.1–153.7	*n* = 16 0.75 ± 0.29 0.63 0.37–1.25	*n* = 16 40.8 ± 13.2 39.3 24.7–74.1	0.123	0.884

The concentrations are given in ng g^{-1} lipid weight and fresh weight (mean ± standard deviation, (median) and min–max) followed by a Mann–Whitney test to reveal potential differences in parameters (Significant *p*-values are in bold).

These recovery values were similar to those reported by other researchers; e.g., Kobuke et al. (1987) reported 83% in coho salmon eggs. The thyroid hormone content of 5 g of muscle of each fish sample was determined by multiplying the thyroid hormone content of 25 μl subsample by 20 (500 μl total sample volume divided by 25 μl subsample tested for hormone content) and dividing by the weight of the excised muscle of each fish (Table 1). Some samples were below the limit of detection have been ignored in the following statistical analysis. No statistical differences of these results in relation to the origin or sex could be revealed by Mann-Whitney tests. No significant relationship of length and weight on the muscular thyroid hormone content was found in the specimens used. (Spearman correlation, $p > 0.05$). This permitted the grouping of all the sampled animals for the following statistical analysis.

3.4. Organochlorine compound analysis

Lipid proportion in the muscles of the European sea bass from aquaculture and from wild ranged around from 0.4% to 3% of the wet weight. Sea bass from aquaculture had a significant higher lipid content in their muscles than those from wild (Mann-Whitney; $p < 0.05$; Table 1). From the 27 PCBs congeners that were analysed only 16 were detected in all the fish (IUPAC 28, 52, 44, 70, 66 + 95, 101, 112, 110 + 77, 149, 118, 143, 153, 138, and 180), the others have been ignored in the following principal component analysis. The 7 ICES (International Council for the Exploration of the Sea) PCBs (IUPAC 28, 52, 101, 118, 138, 153 and 180), the major congeners and most predominant used in the different commercial mixtures of PCBs, represent around 40–60% of all the found congeners according to the origin of the samples (Table 1).

Males and females were regrouped for the analysis because no significant difference could be detected (Mann-Whitney; $p > 0.05$). The wild sea bass had a significant higher total PCBs and *pp'*-DDE lipid-normalized concentrations in their muscles than those from aquaculture. No significant differences were observed in the lipid-normalized concentrations of the 7 ICES PCBs, the *pp'*-DDT and the *pp'*-DDD between wild and aquaculture sea bass (Table 1). The comparison of the fresh weight concentrations reveal meanwhile that aquaculture sea bass had a significant higher concentration of the 7 ICES PCBs and total PCBs whereas wild sea bass showed the highest *pp'*-DDE concentrations. No significant differences were observed in the fresh weight concentrations of the *pp'*-DDT and the *pp'*-DDD between wild and aquaculture sea bass (Table 1). The following 7 ICES PCBs were found in wild sea bass in decreasing importance: 153 > 138 > 28 > 101 > 180 > 52 > 118 and in aquaculture sea bass: 153 > 101 > 138 > 118 > 52 > 180 > 28. The pattern of aquaculture sea bass does not reflect specially the contamination pattern of their food: 153 > 138 > 52 > 180 > 101 > 118 > 28.

3.5. Relationship between thyroid parameters and PCB contamination

It is well known that PCB congeners may interact each to other, their effect can be antagonized or exhausted by the presence of the others congeners. The fresh weight pollutant concentrations have been used for this analysis as the thyroid hormone concentrations are not related to lipids and are also expressed in fresh weight concentrations. A principal component analysis permitted us to reduce the 16 PCB variables to 3 principal components. The three components represent 80% of the total variance (Table 2). The first component represent almost all the PCB congeners with a high degree of chlorination (hepta-, hexa- and pentachlorobiphenyls), whereas the second component represent a mixture of PCB congeners of different degree of chlorination and the third component represents mostly the PCB 52 (Table 2). Spearman correlation tests were used to test the effects of the different components on the thyroid parameters. A significant relationship could be observed between the follicle diameter, the epithelial cell height, the T_3/T_4 ratio and the first principal component. No significant relationship could be observed with the second and third component (Table 2).

Table 2
Principal component analysis, with the explained variance by the principal components, the classification of the different PCB congeners into the three principal components and the results of the multivariate Spearman correlation tests (Significant p-values are in bold)

	Lipid weight			Fresh weight		
	Fact. 1	Fact. 2	Fact. 3	Fact. 1	Fact. 2	Fact. 3
% of total variance	39.7	31.3	8.9	54.7	15.6	10.3
Cumulative variance%	39.7	71	79.9	54.7	70.3	80.6
44	0.28	**−0.57**	0.36	−0.06	−0.36	**−0.65**
70	−0.21	**−0.86**	−0.36	−0.49	0.54	−0.62
66 + 95	**−0.71**	−0.47	−0.32	**−0.73**	0.59	−0.28
101	**−0.96**	0.15	0.07	**−0.93**	0.02	0.09
112	0.48	**−0.81**	−0.02	**0.73**	−0.35	−0.29
110 + 77	**−0.9**	−0.09	0.1	**−0.93**	**−0.18**	0.03
118	**−0.9**	0.22	0.12	**−0.93**	−0.18	0.22
143	0.15	**−0.86**	0.13	−0.14	−0.80	−0.28
153	**−0.81**	−0.41	0.07	**−0.95**	−0.22	0.05
138	**−0.69**	−0.67	−0.11	**−0.90**	−0.23	−0.06
180	**−0.68**	−0.13	0.09	**−0.62**	−0.13	0.12
Diameter (μm)	R_S = 0.495	R_S = −0.112	R_S = 0.069	R_S = 0.390	R_S = −0.109	R_S = 0.032
	N = 21	N = 21	N = 21	N = 21	N = 21	N = 21
	p = 0.023	p = 0.630	p = 0.767	**p = 0.049**	p = 0.596	p = 0.877
Cell height (μm)	R_S = 0.527	R_S = −0.212	R_S = −0.358	R_S = 0.536	R_S = −0.387	R_S = −0.439
	N = 21	N = 21	N = 21	N = 21	N = 21	N = 21
	p = 0.014	p = 0.357	p = 0.111	**p = 0.005**	p = 0.051	**p = 0.025**
T_3 (ng g $^{-1}$)	R_S = −0.113	R_S = 0.006	R_S = −0.336	R_S = 0.175	R_S = 0.361	R_S = 0.019
	N = 21	N = 21	N = 21	N = 21	N = 21	N = 21
	p = 0.626	p = 0.978	p = 0.136	p = 0.393	p = 0.070	p = 0.927
T_4 (ng g $^{-1}$)	R_S = −0.400	R_S = −0.317	R_S = −0.467	R_S = 0.580	R_S = −0.098	R_S = 0.189
	N = 9	N = 9	N = 9	N = 9	N = 9	N = 9
	p = 0.286	p = 0.406	p = 0.205	**p = 0.048**	p = 0.762	p= 0.557
T_3/T_4	R_S = −0.800	R_S = 0.283	R_S = −0.367	R_S = −0.427	R_S = 0.308	R_S = 0.287
	N = 9	N = 9	N = 9	N = 9	N = 9	N = 9
	p = 0.010	p = 0.460	p = 0.332	p = 0.167	p = 0.331	p = 0.366

4. Discussion

4.1. Contamination level

The muscle PCB and DDT concentrations obtained in this study were higher than those reported for European sea bass from the Ria de Aveiro, Portugal (Atlantic) (Antunes and Gil, 2004) and the Orbetello Lagoon, Italy (coastal wetland) (Carubelli et al., 2007) and similar to those reported for European sea bass from Ebro Delta, Spain (western Mediterranean) (Pastor et al., 1996) (Table 3). This is in accordance with other studies who reported higher concentrations in persistent organic pollutants in the various levels of the Mediterranean marine wildlife (marine fish and mammals) compared to the levels measured in the same species in the Atlantic (Kilikidis et al., 1981; Marsili and Focardi, 1996; Canli and Atli, 2003; Fossi et al., 2004; Stefanelli et al., 2004; Storelli et al., 2004; Aguilar and Borrell, 2005; Naso et al., 2005).

It is interesting to see, contrary to other studies who report a reduction of DDT and PCB in striped dolphins of the western Mediterranean, typically used as indicators (Aguilar and Borrell, 2005), that contamination levels in sea bass remain similar to levels described 10 years ago (Pastor et al., 1996). This result underlines the importance of accuracy of such studies concerned with these pollutants and that the problem is still of interest. Most of the biological transformation processes in vertebrates metabolise small quantities of DDT into DDE. The DDE percentage is a common indicator of DDT degradation, and therefore of the age of the contaminant input (Aguilar and Borrell, 2005). Thus, this high abundance of DDE (percentage of 85% in wild sea bass) indicates a progressive degradation of the remnant DDT load and the absence of new inputs in the western Mediterranean. The exposure to DDT is likely due to the large amount of DDT that entered in the Aegean Sea as transnational pollution (through River Strymon that comes from

Table 3
Organochlorine pollutant contamination levels in muscles of European sea bass specimens (ng g $^{-1}$ lipid)

	Sum PCB	Sum DDT	Source
Kavalla wild, Greece (n = 13)	806 ± 514	615 ± 348	This work
Thassos aquaculture, Greece (n = 17)	487 ± 136	180 ± 95	This work
Food pellets (n = 1)	140	29	This work
Ebro Delta, Spain (n = 10)	800 ± 50	513 ± 97	Pastor et al. (1996)[a]
Ria de Aveiro, Portugal (n = 10)	155 ± 49 to 294 ± 104	108 ± 43 to 336 ± 132	Antunes and Gil (2004)[b]
Orbetello Lagoon, Italy (n = 13)	369 ± 195		Carubelli et al. (2007)

[a] PCBs (IUPAC Nos. 18, 28, 52, 49, 44, 101, 151, 149, 118, 153, 105, 138, 187, 183, 128, 180, 170, 194) and DDT compounds (p,p'-DDE, p,p'-DDD and p,p'-DDT).
[b] PCBs (IUPAC Nos. 28, 52, 101, 118, 138, 153, 180) and DDT compounds (p,p'-DDE, p,p'-DDD and p,p'-DDT).

F.Y.R.O.M. and Rivers Nestos and Evros that come from Bulgaria) and from the coastal areas of the Aegean Sea previous to the ban, that was applied in Greece in 1977 and in Turkey, as well to the atmospheric input from other contaminated areas (Dassenakis, 2000; Aguilar and Borrell, 2005). However in the case of Strymonikos Gulf the influence of the pollution transferred from F.Y.R.O.M. through River Strymon, seems to be the main factor as it is shown also from the higher levels of organochlorine pesticides found in Strymon River close to the border with the neighbouring country (Golfinopoulus et al., 2003).

PCBs and DDTs are known to occur in aquatic systems and fish accumulate these substances either directly from the surrounding environment or from their diet. The characteristics of organochlorinated compounds and of the aquatic environment as well as the lipid content influence also the bioaccumulation (Herbert and

Keenleyside, 1995; Pastor et al., 1996). The wild sea bass show a significantly higher amount of pollutants in their muscles compared to the sea bass from aquaculture, especially in total PCBs and DDE lipid-normalized concentration. Whereas fresh weight concentrations reveal meanwhile that aquaculture sea bass had a significant higher concentration of the PCBs and wild sea bass showed the highest *pp*′-DDE concentrations. The lipid proportion in the muscles of the sea bass ranged around 1–3% of the wet weight. The observed differences in lipid content of the muscles of wild and aquaculture sea bass are certainly related to the higher lipid content of their food in the fish farm (around 10%).The higher lipid-normalized concentrations in the wild sea bass are thus exclusively due to the low lipid percentages.

The observed variability in levels between the two sites may be related to differences in contamination of the diet. The concentrations of total PCBs (calculated as the sum of individual PCBs) in muscles of sea bass were more than the double of total DDT (calculated as the sum of *p*′*p*-DDE, *p*′*p*-DDD and *p*′*p*-DDT). From the DDT compounds, DDE was present in the highest concentration in tissues of all the analysed organisms and fish pellets, representing 60–80% of total DDT.

The PCB patterns did not vary among wild and cultivated sea bass. The hexa- and pentachlorobiphenyls were the dominant congeners. This congeners are usually reported as dominant in marine fishes (Loizeau et al., 2001). They are the most abundant congeners in commercial PCB mixtures, such as Aroclor 1254 and 1260, which were commonly used in European countries.

Some studies have shown that food is the major contributor for PCB accumulation (Thomann and Connoly, 1984; Loizeau et al., 2001). The use of commercial diets, in fish farming and its levels of micro-contaminants, may influence pollutant concentration in cultivated fish (de Boers and Pieters, 1991). The relative distribution of PCB congeners may differ in aquatic organisms because the contamination sources have different congener patterns (Fig. 2).

The PCB congener distribution in muscles of cultured European sea bass specimens resembled that of their diet pellets, which suggest that commercial diet is their major source of PCBs in the cultured sea bass. This is marked by their low PCB concentration, especially for trichlorobiphenyls. They have a lower $\log K_{ow}$ and as consequence, they have a lower propensity to leave the aqueous environment for organic compartments. Moreover they are usually more rapidly metabolised than higher chlorinated congeners because the presence of more unsubstituted ring position on their biphenyl rings available for metabolic attack (Pastor et al., 1996). So it is possible that these compounds are lost during the processing (boiling, pressing, drying, grinding) of the whole fish (usually small pelagic fish or bycatch), which are then transformed in pellets in order to have a protein-rich meal mainly used as aquaculture feeds for carnivorous aquatic species (Antunes et al., 2007).

4.2. Thyroid histology

Morphologically, the fish thyroid develops basically is similar to other vertebrates (Raine et al., 2005). A minor difference is that fish do not have a compact gland, but have their thyroid tissue dispersed along the ventral aorta, which anatomically is very similar to the position of the gland in mammals. The thyroid tissue is composed of numerous follicles which contains colloid. These follicles are composed of a single layer of epithelial cells which may vary in height and form. The nucleus is spheroidal, centrally situated, poor in chromatin and contains one ore more nucleoli. The Van Gieson staining method permitted to highlight the connective tissue, but due to the lack of organisation to a distinct organ it is difficult to associate the connective tissue whether to the thyroid follicles, the capillary vessels or other neighbouring structures. The histomorphometrical analysis between the European sea bass from wild and aquaculture revealed some morphological differences (Fig. 3). The wild sea bass showed larger follicles formed by a thicker epithelium compared to the cultured sea bass. The

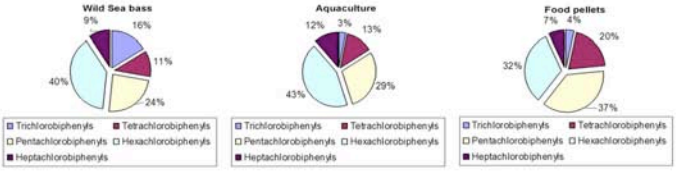

Fig. 2. Variation in the PCB contamination patterns.

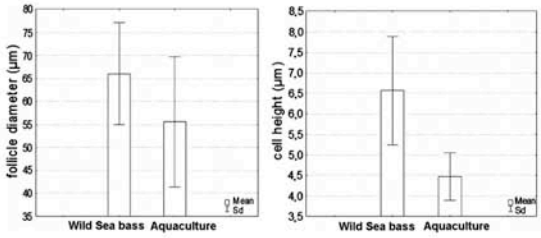

Fig. 3. Differences in thyroid morphometry (follicle diameter and epithelial cell height) in wild and aquaculture sea bass muscles.

follicle diameters measured in sea bass are in good agreement with those described previously for fish and mammals (Zhou et al., 2000).

4.3. Thyroid hormone level

Muscle thyroid hormone concentration was another tested thyroid parameter. Our T_4 results are comparable to other investigations of thyroid hormone concentration in eggs and larvae of other fish species but the T_3 concentrations were far below the normally encountered concentrations in eggs (Power et al., 2001). It seems that the concentrations vary among the different species. The thyroid hormones play an important role in fish embryogenesis and larval development so it is normal to find high concentrations of the biologically active form T_3. T_4 and T_3 detected in eggs are of maternal origin as thyroid follicles are absent from the embryo. The thyroid hormone balance in eggs probably reflects that of the maternal plasma (Power et al., 2001).

4.4. Relationship between thyroid parameters and PCB contamination

Several studies described adverse effects of organchlorinated contaminants on the thyroid function (Brouwer et al., 1989; Schumacher et al., 1993; Hall et al., 1998; Rolland, 2000; Zhou et al., 2000; Braathen et al., 2004; Brown et al., 2004; Debier et al., 2005; Das et al., 2006; Boas et al., 2006). The most innovative aspect of our approach is the simultaneous use of two alteration endpoints. Muscle thyroid hormone concentration and histological assessment of thyroid follicles (follicle diameter and epithelial cell height) were used to gauge alterations in thyroid function.

A significant relation between mean diameter of follicles, epithelial cell height, the T_4 concentration and the muscular PCBs and DDTs fresh weight concentration was observed through multivariate statistical analysis.

The follicle diameter and the epithelial cell height increases with higher PCB and DDT fresh weight concentrations (Fig. 4A). The size of the follicles and the form of the follicular cells gives an indication of the secretary activity of the gland. Thyroid gland dominated by small follicles lined by cuboidal or columnar cells can be classified as highly active. Whereas low active glands show large follicles lined by low or flattened epithelial cells (Hallgren et al., 2002). These correlations support the hypothesis that the contamination of organochlorinated compounds may induce a hyperactivity of the thyroid tissue indicated by the hypertrophy of follicular epithelial cells.

We observed an increase in T_4 concentration while the T_3 remains constant (Fig. 4B). The thyroid systems of fish and mammals are similar in many respects, with one major difference. The mammalian system is driven primarily trough the central brain-pituitary-thyroid axis that regulates thyroid secretion of both T_4 and T_3. In fish, the thyroid system does not appear to be driven primarily by the central brain-pituitary-thyroid axis. Instead, the central brain-pituitary-thyroid axis in fish has the primary role of ensuring T_4 homeostasis. T_3 production and homeostasis is regulated in peripheral tissues by conversion of T_4 to T_3 by deiodination (Brown et al., 2004). T_3 has been found to exert no significant feedback on TSH release.

It appears that the organochlorinated pollutants (specially the higher chlorinated PCBs and DDT metabolites) induce a hyperactivity of the thyroid follicles witch results in an increase of the T_4 concentrations while the T_3 concentration, which is regulated by the deiodination in peripheral tissues, is not affected. Studies have demonstrated that massive experimental increases of T_4 in fish did not increase T_3 levels, concluding that increases in T_4 do not drive T_3 production (Brown et al., 2004).

The effect of PCB exposure on peripheral thyroid hormone levels is well documented in laboratory animals and wildlife. Histopathological changes of the thyroid indicative of hyperactivity were found after exposure (Hallgren et al., 2002). One of the most consistent findings is that PCB exposure disturb the levels of circulating thyroid hormones, especially T_4 (Hallgren et al., 2002). In marine mammals, significant decreases of T_3 and/or T_4 were found in sea lions (Debier et al., 2005), polar bears (Skaare et al., 2002) and seals (Brouwer et al., 1989) and histological changes of thyroid glands related to exposure level were found in seals (Schumacher et al., 1993) and harbour porpoises (Schnitzler, 2005; Das et al., 2006). Among many different pesticides, the thyroid-disrupting effects of DDT are the most studied, DDT exposure of birds decreases T_4 or increased thyroid weight and reduced colloid content of the follicles (Jefferies and French, 1969). Blubber concentration of DDT correlated negatively to T_3 in seals (Hall et al., 1998).

Similar results were observed in different fish species. Changes in thyroid histological appearance and plasma thyroid hormone levels were reported in coho salmon (*Oncorhynchus kisutch*) and chinook salmon (*Oncorhynchus tshawytscha*) collected in Great Lakes (Leatherland, 1993). Salmonids were fed by Aroclor mixtures to determine if PCBs caused these changes, but no conclusive results could be obtained (Leatherland and Sonstegard, 1978; Leatherland and Sonstegard, 1980). Given the equivocal impact of experimental exposure of PCB mixtures on trout and salmon thyroid measures, it was concluded that other factors than PCBs may cause thyroid disruption of the Great Lakes salmon (Leatherland, 1993).

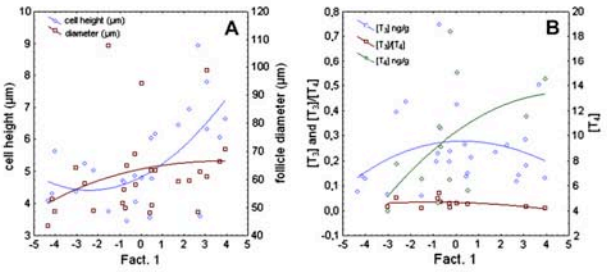

Fig. 4. (A) Correlation between high chlorinated PCB congeners (Fact. 1) fresh weight concentrations and the histological thyroid parameters (epithelial cell height and follicle diameter). (B) Correlation between high chlorinated PCB congeners (Fact. 1) fresh weight concentrations and the muscular thyroid hormone concentrations.

Almost all studies have reported some influence on thyroid cascade that is, therefore, a sensitive biomarker of exposure. However, the interpretation of the thyroid changes and the assessment of effect is more complex because it is difficult to distinguish between direct and indirect xenobiotic actions on the thyroid cascade, which has a considerable capacity to compensate for abuses that otherwise would disrupt thyroid hormones homeostasis. Indeed, a xenobiotic-induced change in fish thyroid function has yet to be causally linked conclusively to decreased fitness or survival (Brown et al., 2004).

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Joseph G. Schnitzler, Ursula Siebert, Paul D. Jepson, Andreas Beineke, Thierry Jauniaux, Jean-Marie Bouquegneau, Krishna Das **Harbour porpoise thyroids: histological investigations and potential interactions with environmental factors** *Journal of Wildlife Diseases 44 (4)*

Joseph G. Schnitzler, Emmanuil Koutrakis, Ursula Siebert, Jean Pierre Thomé, Krishna Das. **Effects of persistent organic pollutants on the thyroid function of the European sea bass *(D. labrax*), an endocrine disruption?** *Marine Pollution Bulletin 56: 1755-1764*

Joseph G. Schnitzler, Thomé JP, Lepage M, Das K: **Organochlorine pesticides and polychlorinated biphenyl residues in wild sea bass (*Dicentrarchus labrax*) off European estuaries**. *Marine Pollution Bulletin* (submitted)

Joseph G. Schnitzler, Klaren PHM, Thomé J-P, Das K: **Thyroid dysfunction in sea bass (*Dicentrarchus labrax*): Part 1: Evaluation of the potential impact of disrupting chemicals in wild individuals from coastal regions near several European rivers mouths**. *Environmental Pollution* (submitted)

Joseph G. Schnitzler, Klaren PHM, Celis N, Blust R, Covaci A, Dirtu AC, Das K: **Thyroid dysfunction in sea bass (*Dicentrarchus labrax*). Part 2: Underlying mechanisms and effects of polychlorinated biphenyls on thyroid hormone physiology and metabolism.** *Environmental Pollution* (submitted)

CONFERENCE ABSTRACTS

Schnitzler J., Klaren PHM., Celis N., Blust R., Covaci A., Dirtu A., Thomé JP., Das K. **Polychlorinated Biphenyls affect Histological Appearance of European sea bass (*Dicentrarchus labrax)* Thyroids** 2010, 17th Benelux Congress of Zoology, Ghent, Belgium (Oral presentation)

Schnitzler J., Klaren PHM., Celis N., Blust R., Covaci A., Thomé JP., Das K. **Effects of Polychlorinated Biphenyls on Thyroid Hormone Physiology and Metabolism of European sea bass (*Dicentrarchus labrax*)** 2010, SETAC Europe: 20th Annual Meeting Seville, Spain (Oral presentation)

Schnitzler J., Klaren PHM., Thomé JP., Das K. **Approaches for assessing potential impacts of thyroid hormone disrupting chemicals in wild sea bass (*Dicentrarchus labrax*)** 2010, SETAC Europe: 20th Annual Meeting Seville, Spain (Poster presentation)

Jauniaux T., Berguerie H., Camphuysen K., Daoust P-Y., Drouguet O., Ghisbain T., Garcia-Hartmann M., Grondin A., Haelters J., Jacques T., Kiszka J., Leopold M., Pezeril S., **Schnitzler J**. and Coignoul F. **Causes of death of harbor porpoises (*Phocoena phocoena*) stranded on the on the continental coastline of the southern North Sea (Belgium, France, and Dutch coasts) between 1990 and 2007** 2008, ICES conference Halifax, Canada, september 2008

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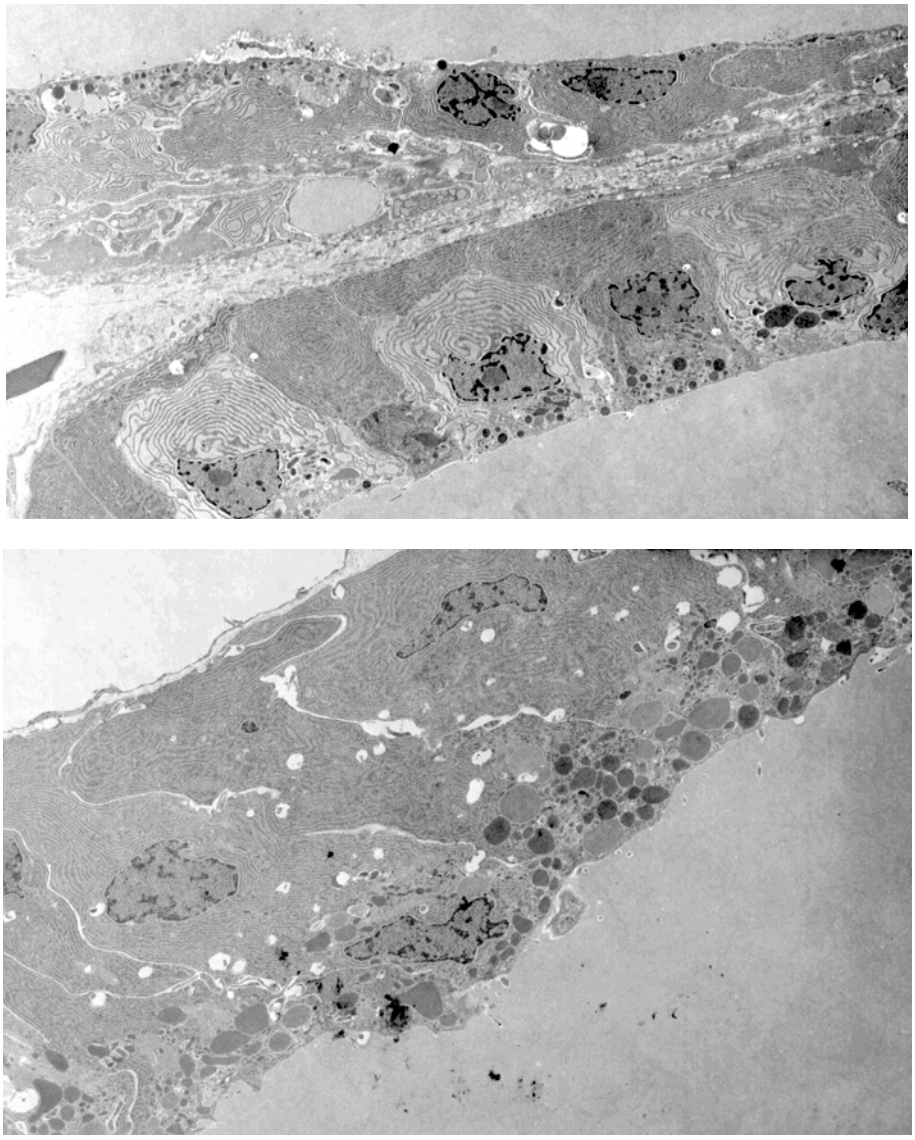
Schnitzler J., Koutrakis E., Thomé JP, Siebert U., Das K. **Effects of persistent organic pollutants on the thyroid function of seabass (*Dicentrarchus labrax*), an endocrine disruption?** 2007, SETAC Europe 17th Annual Meeting; Porto, Portugal (Oral presentation)

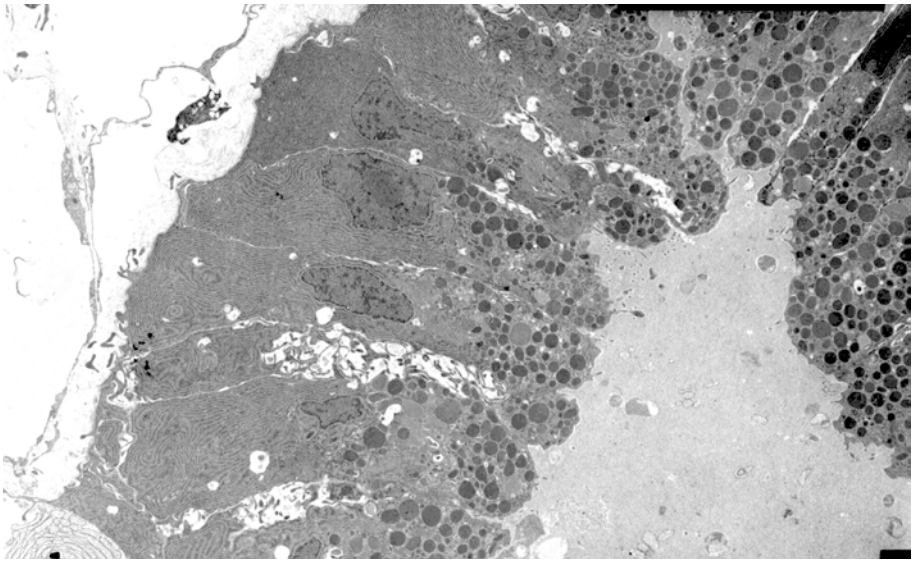
Jauniaux T., Bergerie H., Camphuysen K., Daoust P-Y., Drouguet O., Mardik L., **Schnitzler J**., Coignoul F. **Lesions observed on harbor porpoises (Phocoena *phocoena)* stranded on the Dutch coast in 2006.** 2007, 17th *Biennial Conference on the Biology Of Marine Mammals,* Cape Town, South Africa, 29 november - 3 december, 2007

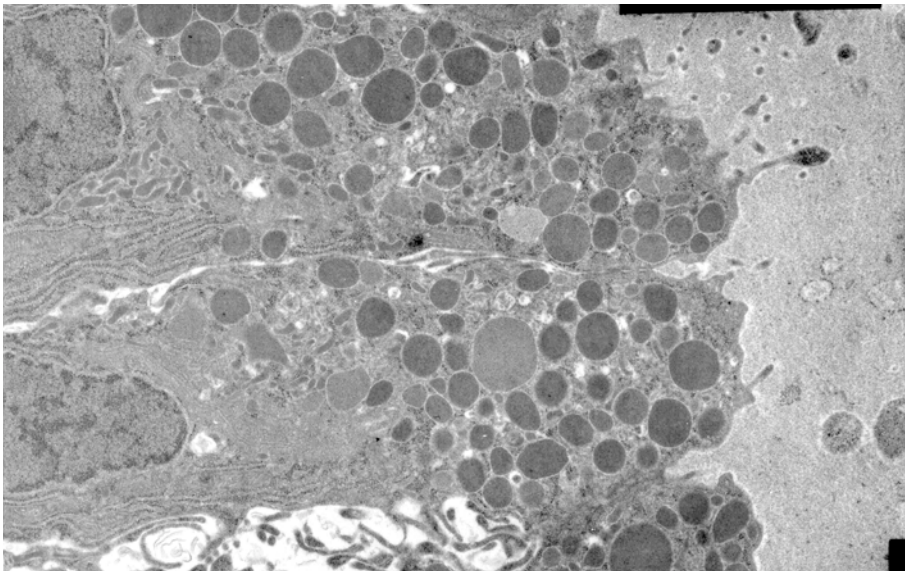
Schnitzler J., Koutrakis E., Siebert U., Das K. **Endocrine disruption in seabass (*Dicentrarchus labrax*): effects of persistent organic pollutants on their thyroid function** 2006, 13th Benelux Congress of Zoology, Leuven, Belguim, (Oral presentation)

Schnitzler J., Das, K., Beineke A, Jauniaux, T., Covaci, A., Jepson, P., Baumgärtner W., Siebert, U. **Interfollicular fibrosis and organohalogens in the thyroid of the harbour porpoise (*phocoena phocoena*) of the British and Belgian coasts.** 2005, 12th Benelux Congress of Zoology, Wageningen, The Netherlands, (Poster presentation)

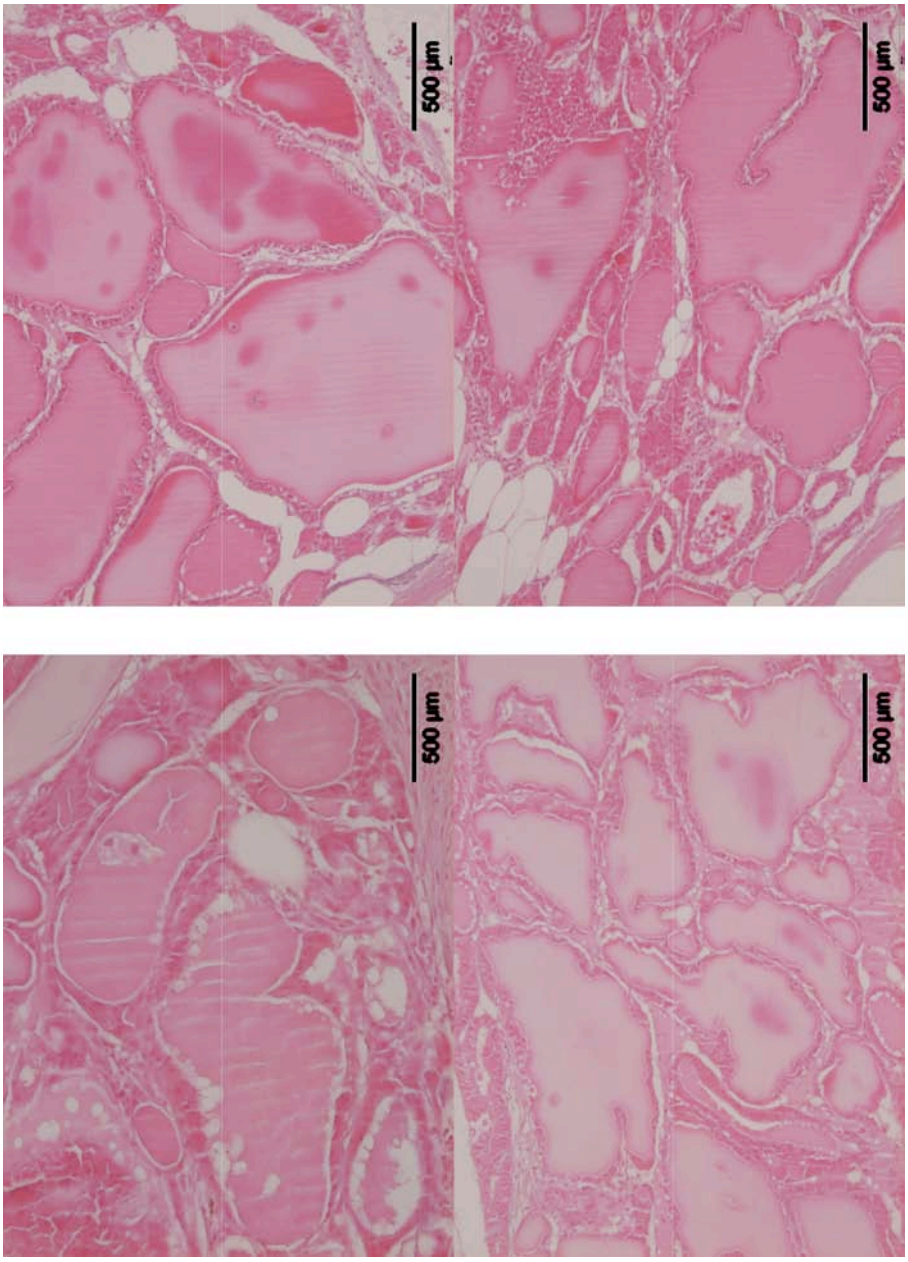


Colour Figure 1: Thyroid follicular cells of sea bass exposed to 1 µg g^{-1} Σ [7 PCBs] in food (x2000 and x6000), between two smaller follicles, we can see few apical cytoplasmic processes extending into follicular lumen, well developed rough endoplasmic reticulum and few colloid droplets and lysosomal bodies

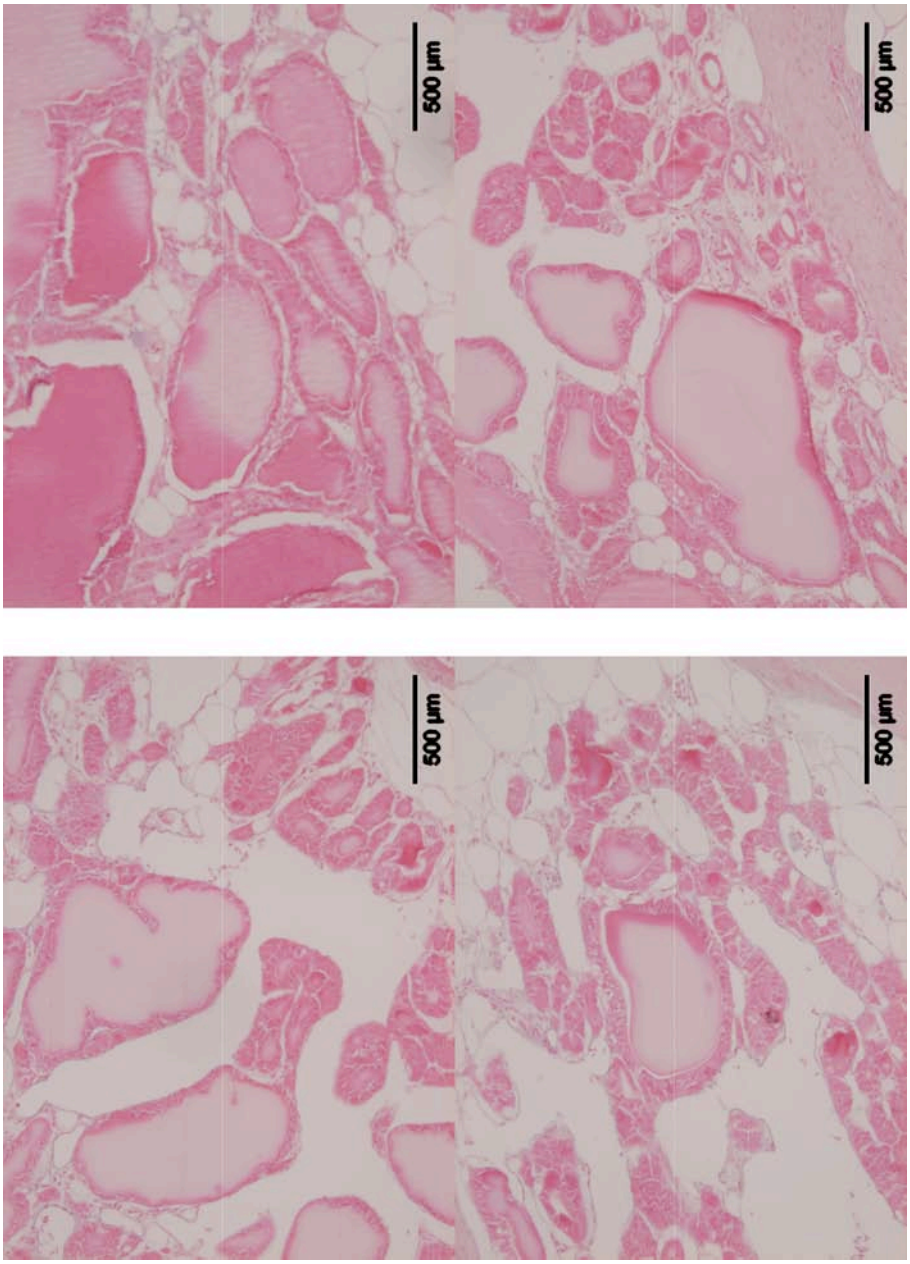




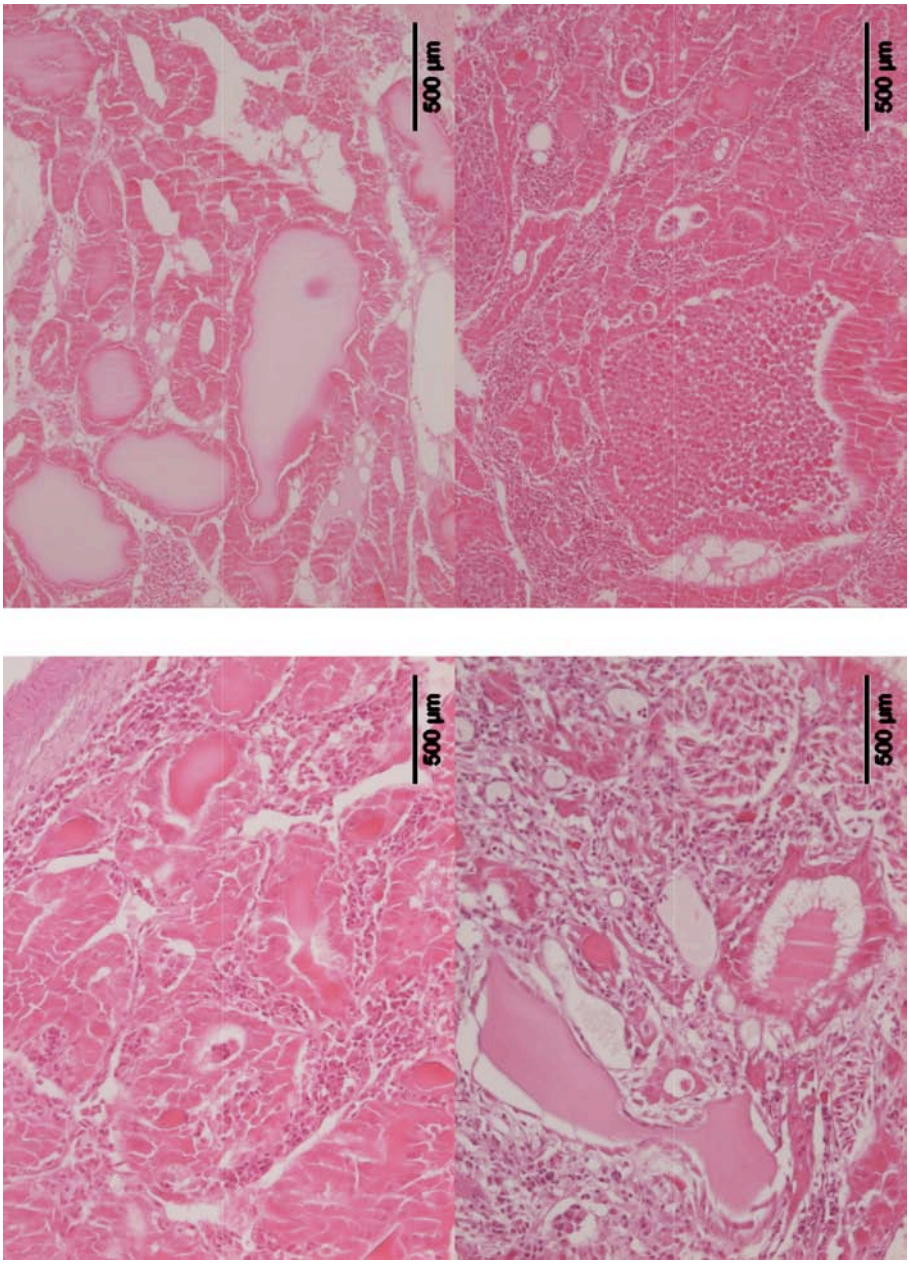
Colour Figure 2: Thyroid follicular cells of sea bass exposed to 1 µg g^{-1} Σ [7 PCBs] in food (x2000 and x6000), of large follicle, we can see apical cytoplasmic processes extending into follicular lumen, dilated profiles of rough endoplasmic reticulum and numerous large colloid droplets and lysosomal bodies



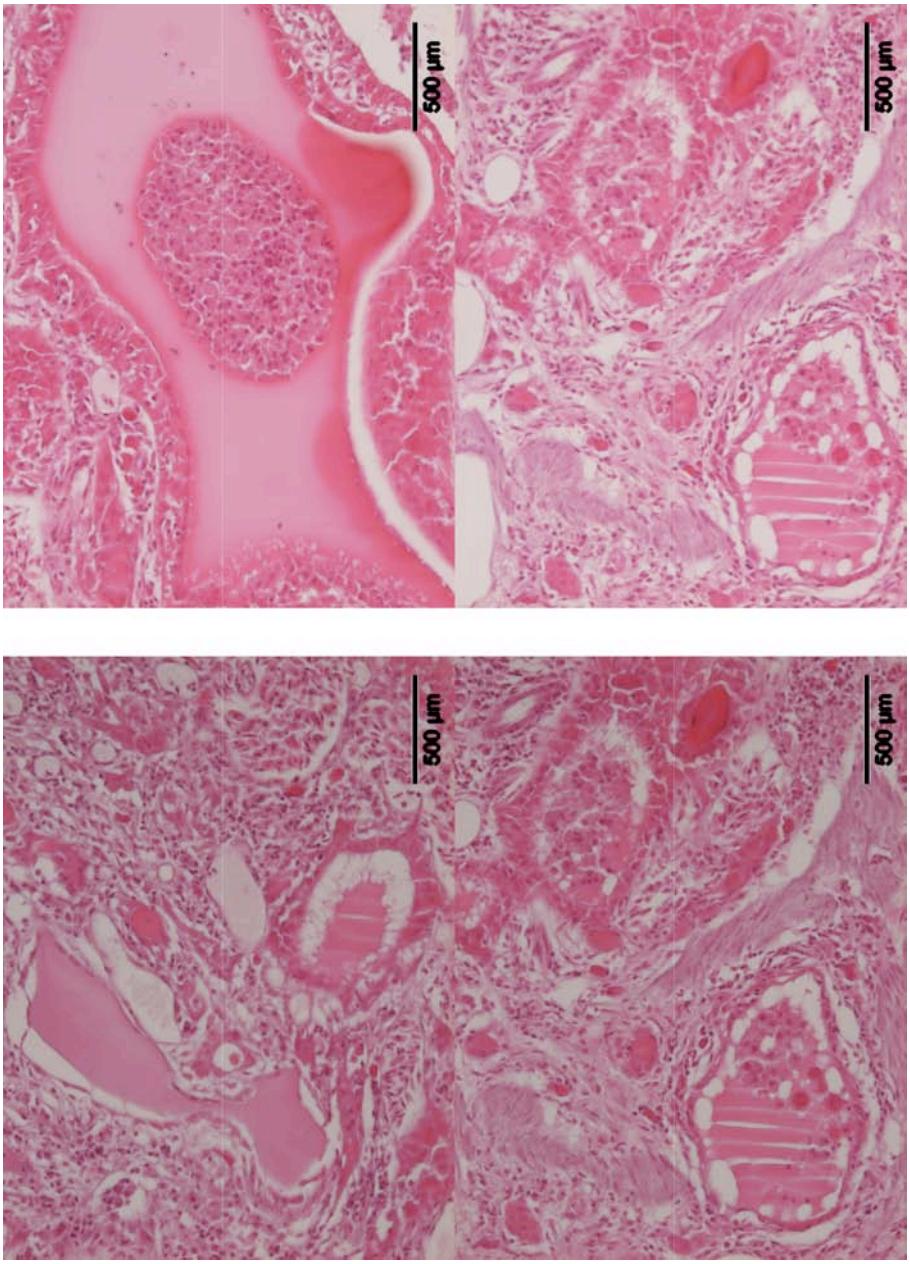
Colour Figure 3: Longitudinal section of European sea bass thyroid tissue in subpharyngeal area (H.E. staining) from control group



Colour Figure 4: Thyroids from animals exposed to 1 µg.g^{-1} dw [7 ICES PCBs] in food pellets revealed remarkable heterogeneity of thyroid follicle sizes.



Colour Figure 5: Thyroids from animals exposed to 10 µg.g^{-1} dw [7 ICES PCBs] in food pellets revealed an enlargement of the interstitial tissue between follicles and degenerated colloid. The follicles appeared in lower number and the tissue seems disorganized.



Colour Figure 6: Thyroids from animals exposed to 10 µg.g^{-1} dw [7 ICES PCBs] in food pellets revealed an enlargement of the interstitial tissue between follicles and degenerated colloid. The follicles appeared in lower number and the tissue seems disorganized.