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Moisés Sánchez-Fortún Burriel

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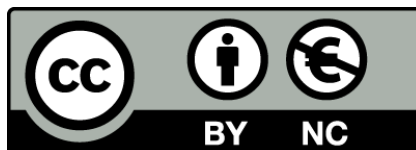




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From rice fields to seabirds: an integrative approach to study mercury dynamics in the Ebro Delta

Moisès Sánchez-Fortún Burriel



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**From rice fields to seabirds:
an integrative approach to study mercury dynamics in
the Ebro Delta**

Memòria presentada per

Moisés Sánchez-Fortún Burriel

per optar al grau de Doctor per la Universitat de Barcelona

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Mercury pollution is of great concern for the scientific community due to their ubiquity and persistence in the environment. Its bioaccumulation and biomagnification capacities along food webs enhance their toxicity both in humans and wildlife. The Ebro Delta is one of the most important wetlands in the NW Mediterranean, both as a rice cultivation area and a biodiversity hotspot. The activity of an historical chlor-alkali plant upstream the Ebro river mouth has increased anthropogenic-derived mercury within its delta and neighboring coastal areas. Industrial operations ceased in 2017, but legacy mercury is still impacting the Ebro ecosystems. Moreover, remediation plans included the dredging of toxic sediments were performed in 2013-2014, although the mid-term impact of such activities have never been evaluated in depth. In rice fields, mercury sinkage and methylation have been reported to occur. Also, mercury reaching coastal areas can be incorporated in local food webs reaching humans and seabird species breeding there. Monitoring mercury dynamics in these environments should be a priority from both food safety and ecosystem health perspective. In the present thesis, we used an integrative approach to study mercury pollution in the Ebro Delta and marine coastal areas. First, we evaluated mercury dynamics in rice paddies and addressed how different agricultural management practices modify metal dynamics in its different components, from soil to biota. Moreover, an *in-situ* mesocosm experiment was performed to evaluate the bioaccumulation potential of mercury in the rice paddy environment. In addition, we examined the potential of Audouin's gull as a model organism to trace trends in legacy pollution in a 15 year series and the effects of soil remediation operations at the chlor-alkali site on mercury bioavailability. To further examine the mercury sources and its temporal variability, we used mercury stable isotopes to relate metal body burdens in seabirds species to mercury sources in relevant foraging areas (ricefields, mesopelagic and epipelagic marine environment). Our results indicate that legacy pollution is still ongoing in the Ebro Delta and stressed the need to continue studying rice fields as mercury cuvettes for mercury speciation and bioavailability, and the ultimate factors influencing mercury dynamics in such environments. Finally, we strongly recommend a continuous monitoring program using sensitive organisms to follow-up mercury fluctuations and the use of mercury stable isotopes, a promising tool to deepen the understanding of pollution in complex environments.

La contaminació per mercuri és d'un gran interès per a la comunitat científica degut a la seva ubiquïtat i persistència en el medi ambient. La seva capacitat de bioacumulació i biomagnificació a través de les xarxes tròfiques accentuen la seva toxicitat tant en humans com en la vida silvestre. El Delta de l'Ebre és un dels aiguamolls més importants en el NO Mediterrani, des del punt de vista del cultiu de l'arròs i com a *hotspot* de biodiversitat. L'activitat històrica d'una factoria de clor-àlcali aigües amunt de la desembocadura del riu Ebre ha incrementat la presència de mercuri d'origen antropogènic en tot el seu delta i l'àrea costanera al voltant de la desembocadura. Tot i que l'activitat industrial de la planta de clor-àlcali cessà el 2017, el llegat de mercuri segueix impactant els ecosistemes al llarg de la conca del riu Ebre. A més a més, es van realitzar durant els anys 2013-2014 plans de neteja incloent el dragatge de sediments contaminats al voltant de la fàbrica, tot i que l'impacte a mig termini d'aquestes activitats no s'ha avaluat en profunditat. En els arrossars, es coneix que s'acumula mercuri i es metila. A banda, el mercuri que arriba a les àrees costaneres pot ser incorporat a les xarxes tròfiques locals, arribant als humans i les aus marines que crien en aquestes àrees. Així doncs, monitoritzar les dinàmiques del mercuri en aquests ambients és necessari, des d'un punt de vista de seguretat alimentària i de salut dels ecosistemes. En aquesta tesi, hem utilitzat una aproximació integrativa per estudiar la contaminació per mercuri en el Delta de l'Ebre i les àrees marines costaneres. Primerament, hem avaluat les dinàmiques d'aquest metall en els arrossars i estudiat com diferents pràctiques agrícoles modifiquen la dinàmica del mercuri en diferents components del sistema de l'arrossar, des del sòl fins a la biota. A més, un experiment de mesocosmos *in-situ* es va realitzar per avaluar el potencial de bioacumulació de mercuri en els arrossars. També hem examinat el potencial de la gavina corsa com a organisme model per traçar les tendències de contaminació al llarg de 15 anys i els efectes en la disponibilitat de mercuri de les operacions de neteja dels sòls dutes a terme a la zona de la fàbrica clor-àlcali. Per estudiar més a fons les fonts de mercuri i la seva variabilitat temporal, hem utilitzat els isòtops estables de mercuri per relacionar els nivells del metall en aus marines a les fonts de mercuri rellevants per a les seves zones d'alimentació (arrossars, ambients marins epipelàgics i mesopelàgics). Els resultats obtinguts indiquen que el llegat de contaminació per mercuri encara perdura en el Delta de l'Ebre i fa patent la necessitat de continuar estudiant els arrossars com a cubetes de mercuri i d'especiació i biodisponibilitat d'aquest, i els factors que influeixen les dinàmiques de mercuri en aquests sistemes. Finalment, recomanem la posada en marxa d'un programa de monitorització continuada utilitzant organismes sensibles a les fluctuacions de mercuri i l'ús d'isòtops estables de mercuri, una eina prometedora per a aprofundir en el coneixement de la contaminació en ecosistemes complexos.



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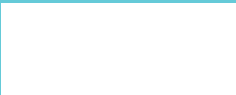
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01 CHAPTER 1

General Introduction



01

GENERAL INTRODUCTION

1. Mercury as a global pollutant

Mercury (Hg) is a widespread global pollutant which has numerous adverse effects on humans and wildlife. Mercury in the environment can enter from both natural and anthropogenic inputs. Natural inputs include biogeochemical processes such as volcanology, biomass burning, etc. On the other side, anthropogenic inputs of mercury in the environment are mainly industrial processes or other human activities such as artisanal gold mining. According to the last UNEP report (UNEP 2019), the Hg derived from human activities has increased the environmental Hg loads up to a 450% throughout human history.

Global Hg emissions in the environment have increased in the last decade, as declines of commercial use in the developed world have been matched by increases in Hg release into the environment by developing countries (Streets et al. 2019; UNEP 2019). Furthermore, future predicted scenarios also indicate an increase in mercury emissions globally (UNEP 2019). This scenario is worsened by the fact that mercury has a high capacity of spreading across environments, because it can travel long distances far from emission sources and has long permanence times in the atmosphere (from months up to a year; Selin 2009; Streets et al. 2019).

Anthropogenic Hg emissions have caused Hg-related poisoning episodes worldwide. Of special importance was the accident that resulted from the deposition of industrial waste containing large quantities of Hg in the Minamata Bay, in Japan back in the 1950s. Humans living in the Minamata Bay area were exposed to large levels of mercury through fish intake, which produced severe neurological impairments including ataxia, speech impairment, loss of visual field, and muscular affections among others. Such changes became known as Minamata disease (Ekino et al. 2007). As a result of this poisoning episode, the Minamata Convention (www.mercuryconvention.org) was signed in 2013 (and came into enforce in 2017), which was conceived to commit the countries to reduce or cease mercury emissions to the environment (UNEP 2013; Kessler 2013). Alongside, other regions, as in Europe, have

implemented nationwide conventions to further prevent mercury remobilization or discharge into the environment due to anthropogenic activities (Ciani et al. 2021).

2. Mercury sources in the environment

Sources of mercury in the environment can be categorized in two groups: natural (or pre-industrial) sources and anthropogenic sources. Natural mercury sources imply the mobilization or release of mercury by natural biotic and abiotic processes, whereas anthropogenic sources of mercury are related to a diverse array of human activities which mobilize and release geologically bound Hg (Ebinghaus et al. 1999; Driscoll et al. 2013; UNEP 2019).

From a complimentary scope, mercury sources can be classified as primary or secondary sources. Primary source emissions, which can be natural or anthropogenic transfer Hg from long-lived lithospheric reservoirs to the atmosphere. Conversely, secondary source emissions (or re-emissions) correspond to the exchange of Hg among surface reservoirs (e.g., re-emission from Hg previously deposited to oceanic surface waters back to the atmosphere) (Driscoll et al. 2013). Re-emissions include legacy mercury pollution in surface reservoirs. Briefly, primary sources increase the Hg levels in surface reservoirs, whether secondary sources distribute them across surface reservoirs. Secondary source emissions are often difficult to classify as anthropogenic or natural, since the primary source of Hg pollution is not always straightforward to determine and in most cases there is a combination between anthropogenic and natural Hg depositions resulting in a Hg budget that is re-emitted (UNEP 2019).

2.1. Natural sources of Hg

Natural sources of mercury include the weathering of rocks or minerals containing mercury such as cinnabar (HgS), geological movements, volcanoes and geothermal sources, which release mercury existing naturally in the Earth's crust (Ferrara et al. 2000; Schuster et al. 2002; Guedron et al. 2006; Bagnato et al. 2017; Sundseth et al. 2017). Vegetal biomass-burning, which also releases mercury in the atmosphere, might be also considered a natural mercury source (Friedli et al. 2009; De Simone et al. 2015, 2017), although global biomass burnings occurring nowadays are predominantly human-induced (Mason 2009). Globally, it is estimated that around 30% of Hg emissions are derived from natural sources (Amos et al. 2013).

2.2. Anthropogenic sources of Hg

Anthropogenic sources of mercury in the environment include an array of industrial processes (e.g., coal burning, chlor-alkali production, cement production), artisanal gold-mining, or dental amalgams (UNEP 2019). Among these, mineral exploitation in artisanal gold-mining practices and fossil fuel combustions are nowadays the main contributors to anthropogenic Hg emissions at a global scale (UNEP 2019). Since the pre-industrial era, human activities have boosted the Hg loads in the environment up to 450%-660%, which has impacted the cycling of this element throughout the globe (Amos et al. 2013; Streets et al. 2017; UNEP 2019) and represent a significant percentage of Hg budgets in the different Earth compartments.

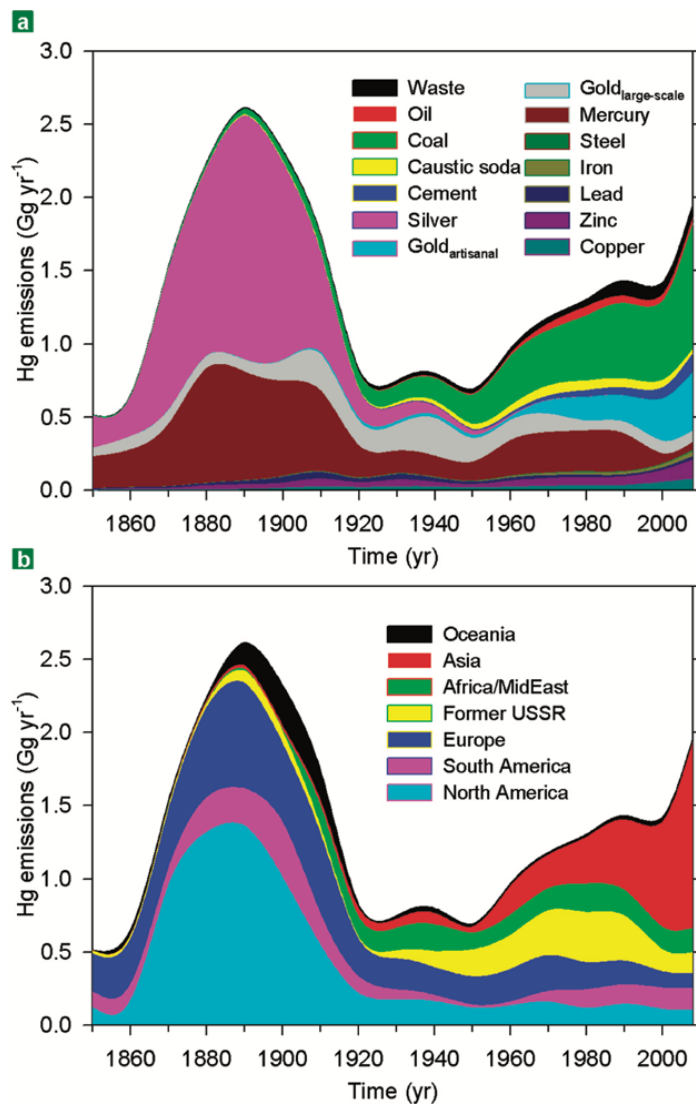


Figure 1 | Temporal trends in Hg emissions to atmosphere by (a) source type and (b) region (From: Streets et al. 2011).

Recent inventories show differential emission patterns among territories and over time for each of the different anthropogenic sources considered (Streets et al. 2011; Fig. 1).

Currently, eastern and southeast Asia territories account for almost 40% - 50% of primary anthropogenic Hg emissions to the atmosphere and around 46% of Hg emissions to aquatic environments (Pirrone et al. 2010; UNEP 2019), mainly due to the industrial-derived emissions. Nonetheless, major regions contributing to Hg emissions from anthropogenic sources vary among the sectors considered, reflecting the socio-economical activities of the area: for instance, artisanal gold mining at large scale predominates in South American countries as a primary Hg source (UNEP 2019).

Regardless the specific source of mercury pollution, nowadays, estimations indicate that globally every year humans dump around 2500 ± 500 Mg of mercury into the environment and that human activities are responsible for the vast majority of Hg re-emissions into the environment (UNEP 2019; Fig. 2). Given the high temporal persistence of mercury in the environment, especially in the atmosphere compartment (Selin 2009; Streets et al. 2011) and furthermore, since mercury emissions to the atmosphere can travel long distances, that implies that Hg emissions can have a global impact (Jackson 1997; Durnford et al. 2010; EEA 2018). In this context, the global cycling of mercury means that, even if anthropogenic sources of mercury pollution stopped today, it would take many centuries for mercury levels in the environment to reduce significantly (EEA 2018). This legacy mercury pollution is of highest concern amongst the scientific community, as its effects can extend beyond the cease of the mercury sources. Recent studies report that re-emission of legacy mercury pollution might represent up to 60% of the actual emissions (Amos et al. 2013; Sundseth et al. 2017).

3. Mercury speciation and biogeochemical cycle

One of the main peculiarities of mercury is its speciation variability, which depends on the environmental conditions in which the metal is found (Ullrich et al. 2001; Gaffney and Marley 2014). This allows mercury to be persistent and ubiquitous in the different environmental compartments and in every region in the planet (Driscoll et al. 2013). Furthermore, the different chemical and physical processes that mercury experiences throughout the cycling among Earth's environmental compartments, have an important effect on its chemical properties and reactivity, environmental fate and impacts, and its biological toxicity (Gaffney and Marley 2014). Figure 3 represents a simplified schematic overview of the biogeochemical cycle of mercury, which comprises and is interconnected through all earth compartments (terrestrial, aquatic, atmospheric, biotic).

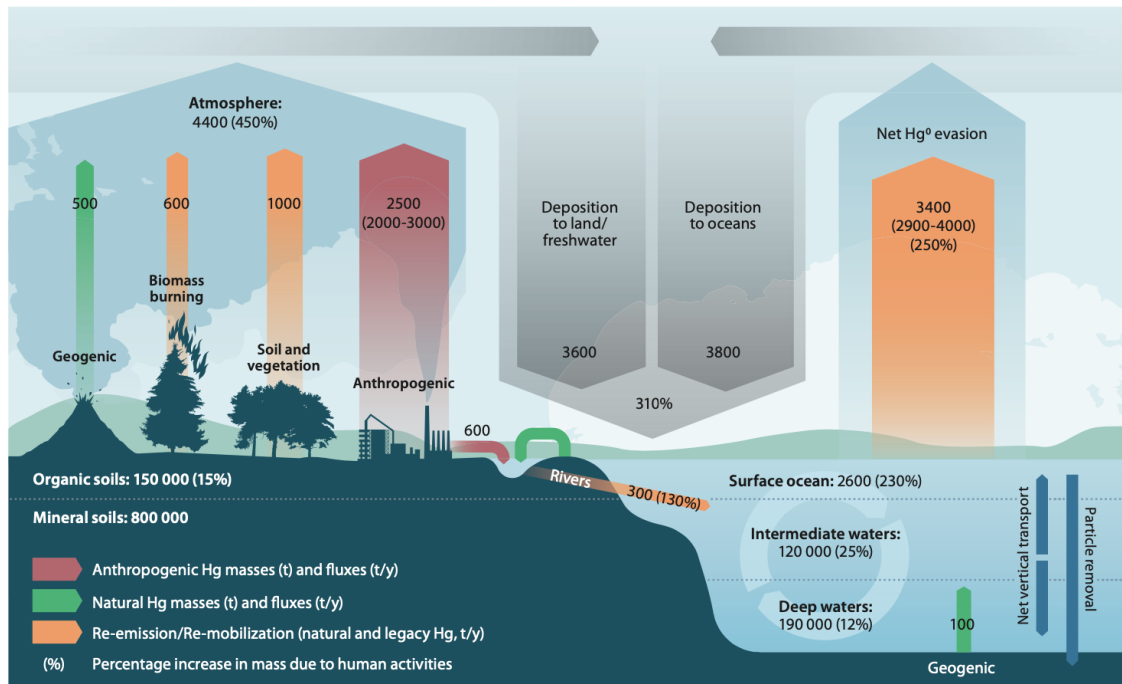


Figure 2 | Global Hg budgets showing the anthropogenic impact on the Hg cycle since the pre-anthropogenic period (prior to 1450 AD), showing natural emissions, anthropogenic emissions and re-emissions. Ranges are given in brackets after the best estimate values; percentages in brackets represent the estimated increase in mass of flux due to human activities since the pre-anthropogenic period. (From: UNEP 2019)

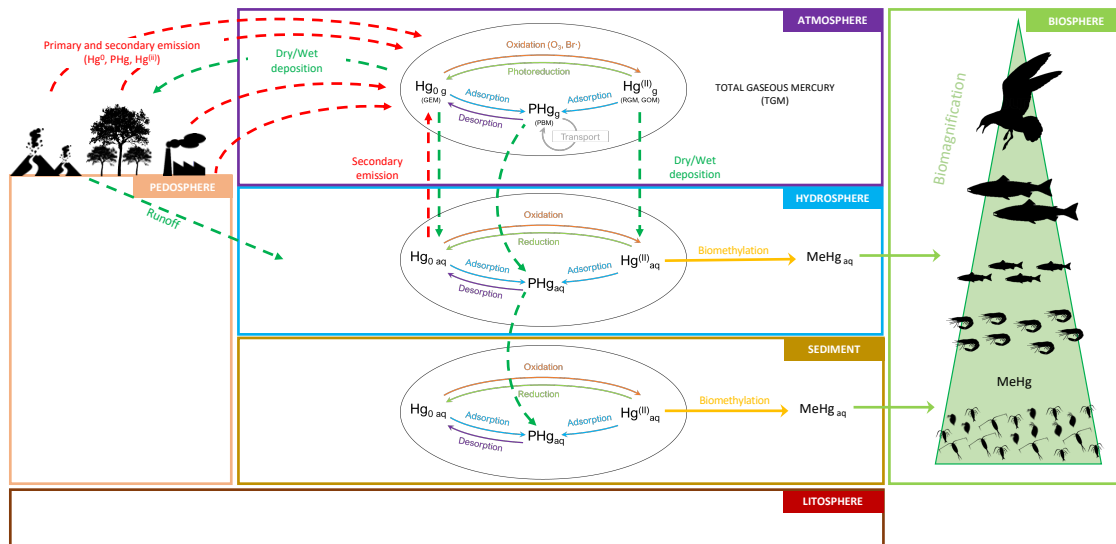


Figure 3 | Biogeochemical cycle of Hg. Depicted the main processes of mercury emission/deposition and Hg species physical and chemical changes in the four main environmental compartments (Atmosphere, Hydrosphere, Sediments and Biosphere) (Adapted from: Boeing 2000; Selin 2009).

The atmospheric compartment has been considered the main distribution pathway for mercury on a global scale (Pacyna et al. 2010; Driscoll et al. 2013). In the atmosphere, mercury is found in three different states: gas phase elemental mercury (Hg^0), gas phase mercury (II) compounds called reactive gaseous mercury or gaseous oxidized mercury (RGM or GOM), and particulate-bound mercury (PHg or PBM), which corresponds to mercury (II) compounds or Hg^0 associated with atmospheric particulate matter (Lindqvist and Rodhe 1985; Lindberg and Stratton 1998; Pirrone et al. 2003). The complete budget of mercury in the atmosphere is known as total gaseous mercury (TGM). The emissions and re-emissions to the atmosphere are mostly Hg^0 (> 95%), with small proportions of Hg(II) in gas phase or particulate-bound mercury (Pirrone et al. 2003; Gabriel et al. 2005; Lindberg et al. 2007; Sprovieri et al. 2016; Streets et al. 2017).

However, the different species of mercury found in the atmosphere can be interconverted one into another by often reversible chemical and physical processes (Lyman et al. 2020). Elemental-gas mercury in the atmosphere can be bound to aerosol particles to become part of the pool of atmospheric particulate mercury or it can be oxidized into mercury(II) compounds by reacting with oxidizing agents present in the atmosphere (e.g., O_3 , NO_3 , H_2O_2 , Cl_2 , Br_2 , OH) (Hall 1995; Lin and Pehkonen 1999; Bergan and Rodhe 2001; Sommar et al. 2001; Calvert and Lindberg 2005; Auzmendi-Murua et al. 2014; Saiz-Lopez et al. 2018). Once oxidized, mercury compounds dynamically partition between the gas and particulate phases (Amos et al. 2012; Feddersen et al. 2012). Oxidized Hg(II) compounds can be reduced to become Hg^0 by photoreduction processes (Saiz-Lopez et al. 2018).

Mercury can persist in the atmosphere from hours up to years (Selin 2009), depending on its chemical form and local environmental conditions (Schroeder and Munthe 1998; Sommar et al. 2001; Lindberg et al. 2007). Particularly, Hg^0 is chemically stable in the atmosphere and highly volatile, and thus it can be transported long distances from the source locations (Durnford et al. 2010), whereas particulate-bound mercury and Hg(II) are not transported such long distances (Schroeder and Munthe 1998). Nonetheless, the ultimate fate of all the mercury emitted to the atmosphere is to deposit to ecosystems. Gas-phase elemental mercury (Hg^0) can be absorbed from the atmosphere by plants through stomata (Wright et al. 2016) or exchanged dynamically with soils (Zheng et al. 2016). Oxidized mercury compounds, in the gas or particulate phase, can be taken up by atmospheric water and deposited in precipitation (i.e., wet deposition) or undergo dry deposition, into terrestrial and aquatic ecosystems (Lodenius 1998; Mason and Sheu 2002; Selin et al. 2007; Driscoll et al. 2013).

Besides deposition from the atmosphere, mercury in water bodies (marine and freshwater) and terrestrial environments can enter from other sources (e.g., runoff, marine hydrothermal vents, etc.; Driscoll et al. 2013). Once deposited into terrestrial or aquatic environments, it can undergo different transformations (Fig. 3). Thus, in aquatic and terrestrial environments mercury occurs in different forms depending on the oxidation-reduction conditions present (Gabriel and Williamson 2004; Gworek et al. 2016). Elemental mercury (Hg^0), divalent Hg(II) complexes and particulate-bound mercury in aqueous phase can be found in aquatic and terrestrial environments.

The evasion of elemental mercury to the atmosphere represents a significant pathway for reducing the level of this toxic element in aquatic ecosystems. Therefore, photo- or bacterial reduction of Hg(II) to Hg^0 in aquatic ecosystems and its posterior transfer to the atmosphere due to evasion contribute to diminishing the levels of mercury in the aquatic environments (Douglas et al. 2012). On the other hand, photo-oxidation in surface waters can transform Hg^0 to Hg(II) species, hampering evasion of mercury from water bodies. Thus, a major fraction of the metal present in water bodies is in form of divalent inorganic mercury Hg(II) (Gworek et al. 2016). Hg(II) and Hg^0 in aqueous phase can be bound to particulate matter present in the system, which allows its transport along the water ecosystems (e.g., rivers, streams, oceanic waters; Cossa et al. 1997; Gworek et al. 2016; Palanques et al. 2020) or its deposition to the bottom sediments (i.e., sea- or riverbed; AMAP 2011; Douglas et al. 2012; Amos et al. 2013). In terrestrial environments, analogous chemical transformations occur between mercury species (Shanley and Bishop 2012; reviewed in Bishop et al. 2020).

Furthermore, aquatic and terrestrial environments are hotspots for the transformation of inorganic mercury (Hg(II)) to organic mercury forms, by the chemical process known as biomethylation (Compeau and Bartha 1987), which essentially, converts Hg(II) to methylmercury (MeHg). Existing as an organic compound, mercury becomes bioavailable and is where most of it transfers to wildlife and humans with bioaccumulation and biomagnification capacities (Kidd et al. 2012). On the other hand, demethylation of MeHg occurs in aquatic and terrestrial environments either by biotically- or abiotically-mediated processes (Robinson and Tuovinen 1984; Oremland et al. 1991; Sellers et al. 1996; Bridou et al. 2010), reducing the bioavailability of mercury to biota. The contribution of methylation and demethylation in MeHg availability in the environment will likely depend on the particular environmental conditions such as pH, redox conditions or salinity, and is subject to temporal and spatial variation (Compeau and Bartha 1984; Hintelmann et al. 2010).

Given the myriad of sources contributing to primary emissions of Hg, legacy re-emissions and its complex biogeochemical cycle, it has been of main importance to find suitable tools to unravel between sources of Hg budgets with the aim to take actions to reduce Hg emissions globally (Wiener et al. 2003). Mercury stable isotopes have been used over a decade in Earth and environmental sciences research to trace sources of mercury in sediments and other environmental compartments (Hintelmann 2012; Blum et al. 2014). Recently, mercury stable isotope research has grown rapidly with over 200 papers using this technique to understand a great diversity of environmental and biogeochemical aspects of mercury (Tsui et al. 2020). Studies on source apportionment have been performed in various animal taxa such as invertebrates (Xu et al. 2016), fish (Gehrke et al. 2011; Cransveld et al. 2017; Maurice et al. 2021), birds (Renedo et al. 2020, 2021) and mammals (Li et al. 2020; Pinzone et al. 2021) including humans (Du et al. 2021).

3.1. Biomethylation and entering of MeHg in the trophic webs

Among the biogeochemical processes depicted in Figure 3, biomethylation is one of the most crucial ones in terms of biological importance, since it transforms inorganic mercury (Hg(II)) in the environment to methylmercury (CH₃-Hg or MeHg), an organic toxic form that is readily bioavailable and can be accumulated into organisms and biomagnified along food webs (Harding et al. 2018).

Inorganic mercury methylation takes place mainly in aquatic and anoxic environments by sulfate-reducing and iron-reducing bacteria (SRB and IRB, respectively) and some archaea (Compeau and Bartha 1985; Fleming et al. 2006; Kerin et al. 2006; Gilmour et al. 2018). In the last years, Parks et al. (2013) reported two gene-clusters (*hgcA* and *hgcB*) present in methylating microorganisms, which appear to be responsible for the capacity of mercury methylation (Gilmour et al. 2013; Podar et al. 2015). The local environmental conditions enhancing biomethylation include the quantity and quality of dissolved organic matter (DOM) and the presence of electron acceptors (e.g., sulfate SO₄²⁻), which promote the bioavailability of Hg(II) and the metabolic reactions leading to the production of MeHg (Gilmour et al. 1992; Bigham et al. 2017). Although the vast majority of research has been performed on biotic mercury methylation processes (e.g., Podar et al. 2015; Bravo et al. 2018; Regnell and Watras 2019), abiotic methylation of inorganic mercury has also been described (Weber 1993; Zhang and Planas 1994; Celo et al. 2006; Munson et al. 2018). Nevertheless, biotic methylation is considered to be faster than abiotic methylation (Choi et al. 1994).

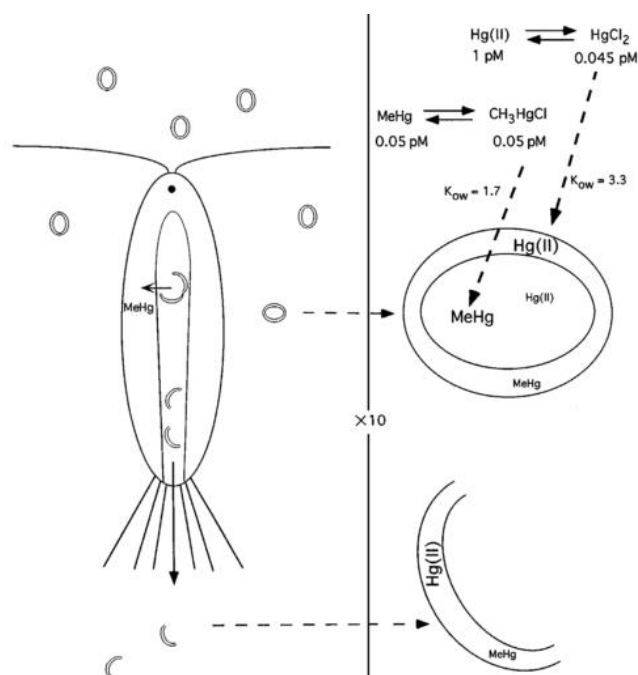


Figure 4 | Model for the absorption of MeHg in phytoplankton through cellular membranes. Note that MeHg mainly accumulates in the cytoplasm of the phytoplankton cells whereas inorganic mercury compounds accumulate in the cellular membranes. (From: Morel et al. 1998)

Phytoplankton, bacteria and protozoans represent the main entrance of MeHg in the lowest levels of the aquatic food web by absorption through cellular membranes (Morel et al. 1998; Pickhardt and Fisher 2007; Schaefer et al. 2014; Fig. 4). Up to 10^4 - 10^5 times the concentration of mercury in ambient water is known to be accumulated in phytoplankton (Mason et al. 1995). Morel et al. (1998) proposed a model in which the transfer of MeHg is more efficient than the transfer of Hg(II) compounds, since the first preferentially accumulates in the cytoplasm of phytoplankton cells, whereas Hg(II) compounds tend to accumulate in the cellular membranes. The accumulation of MeHg in higher trophic level organisms will be driven by the ingestion of MeHg-contaminated food items, rather than direct uptake of MeHg from the surrounding environment. In lower aquatic food webs levels, cytoplasmatic MeHg will be more efficiently absorbed than inorganic mercury (Morel et al. 1998). Furthermore, in higher trophic levels of the food web, MeHg will be efficiently transferred, probably due its lipid-solubility and affinity for thiol groups (-SH) of proteins, which allows it to be retained in muscle tissues of animals (Morel et al. 1998; Ballatori 2002; Harris et al. 2003). Consequently, MeHg biomagnifies along the food web from prey to predator (Watras and Bloom 1992; Lavoie et al. 2013) and bioaccumulates over time in the animal tissues. For instance, Watras and Bloom (1992) estimated that the average proportion of mercury in methylmercury form increases from ca. 10% in the water column to 15% in phytoplankton, to >95% in fish. In this way, MeHg is transferred efficiently in aquatic food webs, achieving high levels in top-predators, such as piscivorous bird species or mammals (Lehnherr 2014), which might result in detrimental effects on the individuals exposed. Trophic transfer and biomagnification of MeHg through food webs has been demonstrated

by the relationship observed between an organism trophic position and its Hg concentration (Bearhop et al. 2000; Power et al. 2002).

4. Effects of mercury to human health

Although not considered top-predators due to the variety of food sources present in the human diet (Bonhommeau et al. 2013), humans are greatly exposed to mercury particularly through the consumption of fish and seafood (Clarkson 2002; Bradley et al. 2017; UNEP 2019; Ferreira-Rodriguez et al., 2021). Mercury can cause severe toxic effects in animal and human health (Clarkson and Magos 2006), and its fate and consequences in the human body will depend on its speciation (Rice et al. 2014). Even in case of accidental ingestion, liquid elemental mercury (Hg^0) and inorganic mercury (Hg(II)) are not easily absorbed through the intestinal tract ($<0.01\%$ for Hg^0 and ca. 10% for Hg(II) compounds; WHO 2003). However, MeHg absorption in humans ranges from 12% - 79% (reviewed in Bradley et al. 2017). Although MeHg in diet is a major route of mercury exposure in the general population (UNEP 2019), elemental mercury in vapor phase can be inhaled and in this case approximately 80% of Hg^0 is absorbed into the blood via the respiratory system (WHO 2003).

Once in the blood system, mercury is distributed to all organs causing an array of different affections. Cellular damage, endocrine disruption effects, teratogenic effects, toxic effects on the reproductive, urinary, cardiovascular and nervous systems among others, have been associated to mercury exposure (Clarkson and Magos 2006; reviewed in Rice et al. 2014). The chronic exposure to MeHg through diet is of major concern for human health, as it is considered predominantly a neurotoxin, which accumulates and affects the central nervous system, accessing the brain through the blood-brain barrier (Friberg and Mottet 1989; Clarkson et al. 2003; Sanfeliu et al. 2003; Díez 2009; Farina et al. 2013; Lohren et al. 2016), causing neurological impairments in speech, vision, hearing or muscular weakness (Ekino et al. 2007; Rice et al. 2014) or affecting negatively the development of the brain, going through the placenta and accumulating in the fetus (Antunes dos Santos et al. 2016).

5. Mercury in aquatic environments

Even in environments with low mercury concentrations, the effect of this toxicant can be disproportionately high if the conditions for inorganic mercury methylation, and bioavailability and biomagnification of MeHg through the food web are favorable. Aquatic environments are prone to have such conditions, and thus, freshwater and marine biota, or

organisms that depend on food webs based in aquatic environments will be more prone to MeHg, and its effects.

5.1. Mercury in freshwater environments

Toxicological studies in freshwater ecosystems (e.g., rivers, streams, lakes, estuaries, wetlands) have pointed out the importance of such environments in the mercury biogeochemical cycle (Chételat et al. 2015; Obrist et al. 2018), both as transformation and transport pathways for mercurial chemical species (Driscoll et al. 2007; Bravo et al. 2018; Demarty et al. 2021). Freshwater environments have been extensively regarded as hotspots for mercury sinking, methylation, bioaccumulation and biomagnification (Chan et al. 2003; Driscoll et al. 2007; Chételat et al. 2015; Backstrom et al. 2020).

Freshwater biota in mercury polluted sites, has been found to have relatively high mercury body burdens. For instance, freshwater fish species in Hg-polluted areas have been found systematically to be a good proxy for environmental mercury levels (e.g., Evers et al. 2008; Monson 2009; Depew et al. 2013; Miller et al. 2013; Nguetseng et al. 2015; Eagles-Smith et al. 2016; Broadley et al. 2019; Backstrom et al. 2020; Donadt et al. 2021).

5.1.1. Rice fields as mercury hotspots

Among freshwater environments, wetland environments referred to continuously or periodically flooded fields (i.e., swamps, peatlands, fresh or saltmarshes) are often linked to high MeHg production rate (Zillioux et al. 1993; Marvin-Dipasquale et al. 2014). Progressively with the worldwide population growth, natural wetlands tend to disappear and are replaced by agricultural farming site such as rice paddies (Elphick et al. 2000; Verhoeven and Setter 2010; Davidson 2014). Indeed, flooded rice paddies are considered as hotspots for mercury methylation, as anaerobic conditions in rice field soils promote methylation of mercury by microorganisms (Rothenberg and Feng 2012). This MeHg formed in the rice paddies can access the rice plants (Meng et al. 2011; Wang et al. 2018; Du et al. 2021) and animals inhabiting these environments (Ackerman et al. 2010a, 2010b; Abeyasinghe et al. 2017; Du et al. 2021). Furthermore, studies comparing MeHg levels in crops close to Hg polluted sites have found higher MeHg accumulation in rice grains than in the other crops studied (e.g., cabbage, tobacco, corn; Qiu et al. 2008). In a recent review, Liu et al. (2019) estimated the global budgets of mercury in various rice-fields ecosystem compartments and

showed how they affected mercury biotransport through rice exportation/importation and the percentage of Hg that ended to human consumption or destined to other uses (Fig. 5).

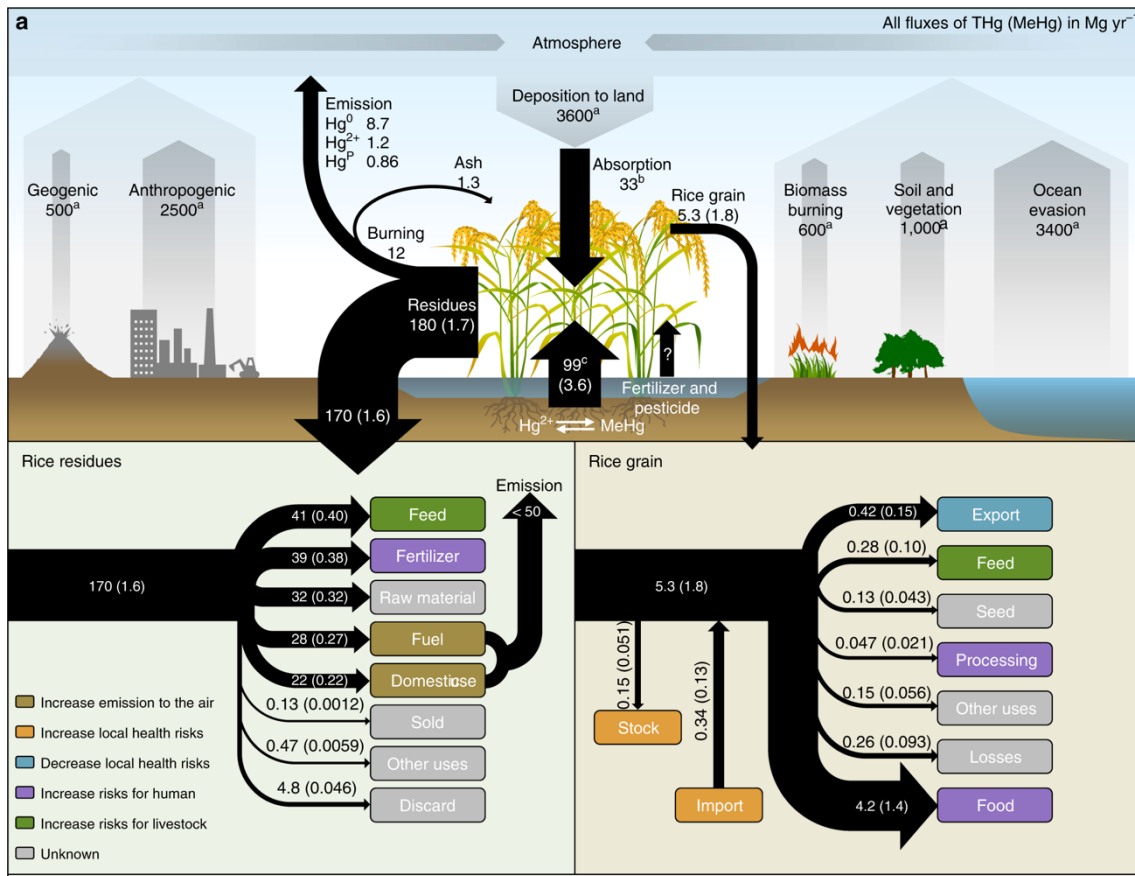


Figure 5 | Global estimated budgets of Hg flow and dynamics through rice paddies ecosystems. (From: Liu et al. 2019).

Thus, although fish and shellfish consumption is considered the main source of mercury for humans (Mergler et al. 2007; McKelvey and Oken 2012; UNEP 2019), some studies suggest that rice consumption might be another route for mercury intake that should not be neglected, especially in populations where rice is a staple-food or in infants being fed with rice-based foods (e.g., Feng et al. 2008; Li et al. 2010; Meng et al. 2010, 2011; Cui et al. 2017; Zhao et al. 2019; Liu et al. 2019). This has incentivized a large amount of research towards understanding how mercury cycles throughout rice paddies and is accumulated in rice plants or organisms inhabiting rice fields (e.g., Rothenberg and Feng 2012; Rothenberg et al. 2014; Zhao et al. 2020; Du et al. 2021), and the factors and environmental conditions affecting mercury transfer from paddies soils and water to both plants and fauna in rice fields are an active field of research (Zhao et al. 2020).

Rice fields are complex ecosystems in which the biogeochemical cycle of Hg includes several ecosystem compartments (e.g., soil, water, biota) and their interactions, as well as multiple physical, chemical and biological transformation processes (e.g., methylation, demethylation, oxidation, reduction, bioaccumulation, biomagnification). And, in addition, these processes are influenced by different biotic and abiotic factors that might modify the fate of mercury within the rice field ecosystem (Fig. 6).

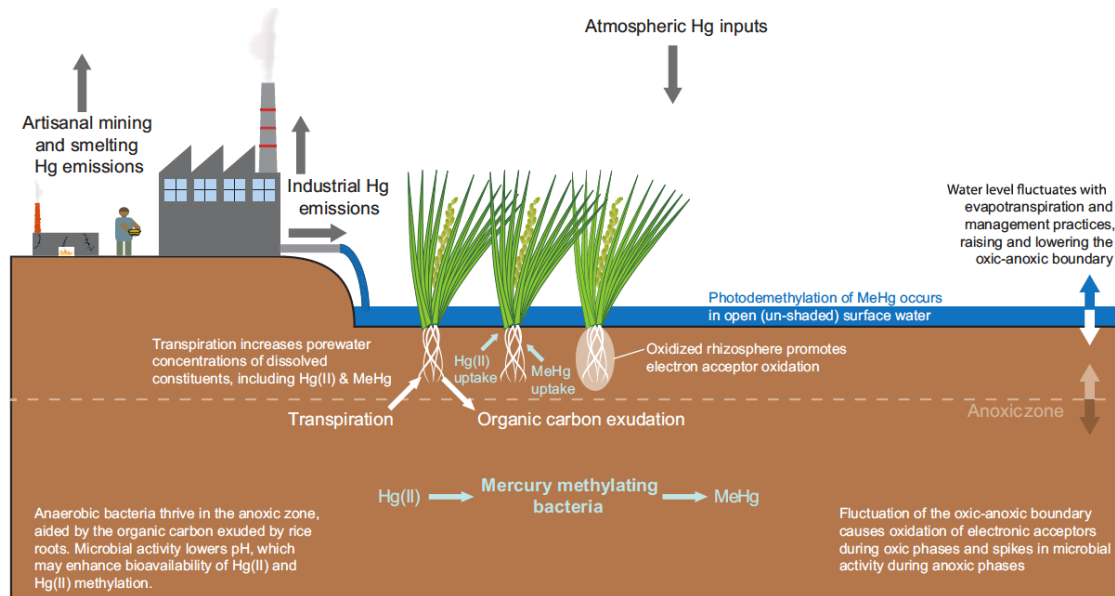


Figure 6 | Factors affecting mercury methylation in rice paddies (From: Rothenberg and Feng 2012).

5.1.1.1. Mercury methylation in rice fields

Inorganic mercury in rice paddies comes mainly from irrigation water from areas subject to industrial pollution (Dai et al. 2013; Palanques et al. 2020) or atmospherical deposition (Rothenberg et al. 2014). Once mercury enters the rice paddy system it might be transformed into other mercurial species, such as methylmercury, which eventually accumulates in rice plants and biomagnifies along rice field food webs.

Rice fields soils are considered as a hotspot for mercury methylation and MeHg formation due to the anoxic conditions attained in flooded paddies, which enhance mercury methylation by anaerobic microorganisms (Meng et al. 2011; Rothenberg et al. 2014; Zhao et al. 2016a). Recently, the development of DNA sequencing technology and the improvement in detecting Hg-methylation genes (cluster *hgcAB*) in bacterial populations has

increased the interest in the characterization of the abundance and composition of microbial methylator communities in these environments (Liu et al. 2018). Hg-methylator microorganism mainly include SRB, IRB and methanogens (Liu et al. 2014, 2018), and the specific composition of bacterial communities influences the capacity of mercury methylation of the ecosystems (Bravo and Cosio 2020).

The activity of Hg-methylator microorganisms will be enhanced/inhibited by the biophysiochemical conditions of the environment (Ullrich et al. 2001). Among them, redox conditions, the presence of electron donors/acceptors, pH and presence, abundance, and composition of organic matter available seem to play an important role.

A relatively low redox potential in anaerobic conditions favors Hg methylation and also inhibits MeHg demethylation (Gilmour and Henry 1991), by stimulating the mobilization of Hg from the solid phase to the aqueous phase, increasing its bioavailability to bacterial methylation (Tang et al. 2019). Thus, in flooded rice fields Hg methylation mainly occurs in anaerobic conditions, which enhance the growth and activity of anaerobic methylators (SRB, IRB and methanogens; Meng et al. 2011). Yet, soil redox potential might change with alternating flooding and drying conditions during the rice plants growth period (Zhao et al. 2016b), what might alter the cycling of Hg in the rice field ecosystem. In this regard, agricultural practices which promote variations in hydroperiods, such as an alternating wetting drying (AWD) cycles might influence MeHg formation (Rothenberg et al. 2016; Tanner et al. 2018, see below). In fact, Peng et al. (2012) observed that the redox potential in rice cultivated under aerobic conditions was higher than that of rice cultivated under flooded conditions, and therefore, Hg and MeHg concentrations and MeHg/THg (%) were higher in the flooded conditions. Nonetheless, when combining cycles of wetting and drying, although it affects the rates of Hg to MeHg transformation and consequently, bioaccumulation of MeHg in biota, both *in situ* and experimental studies, have found discrepancies on the direction of the effect (e.g., Marvin-DiPasquale 2014; Rothenberg et al. 2016; Tanner et al. 2018), which reinforces the fact that although redox conditions might play an important role on mercury methylation in rice paddies, there are other factors which modify Hg availability, methylation and subsequent accumulation by organisms. For instance, under aerobic conditions several studies have found that the activity of Hg-methylators is inhibited (Wang et al. 2014; Tanner et al. 2018), which might result in a decrease of MeHg formation in rice paddy soils.

The presence of electron donors (e.g., S^{2-} and Fe^{2+}) and acceptors (e.g., Fe^{3+} and SO_4^{2-}) regulates the bioavailability of Hg^{2+} and the activity of methylator microorganisms in

rice paddies (Ullrich et al. 2001; Lei et al. 2021). In fact, many studies show that MeHg production is linked to sulphur and iron cycles (e.g., Harmon et al. 2004; Mitchell and Gilmour 2008; Marvin-DiPasquale et al. 2014). Briefly, in rice paddies soils under anoxic conditions and in the presence of S^{2-} , Hg^{2+} might form insoluble mercuric sulphide (HgS) compounds (Yin et al. 2016; Liu et al. 2019), reducing the Hg^{2+} availability for methylation processes, and consequently impairing MeHg formation (Benoit et al. 1999; Jonsson et al. 2014). Nonetheless, HgS can be converted into Hg -soluble compounds (e.g., HgS_2^{2-}) in sulphide excess conditions and under certain redox and pH conditions in the environment (Li et al. 2019; Lei et al. 2021; Li et al. 2022), and therefore promote Hg methylation (but see Benoit et al. 2003; Drott et al. 2007). On the other hand, sulphates (SO_4^{2-}) (e.g., from chemical fertilizers such as K_2SO_4 and $(NH_4)_2SO_4$) can be used as an electron acceptor and reduced to sulfide by SRB under flooding conditions, potentially enhancing SRB activity and consequently increasing Hg methylation (Ullrich et al. 2001). Several studies have found significant correlation between SO_4^{2-} concentrations in rice field soil samples and Hg methylation rates (Rothenberg and Feng 2012; Zhao et al. 2016a, 2016b). Conversely, other studies do reflect that under high sulphate conditions, Hg methylation capacity of rice paddy rhizosphere soils might be lowered due to the high affinity of Hg ions to reduced sulphate compounds and further transformation into insoluble HgS forms (e.g., Wang et al. 2015; Li et al. 2017).

Similarly, iron does seem to have an effect on Hg methylation (e.g., Slowey and Brown 2007; Bravo et al. 2015). For instance, iron reduced form (Fe^{2+}) might sequester sulphide from HgS to form stable iron-sulphur compounds (e.g., FeS , FeS_2 , Fe_2S_4) releasing bioavailable Hg^{2+} to be methylated (Bravo et al. 2015). In addition, in wetland environments, the reduction rate of Fe^{3+} has been observed to positively correlate with MeHg concentrations in soils, which has been explained by the effect on Fe^{3+} in IRB activity (Marvin-DiPasquale 2014).

Thus, although sulphur and iron might play a role on Hg methylation rates by either enhancing/inhibiting Hg mobilization/bioavailability or SRB and IRB activity, to the date, the interaction between sulphur and iron, and Hg in rice fields ecosystems are poorly understood with highly variable results throughout the literature (Zhao et al. 2020), and might be interacting with other environmental variables such as pH, redox conditions or abundance and composition of organic matter present in the environment.

Organic matter concentration and composition significantly affects Hg by having an impact on (i) Hg^{2+} mobilization from HgS to liquid phase (Waples et al. 2005), increasing its

bioavailability (ii) Hg speciation and (iii) controlling microbial Hg methylation activity by providing nutrients to methylator microorganisms (Ullrich et al. 2001; Bravo and Cosio 2020). In rice paddies, soil MeHg concentration has been positively correlated with organic matter content and Hg methylator abundance (Liu et al. 2016; 2018). Other studies observed that rice plants' root-derived organic matter was related to elevated soil MeHg concentrations (Zhao et al. 2018; Li et al. 2022; Strickman et al. 2022). Furthermore, studies evaluating the influence of dissolved organic matter (DOM) from the decomposition of returning straw into rice fields reported that straw returning might enhance Hg methylation, by promoting Hg-methylator microorganisms' abundance and activity (Liu et al. 2016; Tang et al. 2019).

Regarding pH conditions, previous studies have reported that acidic conditions should influence Hg methylation favoring the dissolution of Hg into the aqueous phase, increasing Hg^{2+} bioavailability. And, conversely, alkaline conditions would inhibit Hg methylation by decreasing Hg bioavailability in soils (Ullrich et al. 2001). However, mercury methylation has been observed in field experiments in rice paddies under an array of pH values (e.g., Zhao et al. 2016a; Tang et al. 2018; Lei et al. 2021), and pH was not in all cases a good predictor of MeHg concentrations in soils or rice plants (Rothenberg and Feng 2012).

As pointed out, mercury methylation in aquatic environments and, especially in complex environments such as rice paddies, where many inputs, sources and fluctuating environmental conditions occur, is a multifactorial process which depends on biological, chemical and physical factors and their multiple interactions (Marvin-DiPasquale 2014; Bravo and Cosio 2020). Agricultural practices such as the application of fertilizers and pesticides, or different hydric periods might influence all the above and thus, have an important role on MeHg formation in rice paddies, and ultimately on Hg bioavailability and concentrations in rice plants (Rothenberg et al. 2016).

5.1.1.2. Mercury accumulation and distribution in rice plants

It is widely accepted that rice plants bioaccumulate mercury (Rothenberg and Feng 2012), and this has been of special interest as in some Hg-polluted areas concentrations in grain exceed the permitted Hg levels for food safety (e.g., Li et al. 2017).

With growth, rice plants accumulate both inorganic mercury (IHg) and methylmercury (MeHg) (Zhang et al. 2010; Meng et al. 2010, 2012; Wang et al. 2018). Rice plants mainly accumulate mercury through absorption via roots from the soil or from the

atmosphere through stomata (Meng et al. 2010, 2011, 2012). The different distribution of mercury species (MeHg and IHg) among plant tissues reflects the various sources, mechanisms of uptake and capacity of plant tissues to accumulate IHg and MeHg (Meng et al. 2010, 2012; Zhang et al. 2010; Tang et al. 2020; Liu et al. 2021).

In the case of roots, studies in polluted sites have observed that they can absorb both IHg and MeHg from the soil. However, they tend to bioaccumulate mainly IHg. MeHg, although it is absorbed by the roots from the rizosphere soil, it is efficiently transported to the aerial parts of the plant (Meng et al. 2010, 2011). This specificity for IHg accumulation in roots over the rice plant growth seems due to the presence of phytochelatins, small peptides that chelate heavy metals in plants, which can sequester IHg (but not MeHg) and store it in the vacuoles of root cells (Krupp et al. 2009). Therefore, the majority of MeHg absorbed from the soil via roots will be transported to the aerial parts of the plant, whereas small amounts of IHg will abandon the roots to the above-ground parts (Zhang et al. 2010; Meng et al. 2011, 2014; Rothenberg et al., 2014). Furthermore, demethylation mechanisms of MeHg in roots have been described (Xu et al. 2016; Strickman and Mitchell 2017; Liu et al. 2021), which might promote to some extent a decrease in MeHg levels in plants roots over time.

During the rice plant vegetative phase, the major part of MeHg is stored in the stalk and leaves, but it is transported to the rice grain during its formation (Meng et al. 2011). Indeed, in mature rice plants, MeHg accumulated mainly in the rice seeds and then in roots, stalk and leaves (Zhang et al. 2010; Meng et al. 2011). Conversely, inorganic mercury concentrations are more prominent in roots and leaves, depending on the relative levels of Hg contamination in soil and atmosphere, and then in stalk and grain (Meng et al. 2010; 2012). Furthermore, the distribution of Hg species in the different parts of the rice grain (hull, bran, endosperm) have been studied (Meng et al. 2014). IHg is preferentially retained in the hull and the bran, while MeHg mainly accumulates in the endosperm (white rice). Thus, the hull and bran prevent the translocation of IHg into the endosperm of the rice grain (Meng et al. 2014). Moreover, Rothenberg et al. (2011) suggested that MeHg, as an organic compound, could use membrane transporter pathways to enter the endosperm more efficiently than IHg, promoting the difference in localization of IHg and MeHg within the rice grain. Therefore, most of the IHg is removed by processing the rice grain (i.e., removing the hull and the bran), whereas little MeHg fraction is removed (Meng et al. 2014).

Thus, the paddy soil is the main source for MeHg in rice plants, absorbed by the roots and ultimately accumulated mainly in the rice grain. On the other hand, IHg might come from the soil and accumulates mainly in root cells, or from the atmosphere, entering through

stomata into leaves and accumulating mainly in the aerial parts of the plant (Meng et al. 2010; 2011; 2012). In fact, studies using mercury stable isotopes (Yin et al. 2013; Strickman and Mitchell 2017) have further confirmed these sources, transportation mechanisms and bioaccumulation patterns of mercury species in rice plants.

5.1.1.3. Effect of agricultural management on mercury dynamics in rice paddies

As previously seen, mercury methylation and bioavailability in rice paddies might be influenced by several bio-physicochemical parameters. In turn, these parameters are sensitive to different agricultural practices used to farm rice. Several studies have discussed the impact of agricultural techniques used in rice farming on the bioavailability of mercury (Rothenberg et al. 2011; Rothenberg and Feng 2012; 2014; Liu et al. 2016; Tanner et al. 2018; Tang et al. 2019; 2020).

During 1990's, global climate change and increased water demand have led to develop new techniques using less water without losing crop yields (Rothenberg et al. 2014). Nowadays, alternating wetting and drying (AWD) technique characterized by a drainage just after seeded followed by intermittent wetting and drying periods is widely employed and it is expected to increase in the future (Rothenberg et al. 2014). Experimental studies reported decreased methylation rates in soils of AWD cultivated rice plants (e.g., Rothenberg et al. 2016; Tanner et al. 2018). Moreover, aerobically farmed rice accumulated less MeHg than rice grown under continuously flooded conditions, which was suggested to be driven by a decreased sulphate-reducing bacteria and other methylators abundance, community composition and activity under aerobically conditions (Peng et al. 2012; Wang et al. 2014). However, some studies in agricultural wetlands under AWD regimes have observed an increase in MeHg concentration in rice paddies soils (e.g., Rothenberg and Feng 2012; Marvin-DiPasquale et al. 2014), which correlated with reflooding periods, suggesting that after reflooding soil MeHg spiked due to the revival of sulphate-reducing bacteria (Rothenberg and Feng 2012; Rothenberg et al. 2014). Thus, it is not completely clear how AWD does affect the rice field ecosystem mercury concentrations and methylation, and ultimately its accumulation in rice plants.

The usage of chemical fertilizers and pesticides is a common practice to increase crop yields. Its application does lead to changes in physicochemical properties of soils and microbial communities (Liu et al. 2017) which can also affect the Hg availability and methylation within rice paddies. For instance, nitrogenous fertilizers are one of the most

common types of fertilizers added to soils worldwide (Faostat 2021). Application of nitrogenous fertilizers can result in soil acidification (Liang et al. 2013), what might promote Hg^{2+} bioavailability, but also can inhibit sulfate-reducing bacteria activities and promote the growth and activity of nitrate-reducing bacteria which compete for electron donors with SRB, diminishing MeHg formation (Zhang et al. 2018). Furthermore, nitrogenous and other fertilizers might incorporate sulfates to the soil at the same time (e.g., $(\text{NH}_4)_2\text{SO}_4$, K_2SO_4), which might influence mercury methylation in soils as detailed in the previous section.

Finally, among the agricultural practices having effects on mercury cycling in rice fields, the post-harvest returning of straw material into the fields as an organic fertilizer has recently been detected as a major influential practice on Hg dynamics (Zhu et al. 2015; Liu et al. 2016; Tang et al. 2018; 2019). When rice straw decomposes, it releases large amounts of DOM (e.g., acetate; Marvin-DiPasquale et al. 2014). These compounds enhance the growth and activity of methylator microorganisms, and consequently higher methylation rates and MeHg production (Zhu et al. 2015; Liu et al. 2016), leading to increased MeHg concentrations in soils and in rice grains (Tang et al. 2019).

Overall, the role and significance of the different factors affecting the Hg cycling and dynamics within rice paddies is complex and warrants further research to fully understand how the different factors and their interaction modulate Hg dynamics and which strategies might help to mitigate Hg in these environments, especially regarding agricultural management options.

5.2. *Mercury in marine environments*

The study of Hg in marine and oceanic waters is of special importance, first from a toxicological point of view, as fish and seafood are the main sources of Hg to humans (Sunderland 2007; UNEP 2019), and secondly, due to the impact of metal pollution to environmental health issues (Halpern et al. 2008; Crain et al. 2009).

In marine environments, mercury sources include mainly atmospheric deposition (Wang et al. 2004), and transport from other flowing aquatic systems such as rivers or streams (Cossa et al. 1996; Amos et al. 2014; Palanques et al. 2020; Demarty et al. 2021; Liu et al. 2021). Furthermore, marine environments are the most important sink of Hg on Earth surface (Amos et al. 2013; Ogrinc et al. 2019; Fig. 2; Fig. 7), both from natural and anthropogenic sources (Lamborg et al. 2014).

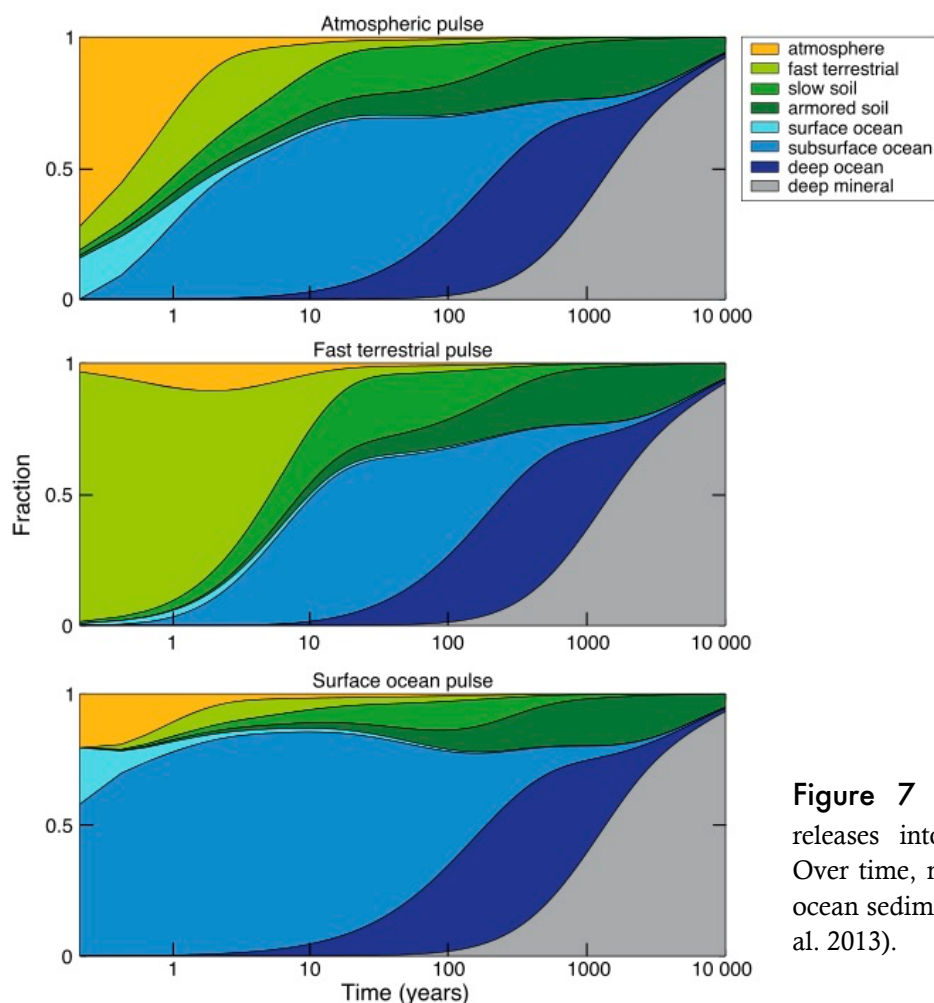


Figure 7 | Fate of mercury releases into the environment. Over time, mercury sinks within ocean sediments (From: Amos et al. 2013).

5.2.1. Methylmercury in marine environments

Mercury in marine environments can be found in four different species: elemental mercury, inorganic Hg(II), methylmercury (MeHg) and dimethylmercury (DMeHg). Among these, MeHg is of special concern for marine biota and human health, due to its bioaccumulation and biomagnification capacity along food webs and its toxicity. The net levels of MeHg in marine ecosystems will be related to MeHg inputs (e.g., rivers, streams) or *in-situ* formation of this species from Hg(II) (e.g., from volcanic activity, atmospherical deposition), and the degradation of MeHg.

Methylmercury might be formed in coastal sediments controlled by bacterial activity (SRB and IRB) and the bioavailability of inorganic Hg, which depends on several factors such as redox conditions, sulfide and organic carbon availability (Benoit et al. 2003; Hammerschmidt et al. 2004). Deep-sea sediments might constitute another suitable environment for biotic mercury methylation, although data on this regard seems to be limited (Black et al. 2012); nevertheless, the methylation rates observed in deep-sea sediments are

much lower than those from coastal sediments (Ogrinc et al. 2007). Finally, Hg might be methylated in the anoxic regions of the water column by methylator microorganisms (Lehnherr et al. 2011).

The degradation of MeHg consists primarily in photodemethylation in the photic area of the water column (Bergquist and Blum 2007; Monperrus et al. 2007; Lehnherr et al. 2011), however factors controlling MeHg degradation rates in marine systems are not fully understood.

5.2.2. Biomonitoring of mercury in marine environments

Anthropogenic impact has been long discussed as a main pressure factor in marine environments (Halpern et al. 2008). In fact, several anthropogenic activities do lead to heavy metal pollution into marine environments (Halpern et al. 2008), including mercury pollution (UNEP 2019). This has generated an urge to monitor and perform longitudinal studies to assess the pollution status of marine environments.

Biomonitoring of marine environments has been performed using several groups of organisms, from algae to marine vertebrates (e.g., algae [García-Seoane et al. 2018], invertebrates [Rainbow 2002], vertebrates [Sanpera et al. 2007]). Since mercury exposure for aquatic organisms occurs mainly via dietary intake and biomagnification some high trophic level predators, such as seabirds, will be more exposed to mercury and integrate information from marine environments and the whole food webs (Furness and Camphuysen 1997; Burger and Gochfeld 2004).

6. Mercury as a local pollutant: The Ebro Delta case

6.1. Mercury in the Mediterranean

The Mediterranean Sea is the largest (2,969,000 km²) and deepest (average 1,460 m, maximum 5,267 m) enclosed sea on Earth. It is formed by two subregions separated by the strait of Sicilia: the western basin, with an area of 0.85 million km², and the eastern basin, with an area of 1.65 million km². Its unique geological history, biogeographical and ecological conditions have promoted its high biological diversity (Coll et al. 2010). It harbors up to 18% of the world's macroscopic marine species, of which 25-30% are considered endemic (Bianchi and Morri 2000), and it is considered a global biodiversity hotspot (Myers

et al. 2000). Yet, biodiversity in the Mediterranean Sea is not distributed uniformly (Coll et al. 2010); it is associated to a productivity gradient from west to east. Therefore, the western Mediterranean basin with highest productivity areas, presents higher species richness values than the eastern basin. In addition, coastal areas and the continental shelf do present greater species richness than deep-sea areas. These patterns are of ecological importance in terms of species conservation (Fig. 8). Furthermore, throughout human history, many civilizations have emerged and declined along its coastal areas. Nowadays, around 480 million people live in the Mediterranean region, from 22 countries along Europe, Africa and Asia. Approximately one-third of the Mediterranean population is concentrated along its coastal areas (UNEP 2017). Thus, the anthropogenic pressure on the coastal and marine area in the Mediterranean region, from resource exploitation to pollution, has been one of the main concerns for environmental programs and policymakers (Coll et al. 2010; UNEP 2017).

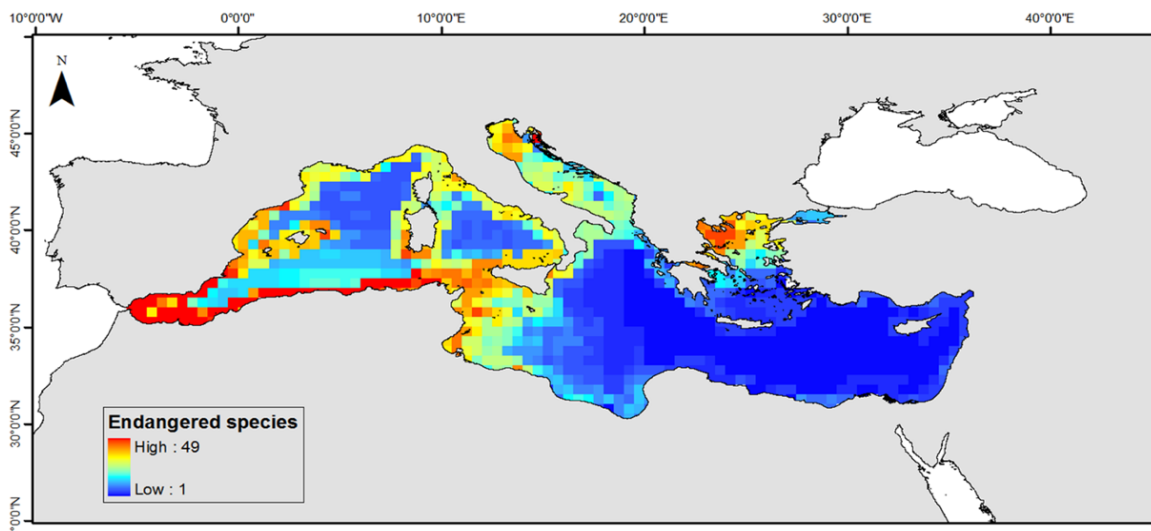


Figure 8 | Biodiversity hotspots for endangered species in the Mediterranean basin (From: Coll et al. 2010).

Regarding mercury pollution, the Mediterranean Sea is affected by Hg contamination due to geochemical processes related to volcanic activity and cinnabar deposits, as well as anthropogenic mercury inputs derived from industrial and agricultural activities (Sunderland and Selin 2013; Salvagio Manta et al. 2016; Streets et al. 2019). Compared to other oceanic areas in the world, the Mediterranean Sea presents anomalously high concentrations of both inorganic mercury and MeHg (Mason and Gill 2005; Gworek et al. 2016). Moreover, overall oligotrophic conditions of the Mediterranean Sea promote the biomagnification of Hg along food webs (Chouvelon et al 2018). Especially, the high MeHg loads in the environment along with biomagnification processes of this Hg species affects the fishes inhabiting these waters. A recent review on the mercury levels in several fish species

in Mediterranean and Atlantic waters (Cinnirella et al. 2019), detected Hg concentrations in several commercial fish species in the Mediterranean superior to those in the Atlantic, and above the allowed concentrations for human consumption. This is of special importance from a food safety point of view, since fish and shellfish consumption is regarded as the primary cause of mercury intake in humans (UNEP 2019).

Moreover, ecotoxicological studies on top-predators in the Mediterranean have reported high concentrations of pollutants (including mercury) on organisms, which have been known to have effects from individual level to population levels (Arcos et al. 2002; Kershaw and Hall 2019; Costantini et al. 2020).

6.2. The Ebro Delta (NW Mediterranean)

Along the Mediterranean coastal area there are 150 wetlands of international importance for biodiversity. One of the most important wetland areas in the Iberian Peninsula and along the western Mediterranean is the Ebro Delta ($0^{\circ}43'N$ $0^{\circ}44'E$; Catalonia, Spain). It is formed by the Ebro River, which with its 910 km long and a drainage basin of ca. 85,362 km² is the river with the highest mean annual flow, and largest hydrographic basin in Spain and one of the main tributaries discharging to the western Mediterranean Sea (Romaní et al. 2010; Fig. 9).

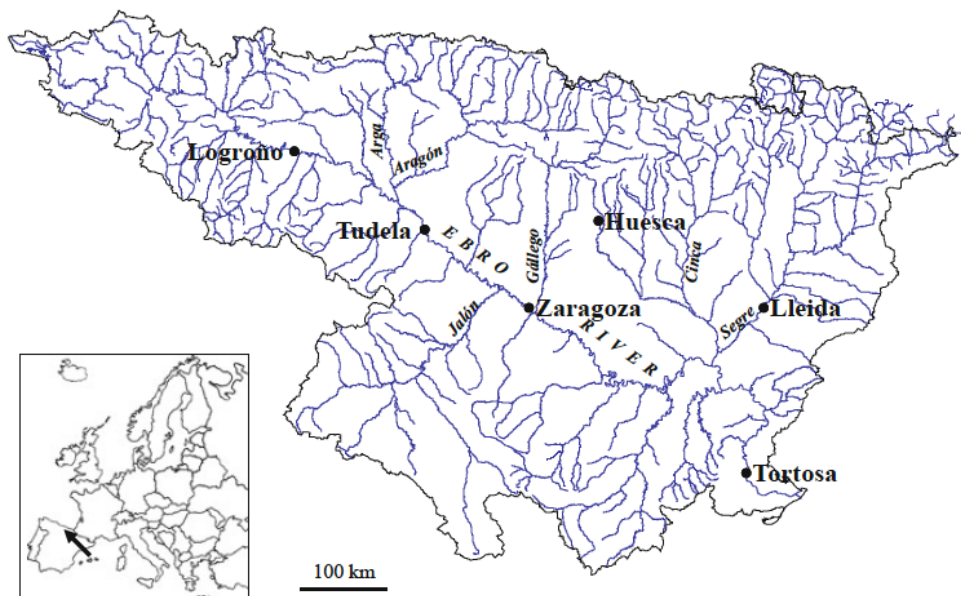


Figure 9 | Map of the Ebro River basin, indicating the main cities and tributaries. In the inset, the position of the Ebro River in the Iberian Peninsula is indicated. The Ebro River forms a delta in its final portion. (From: Romaní et al. 2010).

The Ebro Delta (Fig. 10) comprises 30,000 ha of wetland environments, with almost 75% of the area is occupied by rice fields, 20% is protected as a natural reserve, and 5% is urbanized (Mañosa et al. 2001). Indeed, the Ebro Delta is one of the main rice producing areas in Europe, with a production of about 120,000 tonnes/year (Day et al. 2006). The natural reserve area it is protected as a Natura 2000 Network site (European Union), a Special Protection Area (SPA) under the Birds Directive (Directive 2009/147/EC), and as a Natural Park and Biosphere Reserve (UNESCO, 2013), with ecosystems within the deltaic area (e.g., coastal lagoons, marshes, freshwater springs, etc.) providing habitats for abundant and highly diverse wildlife (Ibáñez and Caiola 2018). Furthermore, the neighboring marine area is a particularly high productivity area within the Mediterranean (Bosc et al. 2004), as a result of the rich-nutrient freshwater discharge from the Ebro river (Cruzado et al. 2002; Salat et al. 2002). It also represents an area with essential habitats for pelagic and demersal fish (Tugores et al. 2019). Therefore, it supports an important fishery activity (Demestre et al. 2008) and it is also a relevant area for marine mammals, seabird colonies, marine turtles and other endangered species (Coll et al. 2010).



Figure 10 | The Ebro Delta. Most of its area is dedicated to the rice farming, one of the main economic activities of the region. (From: Earth Observatory, NASA).

Nonetheless, anthropogenic impacts on deltaic and coastal ecosystems might be influencing ecosystem dynamics and wildlife populations (Ibáñez et al. 2019). Among them,

heavy metal pollution has been documented in the Ebro Delta area (Sanpera et al. 2007; Cotín et al. 2012; García-Tarrason et al. 2013), as it might impact both humans and wildlife.

6.3. *Flix chlor-alkali plant*

Since the late 19th century, the chlor-alkali plant (CAP) at Flix (41°23' N, 0°55' E; 100 km up the Ebro River mouth) has used mercury-cell technology for manufacturing chlorine and other products. This process uses mercury to produce chlorine and sodium/potassium hydroxide by the hydrolysis of brine. As a result, although Hg is theoretically recycled in the production system, part of it is released along with the waste sludges during the process (Brikmann et al. 2014; EuroChlor 2019), promoting the emission of Hg to the environment at the vicinity of the chlor-alkali plant (Maserti and Ferrara 1991; Gonzales 1991; Biester et al. 2002; Hunt 2016). Nonetheless, several studies have demonstrated that most Hg emitted from CAPs is dispersed over long distances and only small amounts are deposited in the vicinity of the plant sites (Jernelöv and Wallin 1973; Lindqvist and Rodhe 1985; Palanques et al. 2020). In Flix, an existing river dam close to the factory site prevents dispersion events (Fig. 11).

In 2003, a study of the Flix chlor-alkali site by Grimalt and collaborators was commissioned to evaluate the presence of toxic compounds in the sediments and waters near the factory (Grimalt et al. 2003). The technical report encountered more than 350,000 tons of toxic waste, containing mainly organochlorine compounds and heavy metals (including mercury), dumped into the riverbed (Grimalt et al. 2003; ACA 2013). In 2012, a protection wall was constructed nearby the Flix chlor-alkali plant limits to enclose and prevent the toxicants coming from the plant reaching the neighboring waters. However, with flooding and level rises from the river, overflowing of the river dam occurred and the pollutants accumulated in the vicinity of the factory were released into the Ebro river being transported downstream (Palanques et al. 2020). Thus, despite plant operations producing chlorine using mercury ceased in 2017, legacy mercury pollution persists and has been impacting downstream Ebro River environments (Carrasco et al. 2011a; Soto et al. 2011; Cotín et al. 2012; Campillo et al. 2019; Palanques et al. 2020). Moreover, during 2013-2014, a sediment remediation plan, including the dredging and disposal of polluted sediments, was implemented to remove toxicants from the riverbed in the vicinity of the factory (Ercros 2019). Nonetheless, the mid-term effects of these activities, which might have led to a remobilization and resuspension of polluted sediments, and a consequent effect on

ecosystems downstream, have been scarcely evaluated (e.g., Vilavert et al. 2015; Blanco et al. 2018; Constenla et al. 2022).

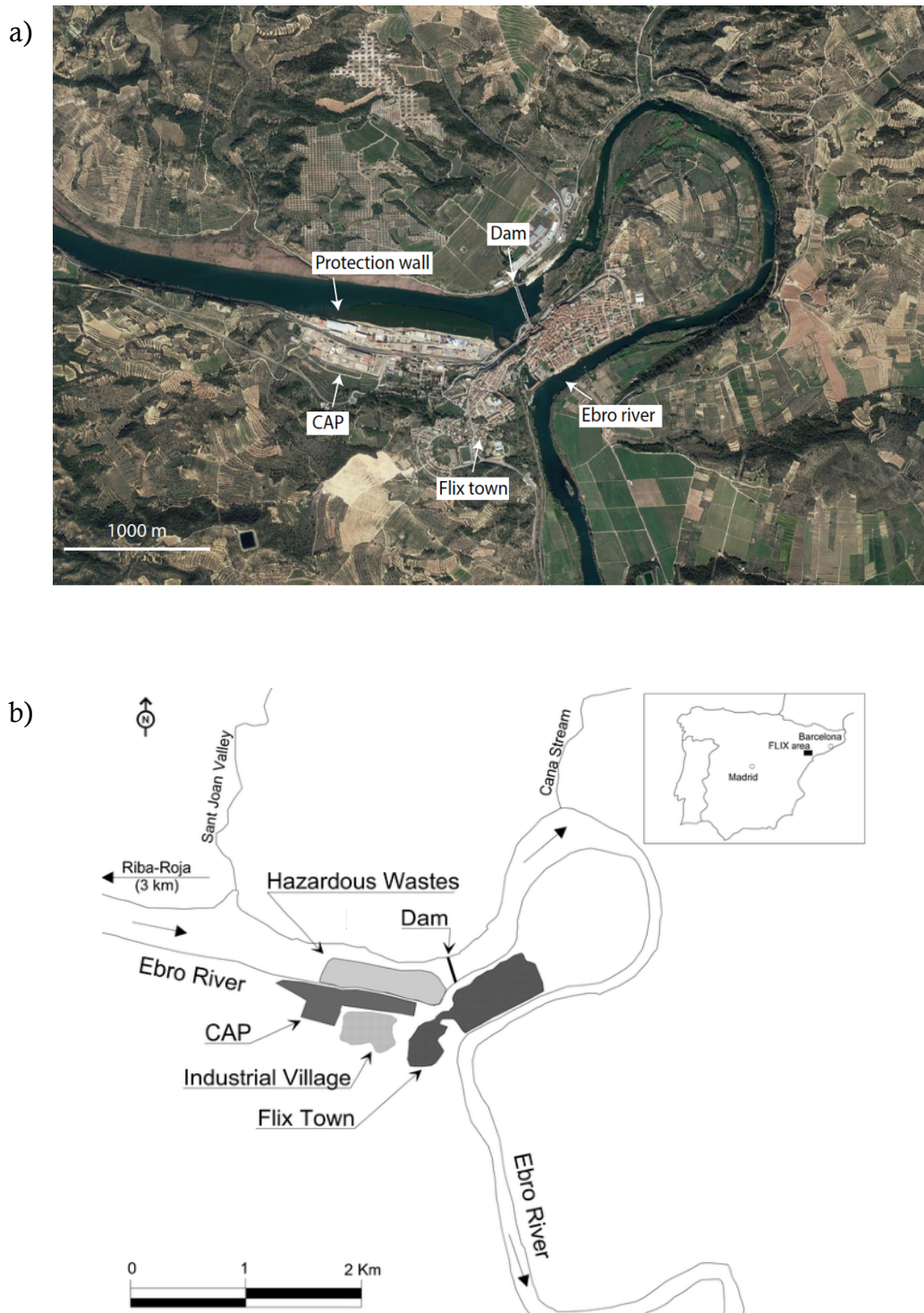


Figure 11 | (a) Aerial view (From: Google Maps) and (b) schematic representation of the chlor-alkali factory site at Flix. The inset shows the location of the Flix reservoir within the Iberian Peninsula (From: Esbrí et al. 2014).

Since rice fields in the Ebro Delta area are flooded using water from the Ebro river (Terrado et al. 2007), pollutants such as mercury might be incorporated into the rice paddy ecosystem, influencing Hg cycling within the fields. In addition, Hg transport by the river would also affect the coastal marine area enhancing mercury loads in the neighboring marine ecosystems (Palanques et al. 2020; Demarty et al. 2021).

6.4. Audouin's gull as a bioindicator species

Audouin's gull (*Ichthyaeetus audouinii*, Payraudeau 1826) is a medium-sized gull species, with adult individuals approximately 42-58 cm in height. Adult individuals present light gray wing upperparts and a distinctive dark-red bill, without marked sexual dimorphism, although males are usually bigger than females (Ruiz et al. 1998; Genovart et al. 2003; Fig. 12). The global population for this species is estimated at 33,000- 46,000 mature individuals (BirdLife International, 2022). It breeds in monospecific colonies from hundreds to thousands of breeding pairs (Burger and Gochfeld 1996). Nesting sites include islands, sandy areas, bare rocks, artificial undisturbed areas (e.g., harbors), low vegetated areas, saltmarshes (BirdLife International, 2022). Although Atlantic breeding colonies have been established in Portugal, it breeds mainly in the Mediterranean area, with important breeding sites along the north-western Mediterranean coast (BirdLife International, 2022; Bécarea et al. 2016; Fig. 13). Among them, the Ebro Delta "Punta de la Banya" colony, has been historically one of the main breeding colonies for this species, as until 2006 it was the breeding site for most of the world's population of Audouin's gull (~15,000 breeding pairs; Genovart et al. 2018), since its establishment in 1981 (Gutiérrez and Guinart 2008; Fig. 13). After 2006, the colony started to lower the number of breeding pairs and colonization and dispersion to other areas occurred, possibly due to mammal predation (Payo-Payo et al. 2018) and reduction of fisheries discards (García-Tarrasón et al. 2015), among other causes. Audouin's gull is categorized as "Vulnerable" by the International Union for Conservation of Nature (IUCN) with a decreasing trend in populations globally (BirdLife International, 2022).

Breeding season starts mid-April with the laying of the eggs, with a modal clutch of 3 eggs that hatch after ~29 days of incubation, until June-July, when juveniles are at fledgling stage. After the breeding season Audouin's gull start the migration towards their wintering grounds in West Africa. They return to the nesting sites during late winter (March) (Pedrocchi and Oro 2005; Sanpera et al. 2007; Bécarea et al. 2016). Nonetheless, a small

wintering population is constrained to the Mediterranean area, mainly in association with fishing harbours (Bécares et al. 2016).



Figure 12 | Audouin's gull (*L. audouinii*) adult individual flying over the Mediterranean. © Marcel Gil-Velasco

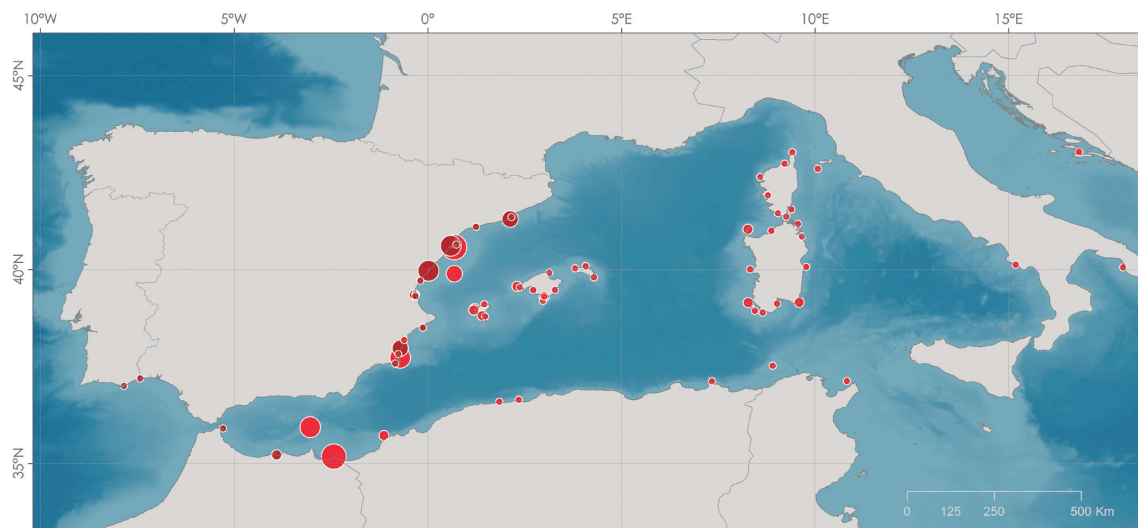


Figure 13 | Audouin's gull breeding colonies along the western Mediterranean. Relative size of the red dots, indicates size of the colony (breeding pairs). (From: Bécares et al. 2016).

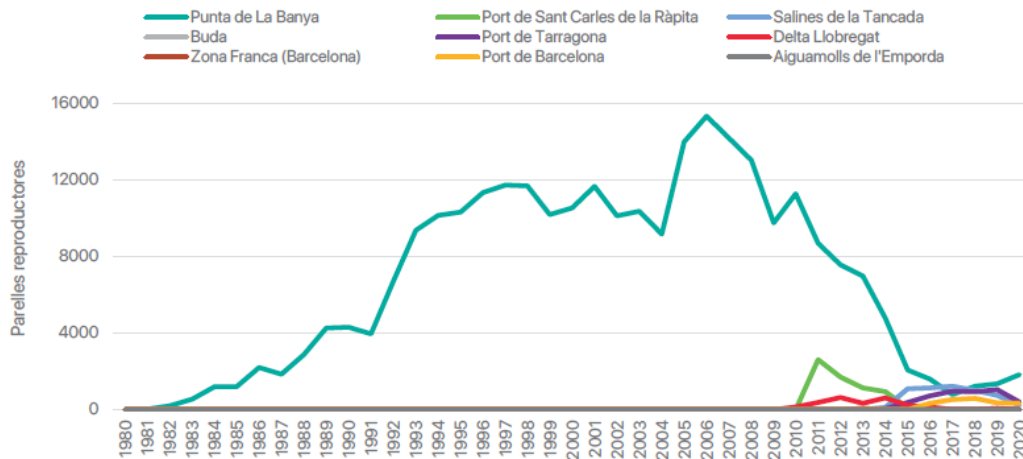


Figure 14 | Evolution in the number of breeding pairs in the breeding colonies of Audouin's gull in Catalonia (Spain). The “Punta de la Banya” colony, since its establishment in 1981, rapidly has increased the number of pairs until 2006, when it started to collapse. (From: Bécars and Gil-Velasco 2020).

During the breeding season in the Ebro Delta, foraging ecology of this species include an opportunistic utilization of marine sources (i.e., fisheries discards), that comprise up to a 75% of the energy obtained from marine resources (Arcos 2001; García-Tarrasón et al. 2013). Fisheries discards are mainly comprised by large proportions of mesopelagic and benthic fish species (Pedrocchi et al. 2002), which occupy higher trophic levels than epipelagic fishes (Arcos et al. 2002), thus potentially accumulating and transferring more Hg to its consumers. Additionally, another important resource in Audouin's gulls' diet is rice field preys (Oro et al. 1996; Ruiz et al. 1996; Pedrocchi et al. 2002), and other terrestrial resources (e.g., insects; Pedrocchi et al. 2002). Rice-field preys include the red crayfish (*Procambarus clarkii*), which is known to bioaccumulate Hg in polluted environments (Suárez-Serrano et al. 2010), transferring it to higher trophic levels of the food webs (Geiger et al. 2005). Therefore, given the trophic ecology of Audouin's gull in the Ebro Delta, this species might serve as an appropriate bioindicator species for mercury pollution in the area, with a legacy Hg-pollution that affects both rice fields and the coastal marine area, which in turn might compromise the ecosystems health.

0

OBJECTIVES

The present thesis aims at improving the knowledge on the mercury pollution problematic in the Ebro Delta area. We made a special emphasis on the rice-field ecosystems because their potential as methylation cuvette of mercury. Moreover, we have fostered in the study of Audouin's gull as mercury bioindicator species for the whole area, given its foraging behavior which encompasses both, marine and rice paddies environments. Most of the fieldwork was conducted in the Ebro Delta (40° 35'N, 0° 42'E; Catalonia, Spain) during the 2017-2019 Audouin's breeding seasons (April-June) along the rice cultivation period (April-September).

The results obtained in this thesis along with previous research, will help to better understand the mid-term impacts of legacy mercury pollution on the Ebro Delta area and contribute to broaden our knowledge about newly developed techniques to monitor Hg in the environment.

Specifically, the following objectives are considered along the following chapters:

- **Chapter 2** explores the dynamics of mercury cycling within rice paddies in the Ebro Delta over the rice farming season. Furthermore, it evaluates the effects of different agricultural practices in rice paddies (use of pesticides and fertilizers, irrigation regimes) on mercury availability and transfer to rice plants and rice fields food webs.
- **Chapter 3** focuses on how mercury accesses and bioaccumulates in fauna inhabiting rice-fields, by means of an *in-situ* mesocosm experiment throughout the rice cultivation season. The effects of agricultural management practices employed in rice farming were evaluated.
- **Chapter 4** evaluates the feasibility of Audouin's gull as a bioindicator on a temporal basis of changes on mercury environmental loads. Following-up a 15-year time series, we investigated how legacy mercury pollution and anthropogenic

activities impacted Hg levels in the breeding colony of Audouin's gull at the Ebro Delta.

- **Chapter 5** deepens in the study of mercury stable isotopes technique and its application to ecological issues. By sampling three species of seabirds from the area (both, gulls and terns) with differing trophic ecologies, we aimed to discern the relationship between mercury bioavailability and habitat use, as well as the contribution of anthropogenic mercury to neighboring coastal areas. Contrasting temporal trends in Hg levels and metal isotope abundance in Audouin's gulls will help to unravel the importance of anthropogenic contributions to bioavailable mercury over time.

Dr. Carolina Sanpera and **Dr. Josep Lluís Carrasco**, co-supervisors of the PhD thesis entitled “**From rice fields to seabirds: an integrative approach to study mercury dynamics in the Ebro Delta**” certify that

the dissertation presented here has been carried out by **Moisés Sánchez-Fortún** in its totality and grants him the right to defend his thesis in front of a scientific committee.

The contribution of the PhD candidate to each manuscript is detailed below, with the impact factor, the quartile, and the current stage of publication:

- **Chapter 2:** Temporal mercury dynamics throughout the rice cultivation season in the Ebro Delta (NE Spain): an integrative approach

Moisés Sánchez-Fortún, Josep Lluís Carrasco, Sergi Díez, David Amouroux, Emmanuel Tessier, Sophie López-Carmona, Carola Sanpera
Environmental Science and Technology (Under Review)
Impact Factor (2020): 9.028 / Quartile (Journal Citation Reports): Q1 (20 out of 274 journals) in the category of “Environmental Sciences”

The PhD candidate MSF has contributed to the conception, study design, data collection, data analysis and scientific writing.

- **Chapter 3:** Effect of different rice farming practices on the bioavailability of mercury: a mesocosm experiment with common goldfish (*Carassius auratus*)

Moisés Sánchez-Fortún, Dolors Vinyoles, Sophie López-Carmona, Josep Lluís Carrasco, Carola Sanpera
Environmental Research (2021)
<https://doi.org/10.1016/j.envres.2021.111486>
Impact Factor (2021): 6.498 / Quartile (Journal Citation Reports): Q1 (36 out of 274 journals) in the category of “Environmental Sciences”.

The PhD candidate MSF has contributed to the conception, study design, data collection and scientific writing.

- **Chapter 4:** Following up mercury pollution in the Ebro Delta (NE Spain): Audouin's gull fledglings as model organisms to elucidate anthropogenic impacts on the environment

Moisès Sánchez-Fortún, Jazel Ouled-Cheikh, Clara Jover, Manuel García-Tarrasón, Josep Lluís Carrasco, Carola Sanpera

Environmental Pollution (2020)

<https://doi.org/10.1016/j.envpol.2020.115232>

Impact Factor (2021): 8.071 / Quartile (Journal Citation Reports): Q1 (23 out of 274 journals) in the category of "Environmental Sciences"

The PhD candidate MSF has contributed to the conception, study design, data collection, data analysis and scientific writing.

- **Chapter 5:** Mercury stable isotopes in seabirds in the Ebro Delta (NE Iberian Peninsula): inter-specific and temporal differences

Moisès Sánchez-Fortún, David Amouroux, Emmanuel Tessier, Josep Lluís Carrasco, Carola Sanpera

Science of the Total Environment (In prep.)

Impact Factor (2021): 7.963 / Quartile (Journal Citation Reports): Q1 (25 out of 274 journals) in the category of "Environmental Sciences"

The PhD candidate MSF has contributed to the conception, study design, data collection, data analysis and scientific writing.

Hereby, we also certify that

none of the manuscripts included in this PhD thesis, partially or in its totality, has not contributed to, nor been part of any other PhD thesis.

Barcelona, 12th May 2022



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02 CHAPTER 2

**Temporal mercury dynamics throughout the rice cultivation season in the Ebro Delta (NE Spain):
an integrative approach**



02

CHAPTER 2

Temporal mercury dynamics throughout the rice cultivation season in the Ebro Delta (NE Spain): an integrative approach

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Under revision:

Environmental Science and Technology

Dinàmica temporal del mercuri als arrossars del Delta de l'Ebre durant el cicle de cultiu de l'arròs: una aproximació integradora

Resum

A les darreres dècades, les entrades de mercuri (Hg) al medi ambient de ma de l'home han augmentat. El Delta de l'Ebre es un àrea important per la producció d'arròs dins la Península Ibèrica. Les activitats industrials que han tingut lloc en el marge del riu i el llegat de contaminació que han suposat aquestes, han fet que els residus contaminats amb mercuri hagin estat transportats fins als ecosistemes deltaics. Els arrossars son ambients propicis per la metil·lació del mercuri, el qual es incorporat a la vegetació i a les xarxes tròfiques locals. En aquest treball hem analitzat els canvis que es donen en la dinàmica del mercuri al llarg del cicle de cultiu de l'arròs en diferents compartiments d'aquest ecosistema com son el sòl, l'aigua, les plantes d'arròs i la fauna. A més a més, hem monitoritzat l'efecte de diferents pràctiques agrícoles (cultiu convencional vs cultiu ecològic) i diferents patrons hídrics (inundació continuada o alternança de períodes d'inundació-sequera) en els nivells de Hg en l'arrossar. Finalment, hem estimat la proporció de metil-mercuri (MeHg) sobre la quantitat de mercuri total en una part de les mostres, ja que el MeHg es la forma més tòxica pels humans i els animals. Els resultats obtinguts indiquen que les diferents pràctiques agrícoles i patrons d'inundació no influencien tant com s'esperava els nivells de HgT en l'aigua, el sòl, les plantes i els organismes de l'arrossar. No obstant això, s'ha trobat una evidència clara de la biomagnificació de mercuri en les xarxes tròfiques d'aquests ecosistemes.

Keywords: Ebro Delta, rice-fields, mercury, methylmercury, *Oryza sativa*

Abstract

During the last few decades, inputs of mercury (Hg) to the environment from anthropogenic sources have increased. The Ebro Delta is an important area of rice production in the Iberian Peninsula. Given the industrial activity and its legacy pollution along the Ebro river, residues containing Hg have been transported throughout the Ebro Delta ecosystems. Rice paddies are regarded as propitious environments for Hg methylation and its subsequent incorporation to plants and rice paddies' food webs. We have analyzed how Hg dynamics change throughout the rice cultivation season in different compartments from the paddies' ecosystems: soil, water, rice plants and fauna. Furthermore, we assessed the effect of different agricultural practices (ecological vs. conventional) associated to various flooding patterns (wet vs. mild alternating wet and dry) to the Hg levels in rice fields. Finally, we have estimated the proportion of methylmercury (MeHg) to total mercury in a subset of samples, as MeHg is the most toxic form for humans and wildlife. We found that different agricultural practices and flooding patterns did not influence as much as expected the THg levels observed in water, soil, plants or fauna. However, we observed clear evidence of Hg biomagnification along the rice fields' food webs.

Keywords: Ebro Delta, rice-fields, mercury, methylmercury, *Oryza sativa*

1. Introduction

Mercury (Hg) is a globally distributed pollutant that has been rising its levels in the environment due to human activities (e.g., artisanal gold mining, industrial waste management, etc.) during the last decades (UNEP 2019). This metal can exert serious negative consequences on the health of both wildlife and humans. Among others, reproductive, neurological, physiological, and teratogenic impairments have been observed on wildlife and humans exposed to Hg (Clarkson and Magos 2006, Díez 2009, Evers 2018).

Mercury is present in all environmental compartments (air, soil, water and biota), and speciation processes during its biogeochemical cycle are highly dependent on the environment physicochemical factors (Ullrich et al. 2001; Driscoll et al. 2013). In the aquatic environment, inorganic mercury (Hg^{2+}) can be methylated by microorganisms at the surface of sediments, mainly sulphate-reducing bacteria (SRB), iron-reducing bacteria (IRB; Podar et al. 2015), and methanogenic archaea (Lázaro et al. 2013), or abiotic processes (Celo et al. 2006) to become methylmercury (MeHg). MeHg is a bioavailable form of mercury and can be bioaccumulated into organisms and biomagnified along food webs (Morel 1998; Gray 2002; Driscoll et al. 2013; Lavoie et al. 2013).

Wetland environments are often linked to high MeHg production rate (Zillioux et al. 1993; Marvin-DiPasquale et al. 2014). Progressively with the worldwide population growth, natural wetlands tend to disappear and are replaced by agricultural farming site such as rice paddies (Elphick et al. 2000; Verhoeven and Setter 2010; Davidson 2014). Although fish and shellfish consumption is considered the main source of mercury for humans (Mergler et al. 2007; McKelvey and Oken 2012; UNEP 2019), some studies suggest that rice consumption might be another route for mercury exposure that should not be neglected (e.g., Feng et al. 2008; Li et al. 2010; Meng et al. 2011; Hong et al. 2016; Zhao et al. 2019; Liu et al. 2019). Indeed, flooded rice paddies are considered as hotspots for mercury methylation, as anaerobic conditions in rice field soils promote Hg methylation by microorganisms (Rothenberg and Feng 2012). This MeHg formed in the rice paddies can impact the rice plants (Meng et al. 2011; Wang et al. 2018) and animals inhabiting these environments (Ackerman and Eagles-Smith 2010; Ackerman et al. 2010, Abeysinghe et al. 2017; Du et al. 2021; Sánchez-Fortún et al. 2021).

Among the factors influencing the bioavailability of mercury in rice paddies, the impact of agricultural techniques used in rice farming (e.g., continuous flooding, alternating wetting-drying periods, use of pesticides) has been evaluated in several studies (e.g.,

Rothenberg and Feng 2012; Rothenberg et al. 2011, 2014; Marvin-DiPasquale et al. 2014; Tang et al. 2018; Tanner et al. 2018). Among them, the alternating wetting and drying (AWD) technique, characterized by a drainage just after seeding followed by periods of intermittent wetting and drying of the field (Bouman and Tuong 2001; Linquist et al. 2014), has been associated with higher MeHg production in agricultural wetlands (Marvin-DiPasquale et al. 2014; Rothenberg et al. 2014). However, other experimental studies (e.g., Rothenberg et al. 2016; Tanner et al. 2018) have observed reductions in MeHg levels in rice plants growth under AWD conditions compared to continuously flooded conditions. This highlights the multifactorial effects of these agricultural techniques on Hg availability and methylation within rice fields (Rothenberg et al. 2016; Tanner et al. 2018; Zhao et al. 2016a; Tang et al. 2020).

Over the last century, the presence of a chlor-alkali plant at the locality of Flix (Tarragona, NE Spain), ca. 100 km upon the Ebro river mouth, has impacted the ecosystems along the river and its delta (Navarro et al. 2009; Suárez-Serrano et al. 2010; Carrasco et al. 2011a, 2011b; Cotín et al. 2012; Palanques et al. 2014; Sánchez-Fortún et al. 2020). During the active period of the plant, around 350 000 tons of toxic waste, containing mainly organochlorine compounds and metals (including Hg), were dumped into the riverbed (Grimalt et al. 2003; ACA 2013). Despite plant operations producing chlorine using mercury ceased in 2017, legacy mercury pollution persists and has been impacting downstream Ebro River basin environments due to the transport of pollutants along the river (Palanques et al. 2020; Sánchez-Fortún et al. 2020).

The Ebro Delta is comprised by 30 000 ha of wetland environments, with almost 75% of its area occupied by rice fields, 20% of its area protected as a natural reserve, and 5% being urbanized (Mañosa et al. 2001). Water from the Ebro river is used to flood continuously or alternatively the rice paddies in the Ebro Delta area (Terrado et al. 2007). Thus, the potential absorption of contaminants from the water-soil system by rice plants in the Ebro delta can lead to higher toxicological risk for wildlife and humans through biomagnification. This is of highest concern in the area, as the Ebro Delta is one of the most important areas in the Iberian Peninsula both as rice farming site and natural reserve (Mañosa et al. 2001).

In this context, the study of mercury dynamics in rice fields provides an estimate mercury budgets in different compartments of the agricultural ecosystems and their interconnectivity. The main purpose of this study is to describe the dynamics of Hg in the rice paddies ecosystems from the Ebro Delta along the rice cultivation season, including the

following compartments: water, soil, rice plants and food webs within rice fields. Additionally, the effect of different agronomic practices used by rice farmers in the Ebro Delta was evaluated regarding the mercury observed in the different environmental compartments considered. Furthermore, mercury speciation analyses to determine the proportion of MeHg were performed for a subset of samples to evaluate the temporal dynamics of MeHg, as a proxy of the bioavailable fraction of mercury in the different compartments of the rice paddies environments.

2. Materials and methods

2.1. Field sampling design

Field work was conducted in the Ebro Delta area (NE Spain, 40°33' N, 00°39' E). During the rice cultivation season of 2017, eight rice fields from the area were randomly selected, which were cultivated by farmers using different agronomical practices, as follows: (i) 4 rice fields using a conventional wet (CW) approach, that is, flooded throughout the cultivation season and using fertilizers and pesticides; (ii) 2 rice fields using an ecological wet (EW) approach, consisting in flooded fields throughout the cultivation season without any usage of fertilizers or pesticides; and (iii) 2 rice fields managed using an ecological dry (ED) approach, where pesticides or fertilizers were not used and the fields were flooded only during half of the rice growing period using a mild alternating wet and dry (AWD) method.

In each of the eight rice fields, we selected 4 sampling points, one in each side of the field, to account for the variability within the field in our measurements. At each sampling point total mercury concentrations ([THg] ng/g dw) were measured for water, soil, rice plants, and fauna inhabiting the rice field (as described below) and the following physicochemical parameters were measured: water pH, salinity (%), conductivity (mS), temperature (°C) and oxygen concentration (mg/l). Furthermore, for animal samples, carbon and nitrogen stable isotopes signatures were measured.

This sampling design was performed three times throughout the rice growing season: (1) in end of June/beginning of July, two months after the rice was seeded; (2) in August, three months after the rice was seeded; and (3) in September, at the end of the rice growing season.

Finally, for a subset of rice plants, fauna samples and soil samples corresponding to the first and third sampling periods (i.e., June/July and September, respectively) from the ecological wet (EW) rice fields, speciation analyses were performed to measure the concentration of MeHg and inorganic mercury in the samples.

2.2. Sample collection and laboratory analyses

2.2.1. Water

For water the determination of the labile fraction of Hg in water, we used the diffusive gradient in thin films (DGT) technique (Turull et al. 2017a; Marrugo-Madrid et al. 2021). DGTs were deployed for 7 days in each sampling point for the first (June/July) and third (September) sampling periods. Upon collection, DGTs were stored at -4°C until analysis. Total mercury (THg) analysis in resin gels were made using a Direct Mercury Analyzer (DMA-80, Milestone Inc.) according to the EPA method 7473 (USEPA 1998), following Marrugo-Madrid et al. (2022).

2.2.2. Soil samples

Soil samples were obtained by extracting a ~5 cm depth core of sediments at each sampling point for each sampling period. Samples were stored at -20°C until analysis. Before the determination of total mercury concentration, samples were homogenized and were oven-dried at 50°C until constant mass. 0.05-0.10 g of soil sample were analyzed for THg concentration as described above.

Mercury speciation analysis was performed in soil samples corresponding to the EW fields on the June/July and August sampling periods to determine the concentration of MeHg in the samples. Mercury species were determined by means of Inductively Coupled Plasma Mass Spectrometry (GC-ICP-MS) after a microwave assisted digestion in HNO₃ 6 M.

2.2.3. Rice plants

For each sampling point at each sampling period, 5-10 rice plants were collected and stored in ziplocked bags at -20°C until analysis. Plants were allowed to thaw before analysis

and were rinsed with distilled water to remove surface contamination. After cleaning, we used stainless-steel scissors to separate each plant in 4 parts: roots, stalk, leaves and grains. For each sampling point and sampling event, each of the plant parts for the 5-10 plants collected was pooled together. They were oven-dried to constant mass at 50°C and then ground to a homogeneous powder with an impactor mill (Freezer Mill 6850, SpexCertiPrepH Inc., Metuchen, NJ, USA) operating at liquid nitrogen temperature. Approximately 0.50 g of powdered plant samples was analyzed for THg concentration as described above.

Rice plant samples from the first and third sampling periods from the EW fields were analyzed for Hg speciation by means of Inductively Coupled Plasma Mass Spectrometry (GC-ICP-MS) dilution after a microwave assisted digestion in HNO₃ 6 M.

2.2.4. Fauna samples

Fauna samples were collected by placing funnel traps for 24 h in each of the sampling points on each sampling period or with aerial nets. Different taxa of invertebrates and small vertebrates were collected (Table 1) and individuals were stored in separate ziplocked bags at -20°C until analysis.

Upon analysis, samples were rinsed with distilled water to remove external contamination. Then, for each of the individuals we recorded the following biometrical measurements: body weight (mg) and total body length (mm). For crayfish (*Procambarus clarkii*) samples, individuals were dissected and hepatopancreas and abdominal muscle tissue were collected for mercury analyses. For each of the dragonfly species collected (*Sympetrum fonscolombii* and *Trithemis annulata*), 2-3 individuals were pooled in each sample to attain sufficient mass for mercury analyses (Table 1). Finally, prior to total mercury determination animal samples were freeze-dried until constant mass and ground to a homogeneous powder using a mortar and pestle or an impactor mill (Freezer Mill 6850, SpexCertiPrepH Inc., Metuchen, NJ, USA). Total mercury analysis was performed as described above.

For carbon and nitrogen stable isotopic analyses, the powdered samples were lipid extracted using 2:1 chloroform:methanol solution following the Folch method (Folch et al. 1957). From crayfish, only abdominal muscle samples were analyzed for carbon and nitrogen stable isotopes. Due to low sample quantities and to avoid sample losses, dragonfly samples were not lipid extracted. Then, 0.30-0.35 mg of powdered samples were placed in tin cups

and analyzed via a Thermo-Finnigan Flash 1112 elemental analyser (CE Elantech, Lakewood, NJ, USA) coupled to a Delta-C isotope ratio mass spectrometer via a CONFLO III interface (Thermo Finnigan MAT, Bremen, Germany). Stable isotope signatures of C and N are expressed using the standard delta notation in ‰ relative to the Vienna Pee Dee Belemnite ($\delta^{13}\text{C}$) and atmospheric N_2 ($\delta^{15}\text{N}$).

Finally, mercury specialization analyses were performed on the subset of samples corresponding to the three sampling periods of EW fields. Mercury species were determined by means of Inductively Coupled Plasma Mass Spectrometry (GC-ICP-MS) after a microwave assisted digestion in HNO_3 6 M, in the case of insect samples, or after a tetramethylammonium hydroxide (TMAH) microwave assisted digestion, in the case of small vertebrates and crayfish samples (Table 1).

2.2.5. Quality control

To assess the accuracy of measurements we analyzed several certified reference materials every 10-15 samples for THg determination and Hg speciation analyses: DORM-4 (fish protein), DOLT-5 (dogfish liver), NIES-13 (human hair), IAEA456 (marine sediment), BCR 414 (zooplankton), ERM CE-464 (tuna fish), GBW 08508 (rice flour), BCR 281 (rye grass). The recoveries ranged from 93-101%, therefore, no corrections were applied to the THg values of the samples prior to statistical analyses (Table S1, Table S2). MeHg concentrations method detection limits for speciation analysis were of 0.1 ng/g dw for sediments, 0.4 ng/g dw for insects, 0.5 for fish, frogs and crayfish, 2.6 ng/g dw for feathers, and 0.1 ng/g dw for plant tissues.

For stable isotope analyses of N and C in fauna samples, international standards (IAEA CH_6 (42‰ of C), IAEA CH_7 (87‰ of C), and USGS 24 (100‰ of C) for ^{13}C and IAEA N_1 and IAEA N_2 (with 21‰ of N) and IAEA NO_3 (13.8‰ of N) for ^{15}N) were applied every 12 samples to calibrate the system. Replicate assays of standards indicated analytical measurement errors of $\pm 0.1\text{‰}$ and $\pm 0.2\text{‰}$ for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, respectively.

2.3. Data treatment and statistical analyses

Data were visually inspected for the presence of outliers and normality. Total mercury concentrations and MeHg concentrations were log-transformed to approximate the values to a normal distribution. All analyses were performed using R v.4.0.3 (R Core Team

2020). Statistically significant threshold was set at $\alpha = 0.05$. Model fits were assessed by visual inspection of residual analyses.

2.3.1. THg concentrations in water, soil and rice plants

For water and soil compartments, linear mixed effects models were fit using the *lme4* package (Bates et al. 2015) to evaluate the effect of agricultural management (CW, EW, ED) and sampling period (June/July, August, September for the soil model, and June/July, September for the water model) as fixed effects on the log THg concentrations determined. The interaction between agricultural management and sampling period was included as fixed effect. Rice field ID was included as a random effect.

For the rice plant samples, linear mixed effects models were fit to evaluate the effect of agricultural management (CW, EW, ED), sampling period (June/July, August, September), plant part (roots, stalk, leaves and grain) on the log THg concentrations. Furthermore, the interaction between plant part and sampling period was included as fixed effect. Rice field ID was included as a random effect.

Pearson correlation tests were performed to evaluate the relationship between THg concentrations in soil and each of the plant parts, including all the periods sampled considering all the agricultural types together.

2.3.2. THg concentrations and stable isotopes of C and N in fauna samples

Due to low sample sizes of collected individuals from each of the rice field types (CW, EW and ED) in each sampling period (Table 1), we pooled the data from the three sampling events for each agricultural management type.

For each of the species collected, differences in $\delta^{13}\text{C}$, $\delta^{15}\text{N}$ and THg concentration among agricultural types was tested using ANOVA with agricultural type as factor, if species were present on the three rice field types. Post-hoc significant differences were further evaluated by using Tukey's honest significant test. If only present in two rice field types, t-tests were used to evaluate differences in $\delta^{13}\text{C}$, $\delta^{15}\text{N}$ and THg among agricultural management types. For these tests, we used only observations with $n > 1$ (Table 1).

Using the $\delta^{15}\text{N}$ values we estimated the mean $\pm$ SD trophic level (TL) for each species sampled according to the following equation (Post et al. 2002):

$$\text{TL}_{\text{consumer}} = \left(\frac{\delta^{15}\text{N}_{\text{consumer}} - \delta^{15}\text{N}_{\text{base}}}{\Delta^{15}\text{N}} \right) + \text{TL}_{\text{base}}$$

where, $\delta^{15}\text{N}_{\text{consumer}}$ is the $\delta^{15}\text{N}$ value for the consumer, $\delta^{15}\text{N}_{\text{base}}$ and TL_{base} represent the $\delta^{15}\text{N}$ value and the trophic level of the organism considered to set the baseline, respectively, and $\Delta^{15}\text{N}$ is the trophic discrimination factor. As baseline organism we selected the coleopteran *Hydrophilus pistaceus*, which feeds on aquatic plants. Thus, $\text{TL}_{\text{base}} = 2.0$, as it is a primary consumer (Post et al. 2002), and $\delta^{15}\text{N}_{\text{base}}$ is the mean value of $\delta^{15}\text{N}$ for *H. pistaceus* in each of the agricultural management types (Table 1). $\Delta^{15}\text{N}$ was set at 3.4, for aquatic food webs (Vander Zanden and Rasmussen 2001).

Log THg concentrations and trophic levels estimated from $\delta^{15}\text{N}$ were used to estimate the trophic magnification factor (TMF) following Borgå et al. (2012). Briefly, log THg was regressed against TL obtaining the parameters for the intercept (a) and slope (b) in the equation $\text{Log} [\text{THg}] = a + b\text{TL}$. Then, the TMF indicating the rate of increase in concentration of THg with trophic position was calculated as $\text{TMF} = 10^b$, where b is derived from the previous linear regression. $\text{TMF} > 1$ indicates magnification of the pollutant along the food web considered (Borgå et al. 2012; Conder et al. 2012).

2.3.3. MeHg proportions in soil, plants and fauna

Mercury speciation analyses for samples in EW fields on the first and third sampling events were used to calculate the proportion of MeHg in samples of soil plants and fauna relative to THg. A descriptive summary of the results is shown in Table 2. Differences in MeHg concentration among the first and second sampling periods for soil samples were evaluated with t-tests.

For rice plant samples, differences in MeHg to THg proportion were evaluated in the third period for all the plant parts (roots, stalk, leaves, grain) using ANOVA with plant part as factor, since grain samples were only available for the third period. Additionally, a linear model was fitted to evaluate the effect of the plant part, sampling event and their interaction on MeHg using only data for roots, leaves and stalks.

MeHg concentrations in fauna from EW rice fields were used to estimate the trophic magnification factor (TFM) for this food web, as described before.

3. Results

3.1. THg concentrations in water, soil and rice plants

Concentrations in the labile fraction of Hg in water were evaluated for a total of $N = 56$ DGTs, which included 32 samples for the CW fields, 16 samples for the EW fields and 8 samples for the ED fields. In the ED fields, we could not deploy the 8 DGTs corresponding to the third sampling period (September) as the field was already dried out prior to harvesting (Table 3).

For soil samples, a total of $N = 96$ soil cores were collected and analyzed for THg concentrations, corresponding to samples for each sampling point for each of the first, second and third sampling events (Table 3).

Analogously, 96 rice plant samples (5-10 plants per sample) were collected, separated in parts, and analyzed for THg, corresponding to each sampling point for the first, second and third sampling events. Since rice plants in the first sampling event (June/July) were in the vegetative phase and only for the second (August) and third (September) sampling events, plants presented the panicle, THg concentration in rice grains was only analyzed on the last two sampling events (Table 3).

For THg concentrations in water by DGT, a significant effect of agricultural types considered for the first sampling event was found ($F_{2,29} = 11.01$, $p = 0.0003$). Post-hoc pairwise comparisons showed significant differences between CW and EW, and CW and ED, but no differences between EW and ED (CW-EW: $p = 0.027$, CW-ED: $p = 0.028$, EW-ED: $p = 1.00$; Fig. 1a). Regarding the third sampling period no differences in water Hg levels between CW and EW fields were detected ($F_{1,21} = 0.87$, $p = 0.36$). Significant differences were observed in mercury concentration in water between the first and the third sampling period in CW ($F_{1,29} = 74.29$, $p < 0.0001$). However, although an increase in THg levels was observed in EW fields from the first to the third sampling periods, no significant differences were detected ($F_{1,13} = 2.23$, $p = 0.16$; Fig. 1a).

Table 1 | Sample size (N) and summary statistics (mean $\pm$ SD) for each of the parameters ($\delta^{15}\text{N}$, $\delta^{13}\text{C}$, [THg]) in each of the species collected during the 2017 rice cultivation season. Data was pooled together for the three sampling events and for each of the agricultural management types. Sample sizes refer to individuals collected and analyzed except for *S. fonscolombii* and *T. annulata*, that refer to pools of 2-3 individuals (see text for details). Statistically significant differences for each species and each parameter among field types ($p < 0.05$) in means from samples with $n > 1$ were evaluated.

Species	N	Conventional Wet (CW)				N	Ecological Wet (EW)				N	Ecological Dry (ED)			
		THg (ng/g dw)	$\delta^{13}\text{C}$ (‰)	$\delta^{15}\text{N}$ (‰)	TL		THg (ng/g dw)	$\delta^{13}\text{C}$ (‰)	$\delta^{15}\text{N}$ (‰)	TL		THg (ng/g dw)	$\delta^{13}\text{C}$ (‰)	$\delta^{15}\text{N}$ (‰)	TL
<i>C. carassius</i>	1	286.8 $\pm$ 0.0	-25.7 $\pm$ 0.0	13.7 $\pm$ 0.0	3.5 $\pm$ 0.0	2	287.4 $\pm$ 61.3	-26.7 $\pm$ 0.0	12.5 $\pm$ 0.0	3.4 $\pm$ 0.0	0	-	-	-	-
<i>C. lateralimarginalis</i>	0	-	-	-	-	2	220.8 $\pm$ 29.8	-28.2 $\pm$ 2.1	11.7 $\pm$ 0.6	3.1 $\pm$ 0.2	0	-	-	-	-
<i>E. sticticus</i>	1	447.4 $\pm$ 0.0	-	-	-	0	-	-	-	-	0	-	-	-	-
<i>H. pistaceus</i>	4	175.4 $\pm$ 101.7	-29.9 $\pm$ 1.7	8.5 $\pm$ 0.7	2.0 $\pm$ 0.2	3	173.4 $\pm$ 49.4	-31.0 $\pm$ 2.7	7.9 $\pm$ 0.7	2.0 $\pm$ 0.2	1	145.6 $\pm$ 0.0	-29.2 $\pm$ 0.0	5.9 $\pm$ 0.0	2.0 $\pm$ 0.0
<i>M. anguillicaudatus</i>	6	149.0 $\pm$ 17.5	-26.3 $\pm$ 0.8	11.6 $\pm$ 0.7	2.9 $\pm$ 0.2	0	-	-	-	-	0	-	-	-	-
<i>P. clarkii</i>	104	256.9 $\pm$ 129.6 ^a	-28.5 $\pm$ 2.8	10.4 $\pm$ 1.5	2.6 $\pm$ 0.5	20	333.6 $\pm$ 85.4 ^b	-27.7 $\pm$ 1.4	11.2 $\pm$ 1.8	3.0 $\pm$ 0.5	0	-	-	-	-
<i>P. parva</i>	0	-	-	-	-	0	-	-	-	-	1	398.8 $\pm$ 0.0	-23.5 $\pm$ 0.0	13.3 $\pm$ 0.0	4.2 $\pm$ 0.0
<i>R. perezii</i>	13	346.1 $\pm$ 68.2	-23.2 $\pm$ 1.5	11.1 $\pm$ 0.7	2.8 $\pm$ 0.2	12	302.3 $\pm$ 90.7	-23.5 $\pm$ 1.8	10.7 $\pm$ 2.4	2.8 $\pm$ 0.7	5	395.7 $\pm$ 92.3	-23.5 $\pm$ 1.3	9.5 $\pm$ 2.3	3.1 $\pm$ 0.7
<i>S. fonscolombii</i>	5	323.3 $\pm$ 52.3	-27.5 $\pm$ 1.7	9.6 $\pm$ 2.1	2.3 $\pm$ 0.6	3	327.1 $\pm$ 52.0	-27.3 $\pm$ 0.8	9.3 $\pm$ 1.7	2.4 $\pm$ 0.5	1	433.5 $\pm$ 0.0	-26.0 $\pm$ 0.0	6.3 $\pm$ 0.0	2.1 $\pm$ 0.0
<i>T. annulata</i>	0	-	-	-	-	2	350.7 $\pm$ 75.3	-27.3 $\pm$ 1.3	10.8 $\pm$ 1.1	2.8 $\pm$ 0.3	2	372.2 $\pm$ 33.6	-22.9 $\pm$ 3.5	10.2 $\pm$ 1.8	3.3 $\pm$ 0.5

^{a, b} Statistically significant differences ($p < 0.05$).

Table 2 | Sample size (N) and summary statistics (mean $\pm$ SD) for each of the parameters ([THg], [MeHg] and MeHg/THg) in soil, plant parts and fauna samples from ecological wet rice fields. For soil and rice plant samples, data are shown for each sample period. For fauna samples data were pooled among the three sample periods (see text for details). For fauna samples, N is the number of individuals analyzed, except for *S. fonscolombii*, where N is the number of pools (2-3 individuals) analyzed.

Sample type	Species	Sample Period	N	THg (ng/g dw)	MeHg (ng/g dw)	MeHg/THg (%)
Soil		1	8	157.86 $\pm$ 49.83	1.25 $\pm$ 0.019	0.84 $\pm$ 0.26
Soil		2	8	156.19 $\pm$ 42.82	0.99 $\pm$ 0.011	0.65 $\pm$ 0.30
Rice plants – roots	<i>O. sativa</i>	1	8	46.04 $\pm$ 16.94	6.38 $\pm$ 5.31	16.67 $\pm$ 9.79
Rice plants – stalk	<i>O. sativa</i>	1	5	32.10 $\pm$ 7.35	14.90 $\pm$ 0.23	46.38 $\pm$ 15.36
Rice plants – leaves	<i>O. sativa</i>	1	8	36.97 $\pm$ 11.07	10.48 $\pm$ 0.35	28.30 $\pm$ 7.50
Rice plants – roots	<i>O. sativa</i>	3	8	70.38 $\pm$ 21.26	3.84 $\pm$ 0.26	5.71 $\pm$ 3.97
Rice plants – stalk	<i>O. sativa</i>	3	7	3.57 $\pm$ 1.08	0.81 $\pm$ 0.50	24.60 $\pm$ 13.98
Rice plants – leaves	<i>O. sativa</i>	3	8	10.76 $\pm$ 4.45	0.69 $\pm$ 0.011	8.36 $\pm$ 6.47
Rice plants – grain	<i>O. sativa</i>	3	8	2.90 $\pm$ 0.57	1.89 $\pm$ 0.050	65.57 $\pm$ 17.33
Fauna	<i>C. carassius</i>		1	244.04 $\pm$ 0.0	181.91 $\pm$ 0.0	74.54 $\pm$ 0.0
Fauna	<i>H. pistaceus</i>		2	144.88 $\pm$ 2.67	60.60 $\pm$ 1.41	41.74 $\pm$ 9.54
Fauna	<i>P. clarkii</i>		9	368.21 $\pm$ 117.42	357.65 $\pm$ 7.65	97.52 $\pm$ 9.91
Fauna	<i>R. perezii</i>		5	220.02 $\pm$ 75.12	122.70 $\pm$ 2.04	57.61 $\pm$ 14.86
Fauna	<i>S. fonscolombii</i>		2	325.18 $\pm$ 61.95	254.22 $\pm$ 4.25	78.37 $\pm$ 12.47

Table 3 | Sample size (N) and summary statistics (mean $\pm$ SE) for THg concentration (ng/g dw) measurements for each of the sample types (soil, water (DGTs) and rice plant parts), agricultural management type, and sampling period obtained during the 2017 rice cultivation season.

Sample type	Sampling period	Conventional Wet (CW)		Ecological Wet (EW)		Ecological Dry (ED)	
		N	THg (ng/g dw)	N	THg (ng/g dw)	N	THg (ng/g dw)
Soil	1	16	224.94 $\pm$ 41.04	8	157.86 $\pm$ 17.62	8	204.38 $\pm$ 47.17
Soil	2	16	219.29 $\pm$ 18.95	8	156.19 $\pm$ 15.14	8	208.58 $\pm$ 43.13
Soil	3	16	205.79 $\pm$ 19.82	8	147.01 $\pm$ 16.21	8	222.59 $\pm$ 38.59
Water (DGT)	1	16	24.33 $\pm$ 3.66	8	79.41 $\pm$ 23.32	8	85.22 $\pm$ 31.96
Water (DGT)	3	16	162.13 $\pm$ 22.76	8	137.45 $\pm$ 36.69	-	-
Rice plants – roots	1	16	71.45 $\pm$ 9.36	8	46.04 $\pm$ 5.99	8	67.89 $\pm$ 21.45
Rice plants – stalk	1	16	42.96 $\pm$ 6.25	8	36.66 $\pm$ 3.08	8	18.19 $\pm$ 3.85
Rice plants – leaves	1	16	44.87 $\pm$ 9.31	8	36.97 $\pm$ 3.91	8	21.29 $\pm$ 3.43
Rice plants – roots	2	16	47.67 $\pm$ 5.14	8	41.37 $\pm$ 6.30	8	78.23 $\pm$ 14.71
Rice plants – stalk	2	16	9.95 $\pm$ 2.15	8	9.43 $\pm$ 1.50	8	9.19 $\pm$ 1.55
Rice plants – leaves	2	16	15.41 $\pm$ 1.25	8	11.50 $\pm$ 1.02	8	18.87 $\pm$ 2.03
Rice plants – grain	2	16	11.94 $\pm$ 2.35	1	10.79 $\pm$ 0.00	8	12.09 $\pm$ 2.57
Rice plants – roots	3	16	110.08 $\pm$ 8.74	8	70.38 $\pm$ 7.52	8	105.17 $\pm$ 20.57
Rice plants – stalk	3	16	2.66 $\pm$ 0.49	8	3.21 $\pm$ 0.50	8	1.47 $\pm$ 0.34
Rice plants – leaves	3	16	14.76 $\pm$ 0.86	8	10.77 $\pm$ 1.57	8	14.02 $\pm$ 1.02
Rice plants – grain	3	16	3.77 $\pm$ 0.35	8	2.90 $\pm$ 0.20	8	3.94 $\pm$ 0.70

The linear mixed model for the soil samples did not show any significant differences in THg levels among agricultural types ($F_{2,5} = 0.16$, $p = 0.85$) or sampling periods ($F_{2,81} = 1.09$, $p = 0.34$). The interaction term was not significant ($F_{4,81} = 0.52$, $p = 0.72$; Fig. 1b).

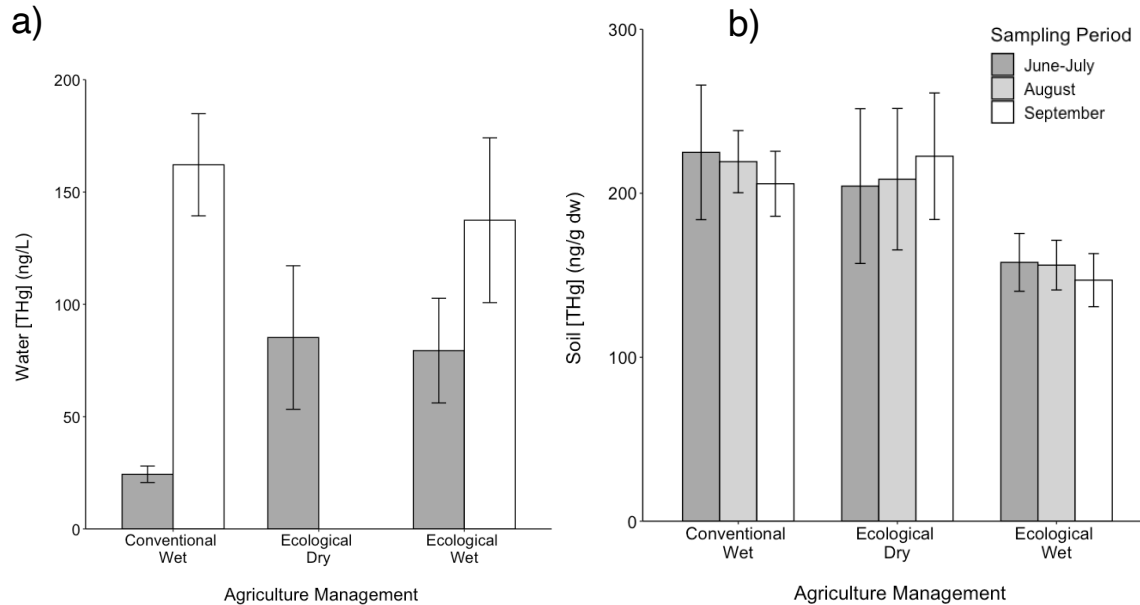


Figure 1 | (a) Water THg concentrations (ng/L) obtained by DGT technique on three sampling periods (June/July, August, September), and (b) soil THg concentrations (ng/g dw) from cores obtained on three sampling periods (June/July, August, September) during the 2017 rice cultivation season from rice fields farmed using three different agricultural management types (Conventional Wet, Ecological Wet, Ecological Dry). Error bars depict the SE of the mean. Significant differences in water THg levels were detected among CW-ED and CW-EW for the first sampling period (June/July) and between the first and third sampling periods in both CW and EW rice fields. Soil THg levels did not differ significantly among agricultural management types or sampling periods.

Regarding the rice plant samples, we found significant differences in THg levels among plant parts ($F_{3,311} = 166.20$, $p < 0.0001$). Pairwise comparisons showed significant differences among all the plant parts pairs (in all cases, $p < 0.0001$), except between stalk and grain ($p = 1.00$), with THg concentrations in roots $>$ leaves $>$ stalk $\approx$ grain (Fig. 2). The model including only the data from roots, leaves and stalk samples for the three sampling periods showed a significant interaction between the sampling period and the plant parts ($F_{4,240} = 56.84$, $p < 0.0001$). Models for each of the plant parts, showed a significant increase in THg concentration in roots over time ($F_{2,78} = 16.41$, $p < 0.0001$), while THg levels decreased in stalks and leaves (stalks: $F_{2,78} = 108.37$, $p < 0.0001$; leaves: $F_{2,74} = 42.80$, $p < 0.0001$). No significant differences in THg levels for each plant part were found among agricultural types (in all cases, $p > 0.10$). Regarding grain samples a significant decrease in THg concentration was observed between the second and third sampling events ($F_{1,45} =$

70.95, $p < 0.0001$), and no significant differences in rice grain THg levels were detected among agricultural management types ($F_{2,5} = 0.010$, $p = 0.99$).

Pearson correlation tests between soil and plant parts THg concentrations showed significant positive correlations between soil and roots, and soil and rice THg levels (soil-roots: $r = 0.47$, $p < 0.0001$; soil-grain: $r = 0.37$, $p < 0.0001$), but not between soil and stalk or leaves THg concentrations (soil-stalk: $r = 0.099$, $p = 0.35$; soil-leaves: $r = 0.16$, $p = 0.13$; Fig. S1).

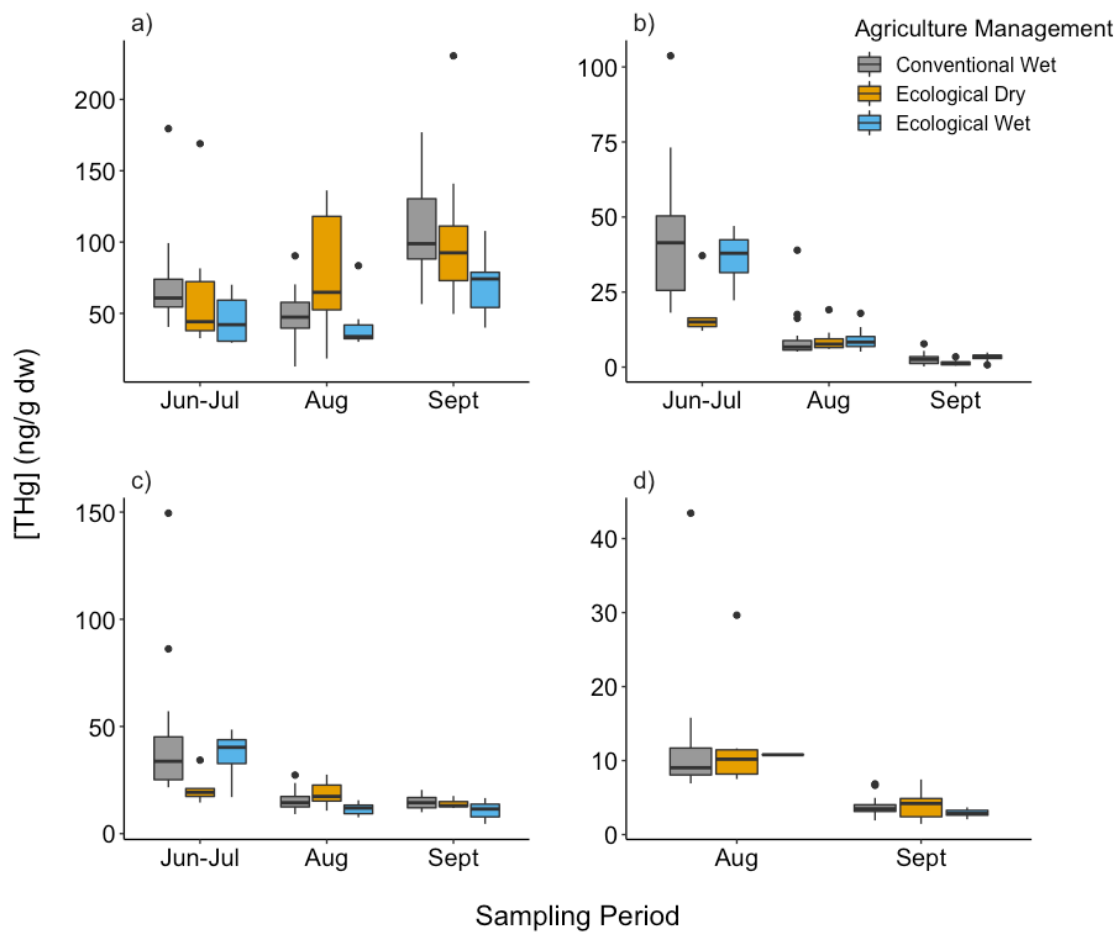


Figure 2 | THg concentrations (ng/g dw) in rice plant parts: (a) roots, (b) stalk, (c) leaves, (d) grain, in three sampling periods over the rice cultivation season from 2017 in the Ebro Delta. Samples from fields farmed using different agricultural management practices (Conventional Wet, Ecological Wet, Ecological Dry) were analyzed.

3.2. THg concentrations and stable isotopes of C and N in fauna samples

A total of $N = 204$ fauna samples corresponding to 10 different species were collected among all the rice fields sampled. Since low samples sizes were obtained, for the statistical

analyses, samples from the three sampling periods were analyzed together for each agricultural type. A summary of the species collected, their trophic ecology, THg levels, $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values, and estimated TL, for each agricultural management type is shown in Table 1. Significant differences in THg, $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ are indicated in Table 1.

For the three types of agricultural management practices (CW, EW, ED) trophic magnification factors considering the species collected in each field type was $\text{TFM} > 1$ (CW: 1.25; ED: 1.53; EW: 1.45), indicating an effective biomagnification of Hg along food webs in each agricultural management type (Fig. 3).

3.3. MeHg proportions in soil, plants and fauna from EW fields

Methylmercury concentrations and proportions were determined for a subset of 16 soil samples and 16 plant samples from the first and third sampling period from EW fields, and 22 fauna samples from the EW fields. From the plant samples, we could not perform speciation analyses on $n=3$ and $n=1$ stalk plant tissue, from the first and third sampling periods, respectively, due to low sample quantity. Results are shown in Table 2.

MeHg to THg proportions in soil samples ranged from 0.37–1.33%. T-test showed no significant differences between first and second sampling period in MeHg to THg proportions on soils ($t = 1.40$, $p = 0.18$).

In rice plant samples, we detected significant differences in MeHg proportions among plant parts on the third period of sampling ($F_{3,27} = 44.82$, $p < 0.0001$). Post-hoc comparisons between plant parts showed differences among MeHg proportions between grain and the other parts of the plant, and between stalk and roots (roots-grains: $p < 0.0001$; roots-leaves: $p = 0.97$; roots-stalk: $p = 0.021$; leaves-grains: $p < 0.0001$; leaves-stalk: $p = 0.06$; stalk-grains: $p < 0.0001$), with grain accumulating higher MeHg proportions compared to the other parts (Table 2). The linear model including only data for roots, leaves and stalks for the first and third period, detected significant differences in MeHg to THg proportions among plant parts ($F_{2,38} = 18.71$, $p < 0.0001$) and between sampling events ($F_{1,38} = 34.00$, $p < 0.0001$). The interaction between plant parts $\times$ sampling period was not significant ($F_{2,38} = 1.31$, $p = 0.28$).

For the rice-fields fauna samples, the proportion of MeHg to THg ranged from 34.99–111.78%, depending on the species (Table 2). MeHg concentrations differed

significantly among species ($F_{4,17} = 17.94$, $p < 0.0001$) but, although positive, the correlation between MeHg concentration and TL was not significant ($r = 0.30$, $p = 0.16$), possibly due to a low statistical power. The TMF for MeHg was of 1.94.

3.4. *Physicochemical parameters of water*

Results of the physicochemical measurements are reported in Table S3.

4. Discussion

The presence of a chlor-alkali industry along the Ebro river has been considered as an important source of Hg in water, soil and biota (Navarro et al. 2009; Carrasco et al. 2011a, 2011b; Cotín et al. 2011, 2012; Sánchez-Fortún et al. 2020). Rice fields environments have been extensively studied as mercury hotspots (Zhao et al. 2020; Tang et al. 2020), since environmental conditions are favorable for microbial growth and Hg^{2+} methylation (Rothenberg et al. 2014; Marvin-DiPasquale 2014), and thus, transference to rice plants (Meng et al. 2011, 2014; Rothenberg and Feng 2012; Wang et al. 2018) and biota inhabiting the rice fields (Ackerman and Eagles-Smith 2010; Ackerman et al. 2010; Sánchez-Fortún et al. 2021; Du et al. 2021). Particularly, the distribution of Hg in rice plants has been of interest since its ingestion might pose one of the main entrance pathways of mercury for humans, especially in areas where rice is a staple-food (Rothenberg et al. 2014; Liu et al. 2019).

Moreover, agricultural management of the fields might vary the physicochemical properties of the rice field environment and thus affect mercury bioavailability and transformation (Ullrich et al. 2001; Tang et al. 2018; 2020), and ultimately its accumulation in plants and fauna (Ackerman et al. 2010; Tanner et al. 2018, Sánchez-Fortún et al. 2021). Among factors influencing Hg bioavailability in rice fields environment, hydroperiod has been studied extensively (e.g., Marvin-DiPasquale et al. 2014; Rothenberg et al. 2014, 2016; Tanner et al. 2018). In the present study we have evaluated how different agricultural management techniques regarding the usage of pesticides (conventional vs. ecological) and the hydroperiod (wet vs. mild AWD) influence the Hg availability for different compartments in the rice fields environments, namely soil, water, rice plants and fauna, and how it varies along the rice growing season.

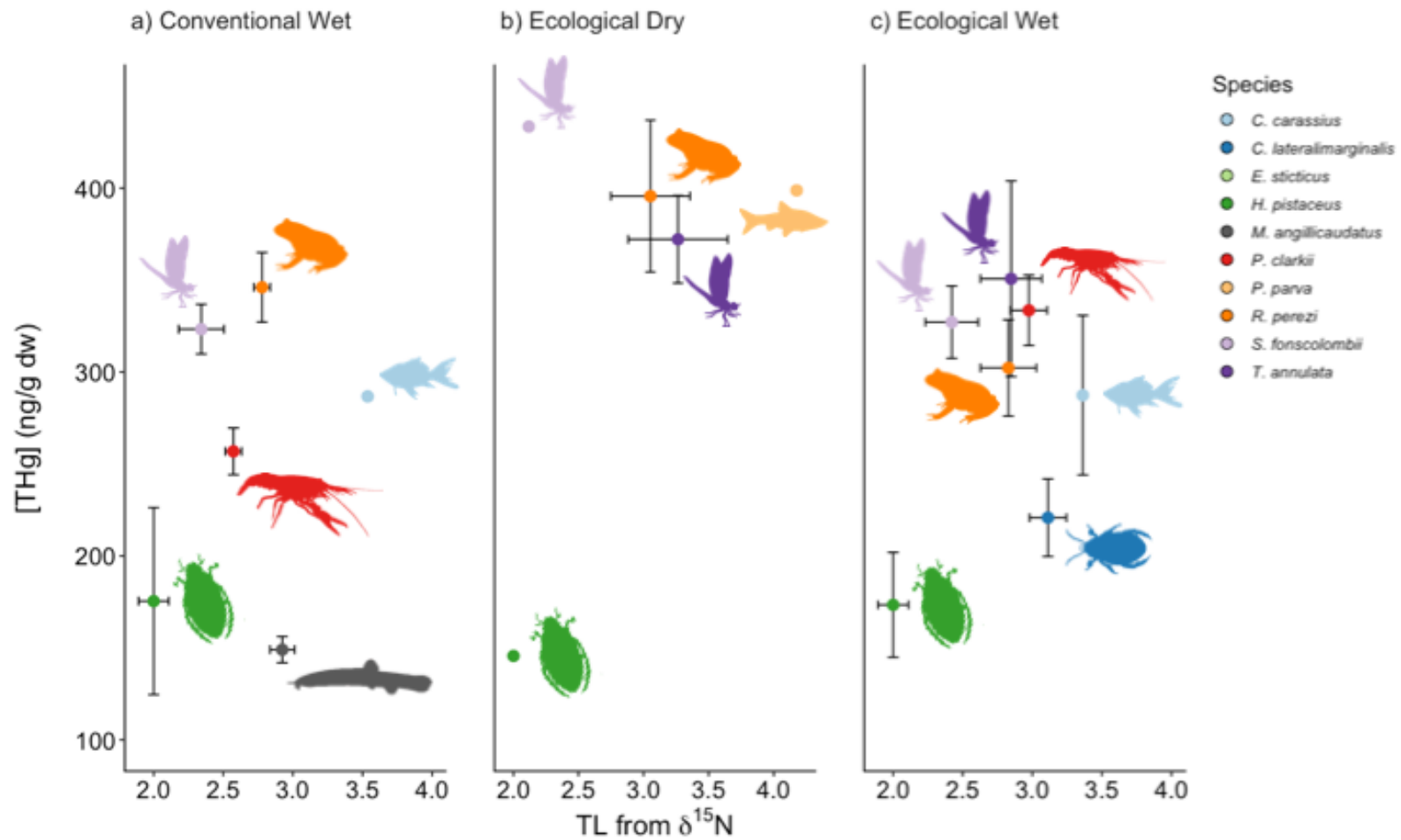


Figure 3 | THg concentrations vs. trophic level (TL) in fauna samples for the three types of agricultural management fields: (a) Conventional wet, (b) Ecological Dry and (c) Ecological wet.. Trophic magnification factors (TMF) for THg were >1 in all three rice field types indicating an effective biomagnification of mercury along the foodweb.

4.1. THg concentrations in water, soil and rice plants

Compared to the other environmental compartments studied, low Hg concentrations in rice fields' water were detected in all the three agricultural types studied, which might be introduced in the rice paddies through irrigation. Indeed, rice fields in the area are irrigated using the water from the Ebro river, which transports significant amounts of Hg due to the legacy mercury pollution from industries along the river (Carrasco et al. 2008; Terrado et al. 2006; Palanques et al. 2020).

Differences among rice field types in THg concentration in water from the first sampling event were detected between conventional and ecological fields, despite the hydroperiod conditions applied to the ecological fields, with higher Hg concentrations in water from EW or ED than CW fields. Nonetheless, these differences might arise from the distance among the rice fields studied, rather than a particular influence of the usage of pesticides and fertilizers.

The Hg concentration in rice paddies water increased for both CW and EW rice fields from the first sampling period to the third. Thus, it might indicate the continuous load of Hg into the rice fields coming from the water sourced to irrigate the fields (Terrado et al. 2006; Palanques et al. 2020). Unfortunately, in ED we could not evaluate the difference in Hg concentration in water between the first and third sampling periods, although we would expect a similar trend in THg concentration over time.

Soil samples Hg concentration did not differ among agricultural types nor among the three sampling periods (Fig. 1b; Table 2). Other studies have found similar results (e.g., Rothenberg and Feng 2012, Zhao et al. 2016b), with THg levels in soil maintained throughout the rice cultivation season. Nonetheless, although THg concentration is maintained throughout the rice growing season, the fraction of Hg bound to organic-matter in paddy soils might change with rice plant growth, making it readily available for methylation and transference to rice plants (Zhou et al. 2015). Based on our results, the application of fertilizers and pesticides does not seem to influence THg concentrations in soil samples (but see Tang et al. 2018, 2019), and both continuous flooding and AWD techniques do not seem to have a great impact on soil THg concentrations (Rothenberg et al. 2016). Thus, although mercury apportionment in the rice fields are continuous through water irrigation (Dai et al. 2013), soil mercury concentrations might depend on different factors such as other sources as atmospheric deposition (Rodríguez et al. 2008), methylation rates

(Zhou et al. 2015; Zhao et al. 2016a; Liu et al. 2019) and physicochemical parameters from the soil (Ullrich et al. 2001; Rothenberg and Feng 2012), and their combination do maintain soil THg levels throughout the rice cultivation season.

Regarding THg concentrations in rice plants (Fig. 2), our results show differences among different plant parts, which reflects the differential capacity of plant tissues to accumulate this metal (Meng et al. 2010; Zhang et al. 2010; Tang et al. 2020; Liu et al. 2021), with roots presenting higher THg concentrations than the aerial parts of the plant (roots > leaves > stalk $\approx$ grain). Nonetheless, THg concentrations in plant parts showed an increase for roots and a decreasing trend in stalk, roots and grains over the rice cultivation season. Decrease in THg concentrations in grain, stalk and leaves is probably due to its dilution because of the increase in biomass with growth (Shu et al. 2016). However, the increase in THg concentration observed in roots might be related to the capacity of roots to sequester inorganic Hg using phytochelatins in the cytosol of root cells and transporting it into the vacuoles (Krupp et al. 2009; Tang et al. 2020); thus, the increase in THg in roots is mainly due to an increase in inorganic Hg forms rather than MeHg.

Furthermore, our results are in accordance with other similar studies (Meng et al., 2010, 2011, 2014; Zhang et al. 2010; Hang et al., 2017) in which higher concentrations in roots and grain were related to Hg levels in soil. In this study, THg levels in soil samples were correlated with those of roots and grain, indicating that soil Hg might be the main Hg source for these two rice tissues (Meng et al. 2010). However, soil THg concentrations did not correlate with THg in leaves or stalk. In this case, field studies in Hg-polluted sites (Meng et al. 2010, 2011, 2012) have also confirmed that aerial parts (leaves and stalks) from rice plants, can accumulate Hg from the air (Marrugo-Negrete et al. 2016). Furthermore, experimental studies using Hg stable isotopes have found that leaves tend to accumulate mercury from the air through the stomata (Tang et al. 2021). Thus, the uncoupling between THg concentrations in leaves and stalk, and soil samples found in our study might be due to the incorporation of Hg from the air on rice plant tissues.

We did not find any significant differences on THg accumulation by each plant part or their trend on THg concentrations over the rice plant growth period among the different agricultural practices studied, implying that neither hydroperiod nor the application of fertilizers and pesticides had an influence on THg levels in the different plant parts. Regarding hydroperiod, Tanner et al. (2018) have reported similar results, with AWD growth rice plants presenting THg concentrations similar to those growth in continuously flooded conditions, but when considering MeHg concentrations they were lower in plants

from AWD treatments. In addition, Rothenberg et al. (2016) reported both decreases in THg and MeHg accumulation by rice plants under AWD conditions compared to continuously flooded conditions, although declined concentrations were dependent on the severity of AWD treatment employed. Also, application of fertilizers and pesticides does not seem to influence THg accumulation in rice plants, although we have a limited sample size and exactly applications were unknown. Moreover, a recent review by Tang et al. (2019) points out that some fertilizers might affect MeHg production in rice paddies soils and thus the overall levels of MeHg in plants. Unfortunately, we did not measure MeHg concentrations for all the agricultural management practices, and thus, we could not evaluate whether in our study MeHg concentrations in rice plants was affected by the alternating wetting and drying management in comparison to continuously flooded conditions or by ecological vs. conventional treatments.

4.2. MeHg concentrations in soil and rice plants in EW fields

Regarding MeHg concentrations in soil from ecological wet fields, they did not differ between the first and second sampling periods, which contrast with other studies that found an increase in MeHg concentration in soil samples over rice growth (Zhou et al. 2015). However, our data does not range the entire rice plants growth, and Zhou et al. (2015) observed the major increase in MeHg in paddy soil samples during the heading stage of the rice plants, which were not attained during the second sampling we performed.

When observing how MeHg proportions were distributed within the plant from ecological wet fields, the grain presented a higher MeHg to THg proportion than the other plant parts, followed by leaves and stalk and finally, roots. Conversely, roots did accumulate mainly inorganic mercury (Table 1). It has been suggested that root uptake of heavy metal by plant from the soil could be controlled by phytochelatins. These peptides composed of cysteines are chelating agents that would serve to sequester inorganic Hg and keep it stored in vacuoles (Meng et al. 2010, 2014; Zhang et al. 2010; Rothenberg et al. 2014; Turull et al. 2017b). Thus, roots can act as a barrier in the translocation of Hg^{2+} to aerial parts but not for MeHg (Meng et al. 2010, 2011; Zhang et al., 2010), that could explain why MeHg is mainly found in grain in several studies (Meng *et al.*, 2010, 2011, 2014; Zhang *et al.*, 2010; Rothenberg et al. 2011), including the present study. Comparing the first and third sampling period, in roots, leaves and stalk, we observe a decrease in MeHg concentrations significant only for leaves and stalk but not for roots. Again, this trend might be due to a biodilution of the metal with an increase in biomass over plant growth (Shu et al. 2016), and in the case of

roots, although not statistically significant, a selective transport of MeHg to the aerial parts and demethylation of MeHg in the roots have been described (Xu et al. 2016; Strickman and Mitchell 2017; Liu et al. 2021), which might be responsible for the decreased MeHg concentration observed in our study over time.

4.3. THg, MeHg concentrations, carbon and nitrogen isotopes in fauna samples

Fauna inhabiting the rice paddies did show significant THg concentrations, regardless the agricultural management practices employed in the rice fields sampled. In general, among different agronomic practices, fauna in ED fields did show THg concentrations higher than EW or CW fields, although not significant differences were detected, possibly due to low statistical power (Table 1). In this regard, Sánchez-Fortún et al. (2021) in a mesocosmos experiment in the same rice fields did found that common goldfish (*Carassius auratus*) exposed to ED fields also tended to accumulate higher THg concentrations than in EW or CW fields.

Thus, hydroperiod seems to have an influence on Hg methylation, bioavailability and accumulation in fauna inhabiting rice fields (Ackerman and Eagles-Smith 2010; Ackerman et al. 2010; Windham-Myers et al. 2014; Sánchez-Fortún et al. 2021). Wetting-drying cycles might enhance MeHg production by transforming Hg to forms that are more easily methylated and changing redox conditions favoring Hg methylation (Marvin-DiPasquale et al. 2014), enhancing bacterial methylation and producing pulses of MeHg after re-flooding (Rothenberg and Feng 2012; Zhao et al. 2016; Tanner et al. 2018). Thus, since fauna mainly accumulates MeHg (Evers 2018; Table 1) we cannot rule out the possibility that higher THg concentrations in fauna from ED fields is due to higher MeHg levels in the environment and in the food web. However, in the present study we only evaluated MeHg levels in soils and fauna for EW rice fields, and thus we cannot firmly conclude whether in our samples MeHg in soils and fauna were higher for ED fields than for those continuously flooded (EW and CW). Moreover, since Hg in fauna is acquired mostly through diet, differences in Hg concentrations in fauna from different fields managed using different agricultural practices could have been influenced by the food sources available in a particular field. Therefore, we determined $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values to evaluate differences in diet resources for each species. Overall, similar $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ were found for the same species among field types (Table 1), nonetheless, limited samples sizes resulted in a low statistical power of the tests used to assess the differences among field types.

Finally, we evaluated the transfer of Hg through the rice field food webs, by sampling organisms from different trophic levels, as estimated from $\delta^{15}\text{N}$ signatures (Post et al. 2002). Regardless the agricultural management applied to the fields (CW, EW, ED) the trophic magnification factor (TMF) was >1 for THg, indicating an effective biomagnification of Hg in all cases (Borga et al. 2012; Conder et al. 2012), potentially representing detrimental effects to higher trophic level fauna depending on ricefields food webs (Cotín et al. 2011; Lavoie et al. 2013; Abeysinghe et al. 2017; Du et al. 2021). Although TMF for ED was slightly higher than in EW and CW fields, it should be interpreted with caution, due to the limited sample size in the present study, and further experimental and in-situ studies are needed to understand mercury transfer along food webs in agricultural ecosystems and their ecological importance. When evaluating TMF for EW fields considering solely MeHg concentrations in fauna samples, the TMF value was higher than for THg, as expected, since biomagnification occurs mainly for organic mercury forms (Lavoie et al. 2013).

4.4. Physicochemical parameters of water

Physicochemical characteristics of the environment have been regarded as one of the main drivers of mercury transformation throughout its biogeochemical cycle, including methylation and bioaccumulation processes (Ullrich et al. 2001; Celo et al. 2006; Lavoie et al. 2013; Lescord et al. 2019). In rice fields, parameters such as pH, oxygen dissolved in water, temperature, salinity and conductivity are known to influence mercury methylation (Ullrich et al. 2001; Rothenberg et al. 2012). In our study, we have measured these parameters in each of the sampling periods (Table S3).

In the fields under study mean pH values ranged from 7.27-7.55 for CW fields, 4.99-7.61 for EW fields, and 6.60-6.86 for ED fields. Likewise, other studies in rice paddies have detected similar pH values in paddies' water (Zhao et al. 2016, Rothenberg and Feng 2012; Sánchez-Fortún et al. 2021). pH values for CW and ED remained constant throughout the rice cultivation season, whereas in EW fields, pH decreased along the rice growing season. Nonetheless, with the available data the measurements of pH do not present relationship with THg concentrations in CW, EW or ED fields, or with MeHg in EW fields for the three sampling periods considered. Thus, in our case, pH might not be mainly influencing mercury dynamics in the rice field compartments studied, and in fact, although in some cases pH conditions might affect Hg methylation (Ullrich et al. 2001), the generalization of these conditions for MeHg production is unclear (Tang et al. 2020) with different environments showing Hg methylation in a relatively wide range of pH conditions (e.g., Braaten et al.

2014). Regarding temperature values, measurements ranged between 24–31°C, and although temperature might have influence on Hg methylation and its accumulation in organisms (e.g., Ullrich et al. 2001), we do not have enough data to evaluate this. Nonetheless, other studies in rice fields (e.g., Rothenberg and Feng 2012) suggested that temperature might not be a main driver of Hg methylation in these environments. Likewise, dissolved oxygen in water, salinity, and conductivity present low variation within the study period, which might indicate a little influence on mercury dynamics in our study.

However, other parameters that have not been measured in the present study such as dissolved organic matter (DOC) abundance and composition, or the presence of electron acceptors as sulfate or iron, which do have an influence in availability of Hg to bacterial methylators and biomethylation processes (Hall et al. 2008; Bravo and Cosio 2020; Li et al. 2022), might be playing a role on the results observed. Thus, we do have limited data available on physicochemical parameters, and in this regard, more experimental and *in-situ* studies need to be performed to further evaluate the consequences of agricultural management practices on environmental physical and chemical properties, and their relationship with Hg cycling in complex environments such as rice paddies (Tang et al. 2020).

Overall, in this study, we demonstrated that in the Ebro Delta (NE Spain), rice fields serve as potential mercury sinks and hotspots for mercury methylation and transference to other environmental compartments (e.g., biota). Also, we evaluated how do THg concentrations in the different compartments studied (water, soil, rice plants, fauna) differ over time regarding the agricultural management employed. Interestingly, we have not found notorious differences among agricultural management techniques on THg concentrations or trends, except for fauna samples, which seemed to be affected by hydroperiod, rather than the use of fertilizers and pesticides. Finally, we have performed speciation analyses to assess the proportion of THg consisting in MeHg, the most toxic and transferrable to biota species of mercury. Unfortunately, we did not analyze MeHg levels in all the compartments studied for all the different agricultural practices employed, and thus, differences in MeHg among agricultural practices was not evaluated. Thus, further *in-situ* studies in the area are needed to evaluate whether techniques such as AWD do have an impact on organic mercury forms, mainly MeHg, in rice paddies compared to continuously flooded paddies and its trends over time on different compartments.

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Supplementary Material

Temporal mercury dynamics throughout the rice cultivation season in the Ebro Delta (NE Spain): an integrative approach

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2.2.5. Quality control for THg, Hg speciation and isotope analyses of N and C

Table S1 | Values of THg (mean $\pm$ SD) obtained for certified reference materials (CRM).

CRM	Reference	n	THg $\pm$ SD (ng/g dw)
NIES-13 (human hair)	This study	34	4390 $\pm$ 127
	Certified values		4420 $\pm$ 200
DORM-4 (fish protein)	This study	16	383 $\pm$ 39
	Certified values		410 $\pm$ 55
DOLT-5 (dogfish liver)	This study	16	422 $\pm$ 98
	Certified values		440 $\pm$ 180

Table S2 | Hg speciation analysis values (mean $\pm$ SD) obtained for certified reference materials (CRM).

CRM	Reference	n	MeHg+iHg $\pm$ SD (ng/g dw)	MeHg $\pm$ SD (ng Hg /g dw)	iHg $\pm$ SD (ng Hg /g dw)
IAEA456 (marine sediment)	This study	2	75.3 $\pm$ 0.3	0.130 $\pm$ 0.0005	75.2 $\pm$ 0.3
	Certified values		77.0 $\pm$ 5.0	0.125 $\pm$ 0.019	76.9 $\pm$ 5.0
NIES-13 (human hair)	This study	1	4409 $\pm$ 47	3776 $\pm$ 39	632 $\pm$ 8
	Certified values		4420 $\pm$ 200	3800 $\pm$ 400	-
BCR 414 (zooplankton)	This study	1	265 $\pm$ 3	200 $\pm$ 2	66 $\pm$ 1
	Certified values		276 $\pm$ 18	-	-
ERM CE-464 (tuna fish)	This study	3	5216 $\pm$ 116	5091 $\pm$ 109	125 $\pm$ 23
	Certified values		5240 $\pm$ 100	5117 $\pm$ 158	-
GBW 08508 (rice flour)	This study		37 $\pm$ 1	28 $\pm$ 1	8.6 $\pm$ 0.3
	Certified values		38 $\pm$ 3	-	-
	Published values (Shi et al. 2005)		38 $\pm$ 3	28 $\pm$ 3	-
BCR 281 (rye grass)	This study	2	16.6 $\pm$ 0.3	0.3 $\pm$ 0.1	16.3 $\pm$ 0.2
	Certified values		16.4 $\pm$ 2.2	-	-

Table S3 | Results of water physicochemical parameters measured over the rice culture season 2017 for fields from the Ebro delta managed using three agriculture techniques (CW: conventional wet; EW: ecological wet; ED: ecological dry).

Rice fields	Physicochemical parameters		June/July	August	September
CW	pH		7.27 ± 0.02	7.47 ± 0.15	7.55 ± 0.18
	Salinity	%	0.05 ± 0.002	0.03 ± 0.01	0.05 ± 0.001
	Conductivity	mS	1.01 ± 0.08	0.64 ± 0.10	1.12 ± 0.04
	O2	mg L ⁻¹	5.07 ± 0.64	3.24 ± 0.15	3.13 ± 0.10
	Temperature	°C	26.88 ± 0.31	30.33 ± 0.29	28.90 ± 0.23
EW	pH		7.61 ± 0.13	5.83 ± 0.28	4.99 ± 0.2
	Salinity	%	0.07 ± 0.001	0.08 ± 0.005	0.07 ± 0.003
	Conductivity	mS	1.23 ± 0.03	1.47 ± 0.1	1.27 ± 0.07
	O2	mg L ⁻¹	5.21 ± 1.01	5.23 ± 0.82	3.56 ± 0.61
	Temperature	°C	24.10 ± 0.10	27.35 ± 0.23	29.44 ± 0.20
ED	pH		6.86 ± 0.21	6.60 ± 0.07	6.82 ± 0.22
	Salinity	%	0.06 ± 0.001	0.05 ± 0.00	0.06 ± 0.003
	Conductivity	mS	1.05 ± 0.03	0.93 ± 0.01	1.13 ± 0.05
	O2	mg L ⁻¹	6.89 ± 0.48	4.65 ± 0.42	5.01 ± 1.25
	Temperature	°C	30.79 ± 0.20	25.16 ± 0.21	26.41 ± 0.11

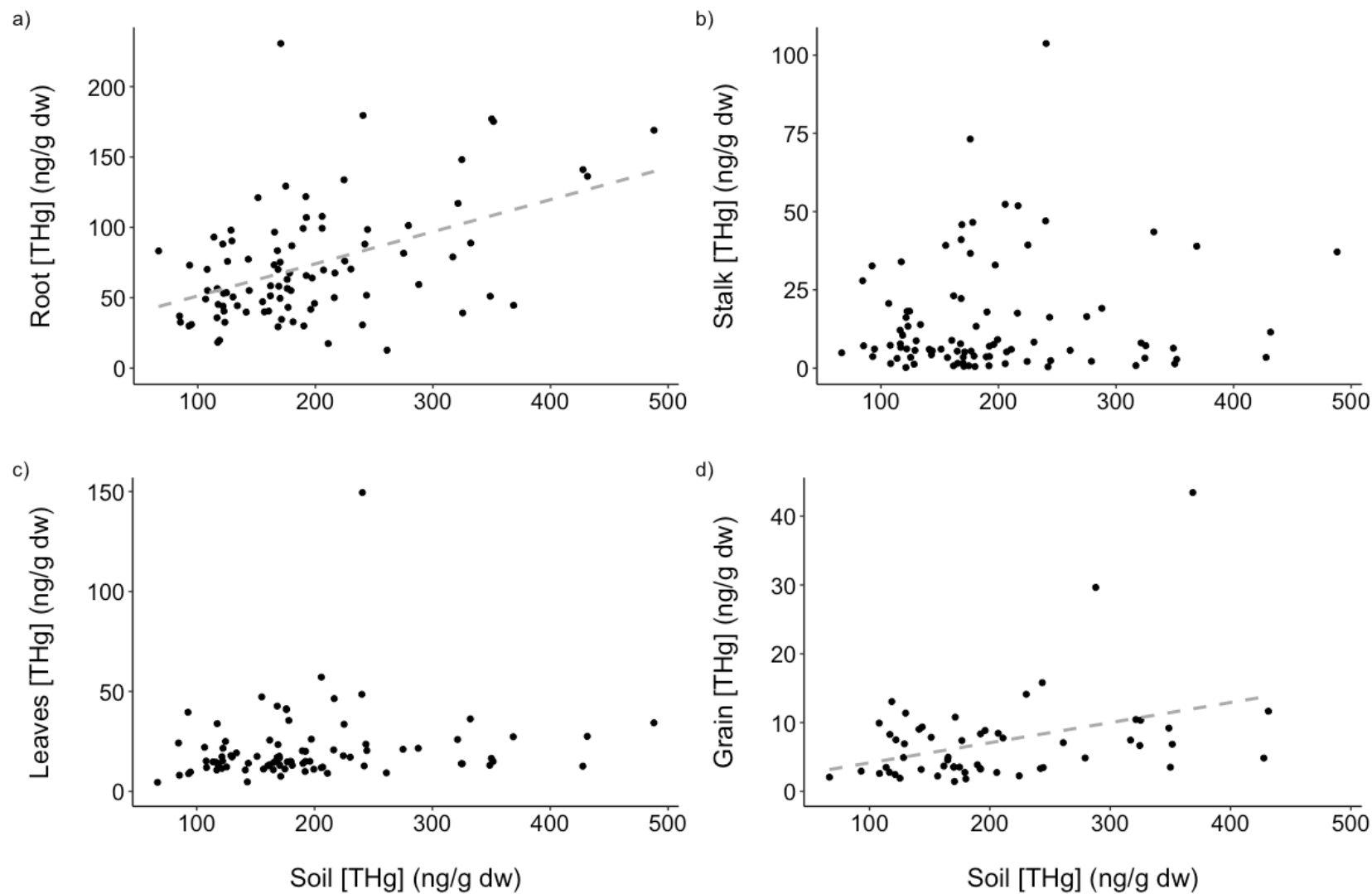


Fig. S1 | Correlations between THg concentration in rice plant parts: (a) roots, (b) stalk, (c) leaves, (d) grain, and THg concentrations in soil samples for all the rice fields under study, and for all the sampling events. The dashed lines represent significant positive correlations found between roots and grain vs. soil THg concentrations.

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03 CHAPTER 3

Effect of different rice farming practices on
the bioavailability of mercury: a mesocosm
experiment with common goldfish
(*Carassius auratus*)



03

CHAPTER 3

Effect of different rice farming practices on the bioavailability of mercury: a mesocosm experiment with common goldfish (*Carassius auratus*)

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Efecte de diferents pràctiques agrícoles utilitzades en el cultiu de l'arròs sobre la biodisponibilitat del mercuri: resultats d'un experiment de mesocosmos amb carpins (*Carassius auratus*)

Resum

Les zones humides, com els arrossars, són ecosistemes rellevants i que constitueixen l'hàbitat de nombroses espècies; alhora també constitueixen una font d'exposició al mercuri, sobretot en la seva forma més tòxica, el metil-mercuri. L'any 2018, durant el cicle de cultiu de l'arròs vàrem dur a terme un experiment de mesocosmos in situ en els arrossars del Delta de l'Ebre (NE Península Ibèrica) per tal d'investigar si es donava bioacumulació de mercuri en els peixos. Per això, es varen exposar carpins (*Carassius auratus*) a diferents tipus de pràctiques agrícoles utilitzades en els arrossars (agricultura convencional vs. ecològica) i irrigació (humit vs. sec) i es varen mostrejar individus mensualment. Varem observar que les concentracions de THg augmentaven al llarg del cicle de cultiu en pràcticament tots els camps estudiats, però aquest augment va ser més acusat en els camps de cultiu ecològic. Així mateix, es varen mesurar les signatures de $\delta^{15}\text{N}$ i $\delta^{13}\text{C}$ per monitoritzar els canvis de dieta dels peixos al llarg del temps, tan dins d'un mateix arrossar, com per detectar diferències entre els diferents arrossars. Els resultats mostraren que no hi havia variacions en la dieta dels peixos al llarg del temps per un mateix arrossar, tot i que sí hi havien diferències segons les diferents pràctiques agrícoles. Així, la irrigació mitjançant un cicle d'alternança humit-sec anava associada a una major biodisponibilitat de mercuri pels peixos i sembla ser un ambient clau per la producció de mercuri que ve influïda pels paràmetres fisicoquímics. Com a conclusió, podem dir que la bioacumulació de mercuri en peixos dels arrossars depèn de varis factors que, a la seva vegada, es traduiran en la biomagnificació del metall en les xarxes tròfiques locals.

Paraules clau: agricultura convencional, agricultura ecològica, bioacumulació, peixos, mercuri total

Abstract

Wetlands such as rice paddies are important ecosystems that provide habitat for a numerous range of species but are considered as a major source of mercury in the most toxic form of methylmercury. An *in situ* mesocosm experiment was conducted during the rice cultivation season of 2018 in rice paddies from the Ebro Delta (NE, Spain) to investigate the bioaccumulation of mercury in fish. Common goldfish (*Carassius auratus*) were exposed to three types of rice-fields subject to different agricultural management (conventional or ecological agriculture) and different hydric practices (wet, dry) and were sampled monthly. Total mercury concentration in fish increased over time for almost all the fields under study, but the mercury increase was particularly higher for fish exposed to ecological fields. We conducted $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ measurements to follow up fish diet changes within or among fields. The results have shown there were no variations in the diet of the fish over time within fields, although differences among management practices were observed, which could explain to some extent the THg variation in fish exposed to each of the agricultural practices. Results indicate that wetting and drying rice fields are a greater source of mercury for fish and a potential hotspot for MeHg production enhanced by physicochemical parameters. Thus, mercury bioaccumulation in fish inhabiting rice paddies depend on several factors what, in turn, could lead to biomagnification across the food webs.

Keywords: Conventional Agriculture, Ecological Agriculture, Bioaccumulation, Fish, Total mercury

1. Introduction

Mercury (Hg) is regarded as a toxic element for humans and wildlife (Clarkson and Magos 2006; Evers 2018). Nonetheless, several anthropogenic activities have taken advantage of this element (e.g., in chlor-alkali industry, as fungicide, pesticide, or in medicine; Clarkson et al. 2003). Hg can be found in all environmental compartments (air, soil, water and biota) and the speciation during its biogeochemical cycle is highly dependent on the local chemical conditions (Leopold et al. 2010; Driscoll et al. 2013). Methylmercury (MeHg) is the most well-known bioavailable and toxic form to humans and wildlife (Clarkson and Magos 2006; Díez 2009; Evers 2018; Mergler et al. 2007). Because of its lipophilic nature, MeHg bioaccumulates in organisms and biomagnifies easily across the food webs (Gray 2002; Driscoll et al. 2013; Lavoie et al. 2013). In the aquatic environment, MeHg can be produced from inorganic mercury (divalent mercury Hg^{2+}) mainly by iron reducing-bacteria (IRB) and sulphate-reducing bacteria (SRB) at the surface of sediments (Compeau and Bartha 1985; Warner et al. 2003; Podar et al. 2015) or in the water column (Eckley et al. 2005) in a process known as methylation. Model experiments have demonstrated that MeHg production by SRB can be enhanced in anoxic conditions with low pH, low salinity, and warm temperatures (Ullrich et al. 2001; Warner et al. 2003). However, complementary studies in natural conditions have shown there is no simple relationship among these physicochemical factors and bacterial methylation rates because of the presence of inorganic and organic complexing agents in water which can reduce Hg^{2+} availability for methylation or stimulate/inhibit microbial activity (Zillioux et al. 1993; Snodgrass et al. 2000; Rothenberg et al. 2014).

Wetland environments referred as continuously or periodically flooded fields (e.g. swamps, peatlands, fresh or saltmarshes) are often linked to high MeHg production rate (Zillioux et al. 1993; Marvin-DiPasquale et al. 2014). Progressively, with growing human population, natural wetlands are being replaced by agricultural farming sites (Verhoeven and Setter 2010; Davidson 2014) such as rice paddies, covering 167 million ha of land in 2017 (FAOSTAT 2020, <http://www.fao.org/faostat/>). As flooded environments, rice paddies are subject to high MeHg production rates (Meng et al. 2011; Wang et al. 2014), which can potentially be accumulated into organisms and be transferred to higher trophic levels in the food web (Ackerman et al. 2010). Besides anthropogenic pollution which can provide inorganic Hg available for methylation, some studies have discussed the impact of agricultural techniques used in rice farming on the bioavailability of mercury (Rothenberg and Feng 2012; Rothenberg et al. 2011, 2014). Indeed, as in natural wetland where water level varies naturally, the type of management practices of agricultural soil (e.g., continuous

flooding, alternance wetting-drying, use of pesticides) can promote microbial Hg methylation (Ullrich et al. 2001; Marvin-DiPasquale et al. 2014; Rothenberg et al. 2014; Tang et al. 2018).

During the 1990s, global climate change and increased water demand have led to the development of new rice farming techniques using less water without losing yields (Rothenberg et al. 2014). Among them, the alternating wetting and drying (AWD) technique is characterized by a drainage just after seeding followed by intermittent wetting and drying periods. However, the use of AWD techniques has been associated with higher MeHg production in agricultural wetlands, highlighting the importance of environmental factors promoted by intermittent flooding such as redox conditions, Hg^{2+} availability, or anoxic conditions enhancing overall mercury methylation (Marvin-DiPasquale et al. 2014; Rothenberg et al. 2014; Tang et al. 2018; but see Tanner et al. 2018).

Hg used as a catalyst in the chlor-alkali industry is considered as one of the main sources of Hg contamination in the aquatic environment (Carrasco et al. 2011a, 2011b). As such, the European Union committed to ban this technology from December 2017 and replace it with better available technologies that do not rely on Hg components (e.g. membrane technology; 2013/732/EU). Nowadays in Spain, chlor-alkali plants using mercury in their production processes have ceased their activity or have made the transition for membrane technology (EuroChlor 2019). Nonetheless, over the past century, the Flix chlor-alkali plant (Tarragona, NE Spain), dumped around 350 000 tons of toxic waste containing more than $436 \mu\text{g g}^{-1}$ of Hg into the Ebro river (Grimalt et al. 2003; Carrasco et al. 2011a). Despite the fact that industrial activities using Hg in this chlor-alkali plant ceased in 2017, legacy mercury pollution persists, and it has already been shown that this pollutant can be transported downstream the Ebro river to its delta (Navarro et al. 2009; Carrasco et al. 2011a; Cotín et al. 2012; Sánchez-Fortún et al. 2020). The Hg dispersion caused by the river flow has resulted in a wide distribution of this metal along the riverbed, thereby affecting fish and shellfish (Carrasco et al. 2011b). Typically, fish tissues and sediment samples upstream the Flix chlor-alkali plant showed lower Hg concentrations than downstream (Navarro et al. 2009; Carrasco et al. 2011a, 2011b). In addition, water from the Ebro river is used to flood continuously or alternatively the rice fields of the Ebro Delta (Terrado et al. 2007). Consequently, the potential absorption of contaminants from the water-soil system by organisms inhabiting rice fields in the Ebro Delta can lead to higher toxicological risk for wildlife through biomagnification. This is of highest concern in the area, as the Ebro Delta is one of the most important areas in the Iberian Peninsula both as rice farming site and as a natural reserve (Mañosa et al. 2001)

The main purpose of this study is to know whether rice paddies in the Ebro Delta are suitable environments enhancing Hg bioavailability and thus promoting bioaccumulation by organisms inhabiting there. Two specific objectives were set: (i) to monitor the temporal dynamics of body mercury in fishes inhabiting the rice paddies and (ii) to understand how different agricultural management practices affect metal bioavailability. With this aim, an *in situ* mesocosm experiment using individuals of a brownish variety of common goldfish (*Carassius auratus*) was carried out in randomly selected rice fields from the Ebro Delta area subject to different agricultural practices. To the best of our knowledge, few studies have investigated the bioavailability and bioaccumulation of Hg in agricultural wetlands using a mesocosm approach *in situ*. In this regard, such experiments will help to understand how Hg bioavailability and bioaccumulation are affected by different environmental conditions.

2. Materials and methods

2.1. Experimental design

The mesocosm study was conducted in the Ebro Delta area (NE Iberian Peninsula: 40°33' N, 00°39' E): comprised of ca. 30.000 ha of wetlands, 75% of the Ebro Delta is used for rice-farming purposes, 20% is protected as a natural reserve and 5% comprises urban areas (Mañosa et al. 2001). During the rice cultivation season of 2018, six rice fields from the area were randomly selected. The rice fields included in the study were cultivated by farmers using different agricultural management methods (2 rice fields per treatment), as follows: (a) conventional wet (CW) approach (flooded throughout the rice cultivation season and using pesticides and fertilizers), (b) ecological wet (EW) approach (flooded throughout the rice cultivation season and without using any pesticides or fertilizers) and (c) ecological dry (ED) approach (without using pesticides and flooded only during half the rice growing period using a mild alternating wet dry, AWD, method). Distance between rice fields managed using the same agricultural practices was ca. 7 km for CW fields and < 1 km for EW and ED fields and all the fields under study layed within a 8 km² area.

For each rice field 4 sampling points (one in each side of the field) were selected and cages (60 cm long x 40 cm wide x 29 cm high) were installed for 60 days (within the months of June/July – August/September, corresponding with the rice growing season in the Ebro Delta). Due to the cultivation schedule of each rice-field and their availability to conduct the experiment, cages were installed in a staggered manner among rice fields in four different days in June/July, ascertaining that rice field plants were at the same stage of development

(i.e., vegetative stage) in all the fields under study. Therefore, four batches of juvenile common goldfish (one for each installation date; approx. 180 individuals per batch) were obtained from an aquaculture industry. From each batch, upon the installation of the cages and before starting the experiment ($t = 0$ days), approximately 20 fishes were randomly selected to know their Hg levels prior to the experiment. Furthermore, 60 fish were assigned to each of the six rice fields, distributed in 15 fishes per cage. Cages were placed in opposite sites within the field, but deeper places were chosen preferably, especially in those rice fields subject to varying water levels (i.e., ED fields) to avoid the lack of water. Before placing them into the cages, goldfishes were weighted (g) and measured (fork-length in cm). Later on, it was intended to collect five fish per cage at 30 days ($t=30$ d) and five fish per cage at 60 days ($t=60$ d) after mesocosm installation, with the five remaining fish being placed as backup in case of the decease of some of the individuals during the experiment. Sampled fish were euthanized using MS-222 Sandoz and frozen at -20°C in zip-locked bags until analysis.

During the installation of the experimental cages and the successive samplings (i.e., 0 d, 30 d, 60 d), physicochemical parameters of the water surrounding the cage were measured (pH, oxygen concentration, salinity, conductivity, temperature).

2.2. Total mercury and stable isotopes analysis

For chemical analysis, fishes were allowed to thaw at room temperature. Then, they were rinsed with distilled water and visually examined for external defects (e.g., malformations, missing parts, scars).

Body condition Index (BCI) was recorded for each fish. Fork-length (mm) and total weight (precision: 0.01 g) before evisceration were measured to determine (BCI) according to the Fulton's condition factor (Froese 2006):

$$\text{BCI} = 100 \times (\text{Total weight} / (\text{Fork-length}^3))$$

where the total weight (with digestive tract) is expressed in g and fork-length in cm. During dissection, the sex of the individuals was recorded if possible. Then, individuals were eviscerated and freeze-dried for 24-48h until constant mass. Eviscerated fishes were entirely powdered using a cryogenic mill (Freezer Mill 6750, SpexCertiPrepH Inc., Metuchen, NJ, USA) to obtain a homogenous sample for Hg analyses. 0.02 g of homogenized sample was analysed to determine the total mercury concentration ($[\text{THg}] \text{ ng g}^{-1}$ dry weight) by means of a Direct Mercury Analyzer 80 (DMA-80, Milestone Inc.). To assess the accuracy of

measurements we analyzed two certified reference materials every 10-15 samples: a dogfish liver certified reference material (DOLT-3, National Research Council of Canada; recoveries ranged from 91 – 96%) and a fish protein certified reference material (DORM-2, National Research Council of Canada; recoveries ranged from 92 – 98%).

The fish samples were also analysed for carbon and nitrogen stable isotopes in order to take into account possible differences in fish trophic ecology across time and across rice-fields. Samples were lipid extracted using 2:1 chloroform:methanol mixture according to the Folch method (Folch et al. 1957), to avoid differences in the isotopic carbon signatures related to differences in lipid content among fish. 0.30-0.35 mg subsamples of lipid extracted fish were encapsulated in tin cups and carbon and nitrogen stable isotopes were analysed at the Scientific Technical Services of the Universitat de Barcelona (Spain) using a Thermo-Finnigan Flash 1112 elemental analyser (CE Elantech, Lakewood, NJ, USA) coupled to a Delta-C isotope ratio mass spectrometer via a CONFLO III interface (Thermo Finnigan MAT, Bremen, Germany). Isotopic signatures of N and C are expressed using the standard delta notation in parts per thousand (‰) relative to the Vienna Pee Dee Belemnite ($\delta^{13}\text{C}$) and atmospheric N_2 ($\delta^{15}\text{N}$). IAEA standards were applied every 12 samples to calibrate the system. Replicate assays of standards indicated analytical measurement errors of $\pm 0.1\text{‰}$ and $\pm 0.2\text{‰}$ for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, respectively.

2.3. Statistical analyses

Total mercury concentration values ([THg]) were log-transformed to approximate a normal distribution. Log THg concentrations in fish were modelled using linear mixed models with the *nlme* package (Pinheiro et al. 2019) including exposition time (0 d, 30 d, 60 d), agricultural rice-farming practices (CW, ED, EW) and the interaction time $\times$ agricultural management as fixed factors. The body condition index (BCI) was also included as a fixed covariate in the model. Rice field ID (CW1, CW2, ED1, ED2, EW1, EW2) was considered as a random factor.

Since for conventional rice-fields fishes were only recovered at 30d of experiment (see “Results” section), we ran two different analyses for the model: (1) using data from all fields (CW, ED and EW fields) until 30d of experiment, and (2) using data from ecological fields (ED and EW fields) along the entire experimental setup (0d, 30d and 60d).

Concerning the stable isotopes of nitrogen ($\delta^{15}\text{N}$) and carbon ($\delta^{13}\text{C}$), a separate linear mixed model with rice field as a random effect was fitted. The model included both covariates along with BCI, exposure time and agriculture management to evaluate their effect on THg concentration in fishes. The interactions between $\delta^{15}\text{N} \times$ agricultural management and $\delta^{13}\text{C} \times$ agricultural management were also included as covariates. Data from all fields (CW, ED, EW fields) and sampling events at 30 and 60 d of exposure was used for this model.

Finally, the effect of the field type (ED or EW) and time (0, 30 and 60d) on $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$, were evaluated fitting linear mixed models for each of the isotopic signatures as response variables, which included the following covariates: BCI, time (0, 30 or 60 days), agricultural management type and the interaction time $\times$ agricultural management. Rice-field ID was included as a random factor. Since fishes exposed in conventional wet fields did not survive until 60 days, these fields were not included in the analyses, as we were interested in evaluating any possible change in trophic ecology throughout the experiment period when fishes were exposed to rice fields, which will be determined by examining the values of the C and N isotopic signatures between 30 and 60 days.

For all the linear mixed models fitted, significant effects were assessed by means of Wald F-test. Post-hoc comparisons were investigated using Tukey's test for multiple pairwise comparisons.

As a quality control, the four fish batches obtained from the aquaculture industry were tested for differences in [THg] using an ANOVA test. Although we assumed that differences in THg levels among fish batches provided by the aquaculture industry would be minimal, differences in log [THg] were found among them ($F(3,64) = 16.53$, $p < 0.0001$; Table 1). Hence, the four batches were considered different groups at $t = 0$ days: one group for each conventional field (Batch 1 and Batch 2), one group for ecologic wet fields (Batch 3) and one group for ecologic dry fields (Batch 4).

All analyses were performed using R v.4.0.3 (R Core Team 2020). Statistically significant threshold was set at $\alpha = 0.05$.

3. Results

We analysed THg concentrations in whole fish ($N = 201$ samples) from six Ebro Delta rice fields subject to different agricultural management practices (2 conventional, 2

ecological dry and 2 ecological wet) at four sampling points each ($N = 24$ sampling points). Descriptive statistics of THg concentration in fish averaged by exposition days and fields are summarized in Table 1. Only for ecological rice fields (EW or ED) fish were recovered after 60 days of experiment because of their death in conventional fields before 60 days (Table 1; Table 2), although fish recovery rates were low for almost all the sites studied (Table 2), possibly due to other stressors besides mercury in the rice field environment causing the death of individuals (Lall and Tibbetts 2009; Harper and Wolf 2009). Most of the mercury in fish has been reported to be methylmercury (MeHg) (e.g., Marrugo-Negrete et al. 2008; Carrasco et al. 2011a, 2011b; Arcagni et al. 2018). Thus, although MeHg concentration was not determined in the present study, we will consider THg concentrations analyzed to serve as a proxy for MeHg.

3.1. THg in fishes from CW, ED, EW fields from 0 to 30 days

The fitted linear mixed model considering all the data on agricultural field types until 30 d of experiment, shows a significant relationship between THg concentrations and BCI ($F(1,152) = 7.85$, $p = 0.006$). Descriptive statistics of BCI of fishes averaged by exposition days and fields are summarized in Table 2. The interaction between time and the agricultural management type was significant ($F(2,3) = 26.15$, $p = 0.012$). Pairwise comparisons of the slopes for each of the agricultural management types showed significant differences between conventional fields and ecological wet fields (CW – ED: $p = 0.011$). Nonetheless, no significant differences were detected between ecological dry and ecological wet fields (ED – EW: $p = 0.10$). Between conventional fields and ecological wet fields we detected marginally significant differences in slopes (CW – EW: $p = 0.052$). THg concentrations increased over time for ecological wet fields and ecological dry fields (EW fields: $t = 4.12$, $p = 0.026$; ED fields: $t = 7.21$, $p = 0.006$), but no significant increase over time for conventional rice-fields was observed (CW fields: $t = 0.032$, $p = 0.98$). In fact, fishes exposed to CW1 increased their THg concentrations in 30 days, but a decrease in THg levels was observed in fishes exposed to CW2 (Fig. 1).

3.2. THg in fishes from ED, EW fields from 0 to 60 days

The model for the ecological rice fields (EW, ED) for the entire experimental setup (0, 30, 60 days) shows that the management regime had a significant effect on THg levels in caged fish ($F(1,135) = 5.54$, $p = 0.020$). Also, the interaction between time and management type was significant ($F(1, 135) = 5.50$, $p = 0.020$). BCI showed a significant relationship with

the THg levels observed in fish ($F(1,135) = 5.32$, $p = 0.023$). THg concentrations increased over time up to 60 days for ecological wet fields (EW fields: $t = 4.84$, $p < 0.0001$). However, THg concentration in fish from ecological dry fields did not show a significant increase over the whole experiment period (ED fields: $t = 0.95$, $p = 0.35$), with a β value for the slope (in log-scale) of 0.0031 (se: 0.0033) possibly as a consequence of a low statistical power, due to the low survival of individuals up to 60 days (Table 2).

After 30 days and 60 days of mesocosm exposure, THg levels in fishes caged was higher than at 0 days, independently the agriculture management employed for almost all fields (Table 1; Fig. 1). At 30-days fishes in ecological (dry or wet) rice paddies have shown higher accumulation of Hg than fishes exposed in conventional wet rice paddies. THg concentration was particularly higher in ED2 after 60 days of exposure (Fig. 1).

3.3. THg, $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ in fishes from CW, ED, EW fields

The model for THg concentration in fishes from all the agricultural type fields for 30 and 60 days including $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ as covariates and its interactions with the agricultural management results in a significant effect of $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ on log-THg values ($\delta^{15}\text{N}$: $F(1, 118) = 17.65$, $p = 0.0001$; $\delta^{13}\text{C}$: $F(1,118) = 5.70$, $p = 0.019$). The interaction between stable isotope values and agricultural management type was significant for both $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ ($\delta^{15}\text{N} \times$ agricultural management: $F(2, 118) = 16.68$, $p < 0.0001$; $\delta^{13}\text{C} \times$ agricultural management: $F(2, 118) = 4.93$, $p = 0.009$, Fig. 2). Pairwise comparisons of the slopes for $\delta^{15}\text{N}$ against THg concentration in fishes showed significant differences between EW fields and both ED and CW fields (EW-ED: $p = 0.036$; EW-EC: $p < 0.0001$). In the EW, the relationship is significantly positive ($t = 2.51$, $p = 0.014$), whereas, in fishes from ED and CW fields we observed a negative association between THg and $\delta^{15}\text{N}$ values (ED: $t = -2.06$, $p = 0.042$; CW: $t = -2.06$, $p = 0.042$; Fig. 2). Regarding the association between $\delta^{13}\text{C}$ and THg, CW fields did not show a significant association ($t = 1.51$, $p = 0.13$), whereas EW and ED fields presented a significant negative association (ED: $t = -2.15$, $p = 0.034$; EW: $t = -3.14$, $p = 0.002$) with no significant differences in pairwise comparisons (ED-EW: $p = 0.44$).

3.4. $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ in fishes from ED, EW fields from 0 to 60 days

Nitrogen and carbon stable isotope signatures results were compared between days of exposure (0, 30 and 60 days) only for ecological fields, as we could not recover any individual

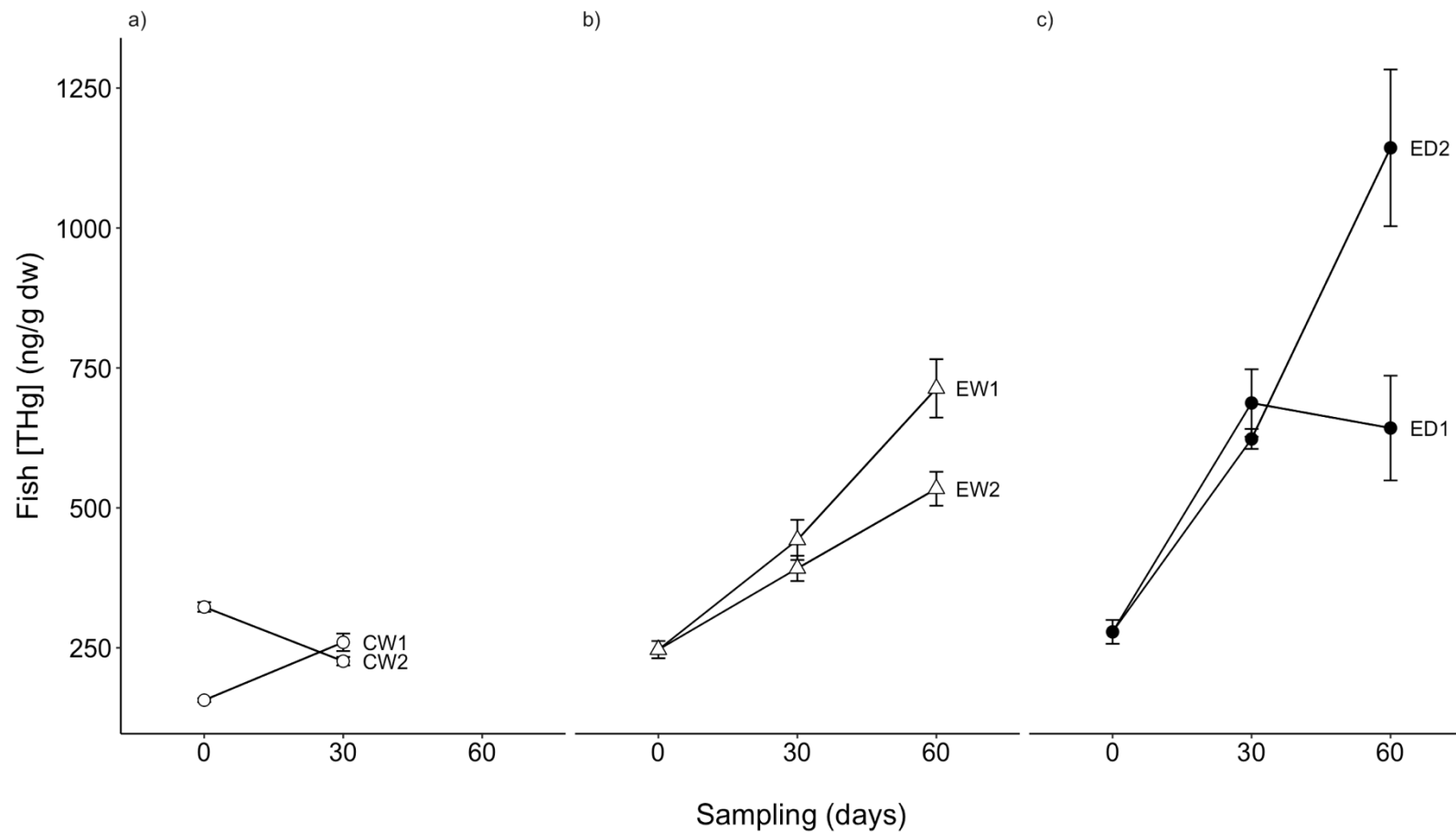


Figure 1 | Whole-body [THg] in *C. auratus* individuals under an *in situ* mesocosm experiment exposed to rice-fields under three different agricultural management practices during the rice cultivation season of 2018 in the Ebro delta area. Two rice-fields were selected for each agricultural management practice: (a) conventional wet fields (CW1 and CW2), (b) ecological wet (EW1 and EW2), (c) ecological dry (ED1 and ED2). Data are represented as mean (points) $\pm$ s.e.m (error bars). For details on samples sizes (N) for each agricultural management practice and for each sampling event see Table 1. See the text for details on the experimental setup.

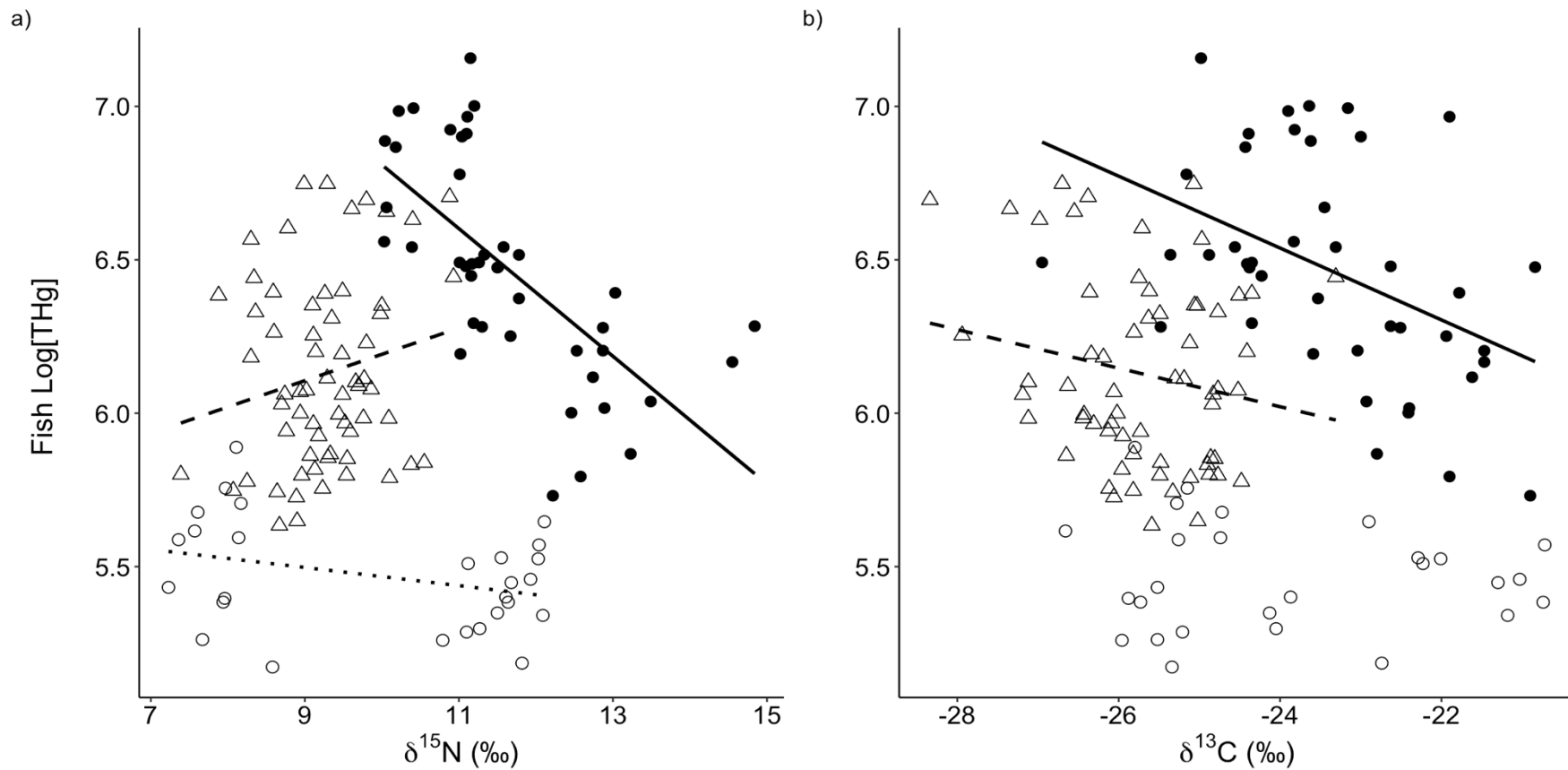


Figure 2 | Whole-body log[THg] (ng/g dw) in *C. auratus* individuals against (a) $\delta^{15}\text{N}$ and (b) $\delta^{13}\text{C}$, for conventional wet (CW1 and CW2) (open circles, dotted regression line), ecological wet (EW1 and EW2) fields (open triangles, dashed regression line) and ecological dry (ED1 and ED2) fields (filled circles, solid regression line). Only data from fishes sampled at 30 and 60 days of experiment are represented (see text for details).

Table 1 | Sample size (N fishes) and mean $\pm$ s.e.m. THg concentrations (ng g⁻¹ dw), $\delta^{15}\text{N}$ (‰) and $\delta^{13}\text{C}$ (‰) values in common goldfish (*Carassius auratus*) batches at 0 days, and caged at 30 days or at 60 days in conventional wet rice fields (CW1 and CW2), ecological wet rice fields (EW1 and EW2) and ecological dry rice fields (ED1 and ED2) from the Ebro delta.

Rice field		0 days after setup*				30 days after setup**				60 days after setup**			
	Batch	N	[THg] (ng g ⁻¹)	$\delta^{15}\text{N}$ (‰)	$\delta^{13}\text{C}$ (‰)	N	[THg] (ng g ⁻¹)	$\delta^{15}\text{N}$ (‰)	$\delta^{13}\text{C}$ (‰)	N	[THg] (ng g ⁻¹)	$\delta^{15}\text{N}$ (‰)	$\delta^{13}\text{C}$ (‰)
CW1	Batch 1	10	156.47 $\pm$ 3.42	7.00 $\pm$ 0.13	-26.06 $\pm$ 0.20	12	259.82 $\pm$ 15.61	7.86 $\pm$ 0.11	-25.47 $\pm$ 0.15	na	na ^a	na	na
CW2	Batch 2	20	322.94 $\pm$ 8.61	12.02 $\pm$ 0.086	-21.15 $\pm$ 0.15	15	226.06 $\pm$ 7.55	11.62 $\pm$ 0.10	-22.69 $\pm$ 0.42	na	na	na	na
EW1	Batch 3	19	244.36 $\pm$ 21.35	11.28 $\pm$ 0.30	-22.03 $\pm$ 0.39	17	442.82 $\pm$ 35.89	9.63 $\pm$ 0.15	-26.29 $\pm$ 0.24	6	713.47 $\pm$ 52.16	9.78 $\pm$ 0.18	-26.45 $\pm$ 0.30
EW2						20	391.9 $\pm$ 22.65	9.02 $\pm$ 0.17	-25.70 $\pm$ 0.18	20	534.2 $\pm$ 30.31	9.02 $\pm$ 0.13	-25.00 $\pm$ 0.091
ED1	Batch 4	19	278.46 $\pm$ 14.82	10.93 $\pm$ 0.51	-22.41 $\pm$ 0.51	17	687.43 $\pm$ 60.21	11.67 $\pm$ 0.29	-22.95 $\pm$ 0.24	11	642.66 $\pm$ 93.54	12.13 $\pm$ 0.54	-22.55 $\pm$ 0.33
ED2						13	623.16 $\pm$ 17.81	11.35 $\pm$ 0.072	-24.38 $\pm$ 0.38	2	1143.25 $\pm$ $\pm$ 140.01	11.12 $\pm$ 0.027	-24.69 $\pm$ 0.29

* Sample sizes refer to the fishes obtained from the aquaculture industries batches. 60 fishes from the same batches were introduced in each field (15 individuals x 4 cages per field).

** Sample sizes refer to the fishes recovered from those introduced in the mesocosm cages for each field (15 individuals x 4 cages per field). See text for details on the experimental design.

^a na: non available data because of the death of all fishes caged before 60 days for conventional fields (CW1 and CW2).

Table 2 | Sample size (N fish) and mean $\pm$ s.e.m. weight (non-eviscerated), fork length and BCI in common goldfish (*Carassius auratus*) batches at 0 days and caged 30 days or 60 days in conventional wet rice fields (CW1 and CW2), ecological wet rice fields (EW1 and EW2) and ecological dry rice fields (ED1 and ED2) from the Ebro Delta.

Rice field	0 d after setup										30 d after setup				60 d after setup				% fish recovery**
	Batch					Mesocosm*													
	Batch	N	Weight (g)	Fork length (mm)	BCI	N	Weight (g)	Fork length (mm)	BCI	N	Weight (g)	Fork length (mm)	BCI	N	Weight (g)	Fork length (mm)	BCI		
CW1	Batch 1	10	5.05 ± 0.53	67.73 ± 2.10	1.58 ± 0.055	60	8.07 ± 0.25	77.09 ± 0.78	1.73 ± 0.020	12	6.06 ± 0.18	75.22 ± 1.20	1.45 ± 0.082	na	na	na	na ^a	20	
CW2	Batch 2	20	3.38 ± 0.23	56.39 ± 1.10	1.85 ± 0.056	60	3.76 ± 0.13	59.90 ± 0.68	1.73 ± 0.026	15	3.39 ± 0.28	59.32 ± 1.14	1.58 ± 0.078	na	na	na	na	25	
EW1	Batch 3	19	2.79 ± 0.10	51.53 ± 0.54	2.02 ± 0.043	60	8.05 ± 0.45	73.68 ± 1.22	1.89 ± 0.022	17	12.41 ± 1.07	81.66 ± 2.04	2.20 ± 0.039	6	13.21 ± 1.75	84.50 ± 2.56	2.13 ± 0.10	42	
EW2						60	8.54 ± 0.32	75.35 ± 0.97	1.94 ± 0.021	20	9.37 ± 0.61	75.29 ± 1.51	2.14 ± 0.035	20	9.59 ± 0.65	78.70 ± 1.68	1.91 ± 0.055	67	
ED1	Batch 4	19	3.50 ± 0.29	60.02 ± 1.45	1.56 ± 0.041	60	3.66 ± 0.14	59.50 ± 0.69	1.70 ± 0.021	17	3.94 ± 0.27	61.01 ± 0.89	1.71 ± 0.076	11	3.63 ± 0.44	59.45 ± 1.76	1.68 ± 0.087	47	
ED2						60	3.77 ± 0.15	60.07 ± 0.75	1.71 ± 0.027	13	3.37 ± 0.34	58.82 ± 1.66	1.61 ± 0.077	2	6.85 ± 0.84	68.29 ± 2.46	2.14 ± 0.034	25	

* Fish introduced in the mesocosm cages for each field (15 individuals x 4 cages per field). See main text for details on the experimental design.

** Reflects the percentage of fish recovered throughout the experiment in relation to the total number of individuals in the mesocom cages at 0d (N = 60 individuals) in each field. We assume that the loss of individuals is due to natural mortality during the experimental period.

^a na: non available data because of the death of all fishes caged before 60 days for conventional fields (CW1 and CW2).

in conventional fields after 60 days of experiment. No significant effect of the agricultural type ($F(1,132) = 0.62$, $p = 0.43$), the BCI ($F(1,132) = 0.0002$, $p = 0.99$), time ($F(2, 132) = 1.20$, $p = 0.30$) was found on the $\delta^{15}\text{N}$ signatures. The interaction of time $\times$ agricultural management had a significant effect ($F(2,132) = 11.24$, $p < 0.0001$) on the $\delta^{15}\text{N}$ values measured in the fishes exposed to ecological rice-fields. Multiple comparisons for each of the agricultural management types showed any significant differences between 0, 30 or 60 days of exposure in ED fields on $\delta^{15}\text{N}$ signatures (0-30d: $p = 0.52$; 0-60d: $p = 0.27$; 30-60d: $p = 0.67$), and in the case of EW fields, $\delta^{15}\text{N}$ signatures differed only between 0-30d ($p = 0.0009$) and 0-60d ($p = 0.001$), but not between 30-60d ($p = 0.99$). If agricultural management types were compared for each of the exposure times, differences between ED and EW fields were observed at 30 and 60 days of exposure (30d: $p < 0.0001$; 60d: $p < 0.0001$).

The fitted model for the carbon stable isotope signatures showed no significant effect of the BCI of the fishes ($F(1,132) = 2.35$, $p = 0.12$) and agricultural management ($F(1,132) = 0.095$, $p = 0.76$). However, there was a significant effect of time ($F(2,132) = 4.01$, $p = 0.020$) on the $\delta^{13}\text{C}$ signatures observed. The interaction time $\times$ agricultural management was significant ($F(2,132) = 3.92$, $p = 0.022$). Multiple comparisons for each of the agricultural management types showed any significant differences between 0, 30 or 60 days of exposure in ED fields on $\delta^{13}\text{C}$ signatures (0-30d: $p = 0.51$; 0-60d: $p = 0.74$; 30-60d: $p = 0.72$). In EW fields only between 0-30d ($p = 0.0012$) and 0-60d ($p = 0.004$), but not between 30-60d ($p = 0.59$). Descriptive statistics of both $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values are summarized in Table 1.

3.5. Water physicochemical parameters in CW, ED, EW fields

Water physicochemical parameters (pH, salinity (%), conductivity (mS), dissolved oxygen (mg l^{-1}) and temperature ($^{\circ}\text{C}$)) recorded over the rice growing season in 2018 are all summarized in the Table S1. Mean pH values ranged 7.5-6 in CW fields, 7.6-5 in EW fields and 6.9-5.1 in ED fields over the experimental period, with a general trend to a decrease in pH within fields over time. Mean salinity (%) and conductivity (mS) presented similar values for all the fields studied, ranging from 0.03-0.08 and 0.64-1.47 over the experiment, respectively. Mean dissolved oxygen (mg l^{-1}) and water temperature ($^{\circ}\text{C}$) varied among field types and sampling events (Table S1). For CW fields (CW1 and CW2) physicochemical parameters were not analyzed at $t = 60$ d as all fishes caged died before 60 days.

4. Discussion

The presence of a chlor-alkali industry along the Ebro river has been considered as an important source of Hg in water, soil and biota inhabiting the deltaic ecosystem (Navarro et al. 2009; Carrasco et al. 2011a, 2011b; Cotín et al. 2012; Sánchez-Fortún et al. 2020). Furthermore, rice paddies considered as ephemeral wetland (Elphick 2000) are often associated with higher production and bioaccumulation of MeHg in rice and organisms due to the fact that environmental conditions are favourable for microbial growth and Hg²⁺ methylation (Rothenberg et al. 2014). Flood regime in rice farming has been identified as a factor influencing this process (Hall et al. 2008; Marvin-DiPasquale et al. 2014; Rothenberg and Feng 2012; Rothenberg et al. 2014).

After one- and two-month exposure in rice fields managed by different agriculture techniques, THg concentration in goldfishes increased over time respect the 0-days fishes at the introduction in cages for all fields except for CW2 (after 30 days exposure; Table 1) and ED1 (after 60 days exposure compared to 30 days of exposure; Table 1). In our experimental setting, THg concentrations had approximately doubled in fishes for at least one of the two conventional and ecological wet fields (CW1 and EW1) and had more than doubled for ED1 and ED2 after 30 days. THg concentrations had almost tripled in EW1 and EW2, and was multiplied by four in ED2 after 60 days compared to the 0 days fishes.

Thus, regardless differences in soil treatment, and eventual differences in food availability, or fish condition (Ackerman and Eagles-Smith 2010), Hg can be accumulated by fishes growing in rice paddies and be transferred to higher trophic levels in the food webs. Hg in fish tissues is found mostly as MeHg (Marrugo-Negrete et al. 2008; Carrasco et al. 2011a, 2011b; Arcagni et al. 2018), which is mainly absorbed by feeding although, to a lesser extent, fishes can also absorb dissolved Hg through the gills (Snodgrass et al. 2000; Gray 2002; Díez 2009). However, MeHg elimination is limited in fishes (Trudel and Rasmussen 1997). Thus, fish growth should be linked with higher concentrations of THg in fish tissues over the time (Snodgrass et al. 2000). In the wild, mercury variations between individuals can be explained by age and metabolic response to detoxification (Gray 2002), nonetheless all the fishes used in this experiment were juveniles. A linear increase was actually observed between THg concentration in fishes and BCI (see Supplementary Materials, Fig. S1). These results suggest that fishes are growing and potentially exposed to MeHg via feeding. Moreover, temperature was optimal for feeding to goldfish throughout the experiment (close to 28°C; Jones 1984; Table S1), except at the beginning of the mesocosm experiment in ecological dry fields, which could be attributed to the placement of the cages within the field,

in areas where plant density was lower, increasing the direct influence of sunlight. Nonetheless, these measurements could be greatly affected by the time of day the water temperature was measured and thus should be interpreted with caution. The major linear increase of THg concentration over the time reflects the constant presence of MeHg in rice paddies selected for the experiment.

4.1. Conventional wet fields Hg dynamics

In conventional wet rice fields, fish mercury concentration increased after 30 days in CW1 field, however, it decreased in CW2 (Fig. 1). Whereas results for CW1 field are in accordance with the overall positive bioaccumulation trends observed in the ecological rice fields, CW2 fishes exposed 30 days to the rice field environment presented a lower concentration of THg in their bodies. Amongst the causes proposed for the decrease in THg concentration over time, could be a dilution effect of THg through growth (Simoneau et al. 2005) and/or to a lower Hg intake. Our findings suggest that lower THg levels in CW2 fishes after 30-days exposure could be related to the observed poorer body condition (Table 2), probably as a result of a decrease of feeding rates due to stressors in the environment (Lall and Tibbetts 2009). In fact, fishes in conventional wet fields did not survive up to 60 days of the experiment. Their death could be related to a lack of nutrient resources or to the presence of pesticides in water affecting their body condition (Lall and Tibbetts 2009; Harper and Wolf 2009; Belenguer et al. 2014; Ccancapa et al. 2016). Accordingly, we found mean BCI at 30 days for these fields were lower than BCI at $t=0$ days (Table 2).

4.2. Ecological wet and dry fields Hg dynamics

Total mercury concentrations in fish exposed to ecological wet fields show a clear linear increasing trend in THg throughout the experiment in the two fields studied.

Conversely, although at 30 days of exposure fishes in ecological dry fields show higher THg levels than fishes at 0 days, there are differences between the two fields at 30 and 60 day. This differences between dry fields could be the result of different Hg dynamics in each field. Furthermore, at 60 days of experiment, for ED fields, sample sizes are low, particularly for the field ED2 (ED1: $N = 11$ individuals, ED2: $N = 2$ individuals) as we are sampling fishes that have survived until 60 days of experimentation. Thus, the results in this case should be interpreted with caution. Furthermore, it is also worth noticing the high variation in THg concentrations of fishes exposed to ED fields throughout the experiment

(Table 1), which might be due to differences in THg levels among sampling points; in two sampling points fishes accumulated twice as Hg compared to the others, possibly due to a bioaccumulation gradient as a consequence of the transport of Hg by flooding the rice field (Ackerman and Eagles-Smith 2010).

Several studies have demonstrated that uptake Hg by fish or other organisms might be enhanced by hydroperiod (Bodaly and Fudge 1999; Snodgrass et al. 2000; Ackerman and Eagles-Smith 2010; Ackerman et al. 2010). When comparing conventional wet, ecological wet and ecological dry fields, the THg concentrations observed in fishes from ED fields are higher than those exposed to EW and CW fields. Since diet is the main source of mercury in organisms, differences in Hg concentrations in fishes exposed to rice-fields managed differently and with different flooding regimes might be influenced by the food sources available in a particular field, with ED fields being more enriched in MeHg or fishes feeding from higher trophic levels.

4.3. THg, $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ in fishes from CW, ED, EW fields

To better understand possible differences between sites in terms of the trophic ecology of the fishes exposed to a particular environment, we analysed $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values in the whole bodies of the fishes (Bodaly and Fudge, 1999).

We found a significant relationship between THg levels and $\delta^{15}\text{N}$, that differs between EW fields, and both ED and CW fields. In the EW, the relationship is significantly positive, as expected from biomagnification processes, in which individuals feeding from higher trophic levels tend to be exposed to higher levels of Hg (Chen et al. 2009; Coehlo et al. 2013). Contrarily, in fishes from ED and CW fields we observed a negative association between THg and $\delta^{15}\text{N}$ values. However, we think that these negative associations are likely a spurious relationship due to the particular physicochemical conditions of each field and each sampling point or the presence of mercury gradients along the fields (Ackerman and Eagles-Smith 2010).

Although only evaluated for ecological fields, $\delta^{15}\text{N}$ values differed between ED and EW field types at 30 days and 60 days of exposure. In both cases, fishes in ED fields were apparently feeding from higher trophic levels, as indicated by the higher $\delta^{15}\text{N}$ values observed (Post 2002) than fishes exposed to EW, which might explain partly the higher Hg levels found in ED fields compared to the other types of rice fields studied.

$\delta^{15}\text{N}$ values were not significantly different after 30 days and 60 days of exposure for ED or EW fields. Thus, fishes exposed in fields managed by the same type of agriculture have not changed their overall trophic level over the period studied (Post 2002). Consequently, this further indicates that bioavailability and bioaccumulation of mercury are the processes driving the increase in THg levels observed in fishes exposed to these fields.

Regarding $\delta^{13}\text{C}$ values, fishes exposed to EW and ED rice-fields presented a significant negative relationship between $\delta^{13}\text{C}$ and THg concentrations. Moreover, we found a similar range in $\delta^{13}\text{C}$ fish values (Table 1). These results suggest that the carbon source of fishes in the different rice paddies under study are similar (i.e., similar feeding habitat; Cherel and Hobson 2007; Garcia et al. 2007). The negative relationship between $\delta^{13}\text{C}$ and THg indicates that to some extent there is an effect of the diet source to the Hg levels in the fishes, which probably is due to the trophic specialization of individuals within the rice paddies (Sharma et al. 2008), as reflected by the wide range in both $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, spanning more than one trophic level (DeNiro and Epstein 1981; Post 2002) in ecological rice-fields.

Altogether, our results support the idea that agriculture management and flooding regime play an important role in the Hg bioaccumulation by fishes, with ecological dry fields possibly enhancing Hg methylation and Hg bioavailability (Rothenberg and Feng 2012; Marvin-DiPasquale et al. 2014, but see Tanner et al. 2018). Besides, some areas within the fields could be more suitable than others for microbial Hg methylation because of local physicochemical conditions (Ullrich et al. 2001).

4.4. Environmental conditions over the rice culture season

When comparing THg levels in fishes exposed to different agricultural management practices, after 30 days, we found that THg accumulation was significantly higher in those exposed in ecological dry fields compared to those in conventional wet fields. When comparing THg values in fishes over the whole experimental period (i.e., 60 days) in ecological rice fields, those fishes exposed to an alternating wet drying (AWD) regime tended to accumulate more THg compared to individuals exposed to ecological wet rice fields (Fig. 1). In this way, it is put forward that water regime was an important factor promoting Hg bioaccumulation in organisms. It has been shown that variations in redox conditions by intermittent flood or new flooding shallow reservoirs can promote microbial biomethylation by sulfate-reducing bacteria (SRB) at sediment anoxic surface (Snodgrass et al. 2000; Marvin-DiPasquale et al. 2014; Rothenberg et al. 2014; Hang et al. 2017). When water level

decreases, anoxic sediment is exposed to oxygen and Hg is released by sediment in the aquatic system (Snodgrass et al. 2000). Therefore, when rice paddies are re-flooded and anoxic conditions recovered, Hg^{2+} dissolved in the water is made more available to be methylated (Snodgrass et al. 2000) and microbial methylation activity is enhanced (Rothenberg and Feng 2012) increasing the MeHg concentration in the environment on paddies using AWD techniques. MeHg produced dispersed in the water column upon re-flooding can then contribute to higher accumulation in fishes.

In general, a decrease in pH was observed over the rice culture season 2018 to an acidic pH range of 6.8-5 (except for ED2 field which increases slightly the pH; Table S1). A possible explanation to this pH decrease over time could be the increase of sulphates (SO_4^{2-}) by oxidation of sulphides in the rhizosphere, which in turn promotes favourable conditions for SRB to methylate Hg over the rice growing season (Rothenberg and Feng 2012; King et al. 2000; Ullrich et al. 2001). Therefore, besides bioaccumulation related to the time of exposure of the individuals to the rice fields, physicochemical conditions of the environment seem to enhance Hg methylation rates over the rice growing season. However, this idea has not been tested in our study and other factors such as an increase in organic matter in decomposition could be causing a decrease in the water pH over time (Hall et al. 2008). As for salinity and conductivity values measured in the fields, values remained constant along the duration of the mesocosm experiment, which suggest a weaker implication in THg accumulation by organisms in our *in situ* experiment.

Our study demonstrates that rice-fields in the Ebro Delta are mercury hotspots for mercury bioavailability. Previous mesocosm experiments within agricultural wetlands using fishes or aquatics invertebrates have already shown that higher levels of Hg are accumulated by organisms in agricultural wetlands than in natural wetlands (Ackerman and Eagles-Smith 2010; Ackerman et al. 2010). Our study highlights that agriculture type management and overall flood regime can play a role on this observation. THg accumulation by fishes caged was higher in fishes exposed to fields with a periodically flood regime. Alternance between flood and dry period could promote disturbance of physicochemical parameters and involve periodic Hg biomethylation during the rice cultivation cycle (Rothenberg and Feng 2012). Furthermore, dietary differences as revealed by stable isotope analyses between different agricultural management types, might be also influencing the levels of THg levels observed in the organisms. Although it is difficult to estimate a degree of influence among the ultimate causes of the differences observed in Hg bioaccumulation of organisms inhabiting in complex aquatic systems such as rice-fields, it is clear that rice-paddies in the Ebro Delta area are

environments suitable for Hg bioaccumulation, enhancing the mercury exposure for higher-trophic level wildlife depending on these dietary sources.

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Competing interests

No competing interests declare

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Supplementary Material

Effect of different rice farming practices on the bioavailability of mercury: a mesocosm experiment with common goldfish (*Carassius auratus*)

M. Sánchez-Fortún, D. Vinyoles, S. López-Carmona, J. L. Carrasco, C. Sanpera

Table S1 | Results of water physicochemical parameters measured over the rice culture season 2018 for fields from the Ebro delta managed by three agriculture types (CW1, CW2: conventional wet; ED1 and ED2: ecological dry; EW1 and EW2: ecological wet).

Rice fields	Physicochemical parameters		0 days after setup Mean $\pm$ s.e.m.	30 days after setup Mean $\pm$ s.e.m.	60 days after setup Mean $\pm$ s.e.m.
CW1	pH		7.27 $\pm$ 0.02	7.47 $\pm$ 0.15	na ^a
	Salinity	%	0.05 $\pm$ 0.002	0.03 $\pm$ 0.01	na
	Conductivity	mS	1.01 $\pm$ 0.08	0.64 $\pm$ 0.10	na
	O2	mg l ⁻¹	5.07 $\pm$ 0.64	2.24 $\pm$ 0.15	na
	Temperature	°C	26.88 $\pm$ 0.31	30.33 $\pm$ 0.29	na
CW2	pH		7.02 $\pm$ 0.29	5.95 $\pm$ 0.3	na
	Salinity	%	0.05 $\pm$ 0.002	0.05 $\pm$ 0.003	na
	Conductivity	mS	0.92 $\pm$ 0.04	1.06 $\pm$ 0.06	na
	O2	mg l ⁻¹	5.63 $\pm$ 1.07	4.3 $\pm$ 0.61	na
	Temperature	°C	30.61 $\pm$ 0.48	30.80 $\pm$ 0.21	na
EW1	pH		7.61 $\pm$ 0.13	5.83 $\pm$ 0.28	4.99 $\pm$ 0.2
	Salinity	%	0.07 $\pm$ 0.001	0.08 $\pm$ 0.005	0.07 $\pm$ 0.003
	Conductivity	mS	1.23 $\pm$ 0.03	1.47 $\pm$ 0.1	1.27 $\pm$ 0.07
	O2	mg l ⁻¹	5.21 $\pm$ 1.01	5.23 $\pm$ 0.82	3.56 $\pm$ 0.61
	Temperature	°C	24.10 $\pm$ 0.10	27.35 $\pm$ 0.23	29.44 $\pm$ 0.20
EW2	pH		7.49 $\pm$ 0.17	7.19 $\pm$ 0.31	5.27 $\pm$ 0.07
	Salinity	%	0.06 $\pm$ 0.003	0.04 $\pm$ 0.004	0.07 $\pm$ 0.003
	Conductivity	mS	1.26 $\pm$ 0.05	0.81 $\pm$ 0.06	1.25 $\pm$ 0.06
	O2	mg l ⁻¹	3.18 $\pm$ 0.42	8.08 $\pm$ 0.55	4.86 $\pm$ 0.66
	Temperature	°C	24.69 $\pm$ 0.08	28.97 $\pm$ 0.26	29.43 $\pm$ 0.28
ED1	pH		6.86 $\pm$ 0.21	6.6 $\pm$ 0.07	6.82 $\pm$ 0.22
	Salinity	%	0.06 $\pm$ 0.001	0.05 $\pm$ 0.00	0.06 $\pm$ 0.003
	Conductivity	mS	1.05 $\pm$ 0.03	0.93 $\pm$ 0.01	1.13 $\pm$ 0.05
	O2	mg l ⁻¹	6.89 $\pm$ 0.48	2.65 $\pm$ 0.42	5.01 $\pm$ 1.25
	Temperature	°C	30.79 $\pm$ 0.20	25.16 $\pm$ 0.21	26.41 $\pm$ 0.11

ED2	pH		6.38 ± 0.21	5.13 ± 0.06	6.67 ± 0.29
	Salinity	%	0.07 ± 0.005	0.07 ± 0.002	0.07 ± 0.008
	Conductivity	mS	1.16 ± 0.07	1.35 ± 0.03	1.21 ± 0.17
	O2	mg l ⁻¹	3.80 ± 0.30	6.76 ± 0.2	1.70 ± 0.23
	Temperature	°C	31.73 ± 0.40	24.46 ± 0.17	25.97 ± 0.30

^ana: non available data because of the death of all fishes caged before 60 days for conventional fields (CW1 and CW2).

Relationship between THg and BCI in *Carassius auratus* in the mesocosm experiment

In order to assess the relationship between log[THg] and body condition index (BCI, see main text for details) we fitted three separate linear mixed effects models for each of the sampling periods (0, 30 and 60 days after experimental setup). The models included the body condition index (BCI), agricultural management (CW, EW, ED) and the interaction BCI $\times$ agricultural management as fixed factors. Rice field ID (CW1, CW2, ED1, ED2, EW1, EW2) was considered as a random factor.

The linear mixed effects model at 0 days after setup showed no effect of the interaction BCI $\times$ agricultural management ($F(2,62) = 2.26$, $p = 0.11$). After excluding the interaction term from the model, BCI showed no effect on log[THg] in fishes at 0 days ($F(1,66) = 0.30$, $p = 0.59$; **Fig. S1**), with a β value for the slope (in log-scale) of 0.08 (se: 0.15). At 30 days after setup, we did not find an effect of the interaction BCI $\times$ agricultural management ($F(2,85) = 0.73$, $p = 0.49$; **Fig. S1**). After excluding the interaction term from the model, BCI showed a significant effect on log[THg] in fishes at 30 days ($F(1,87) = 8.05$, $p = 0.0057$), with a β value for the slope (in log-scale) of 0.28 (se: 0.098). Finally, at 60 days after the experimental setup (with fish recoveries only for ED and EW fields), we did not find an effect of the interaction BCI $\times$ agricultural management ($F(1,33) = 2.22$, $p = 0.15$). After excluding the interaction term from the model, BCI did show a positive relationship with log[THg] in fish, with a β value for the slope (in log-scale) of 0.25 (se: 0.21), although it was not statistically significant ($F(1,34) = 1.47$, $p = 0.23$; **Fig. S1**), possibly due to a lack of statistical power of the analyses, as a consequence of the low survival of fish up to 60 days in the mesocosm experiment.

These results are in accordance to our expectations, as fishes exposed to rice-fields (30 and 60 days) and thus, to an environment with mercury bioavailability present a positive relationship between BCI and THg levels, which indicates that fish are growing and potentially being exposed to Hg via feeding. Conversely, at 0 days (fish not exposed to rice-field environments) there is no positive relationship between BCI and THg levels.

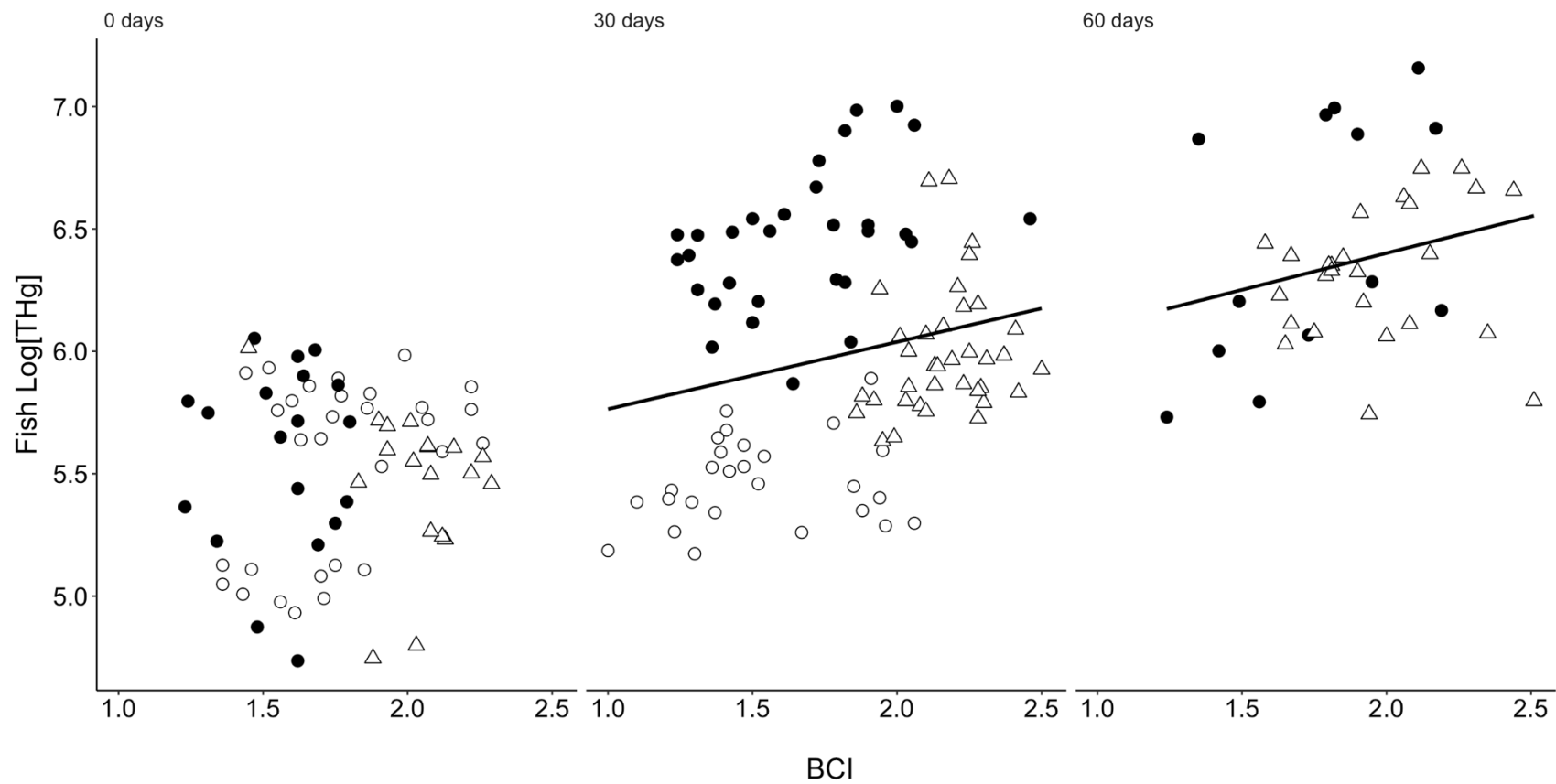
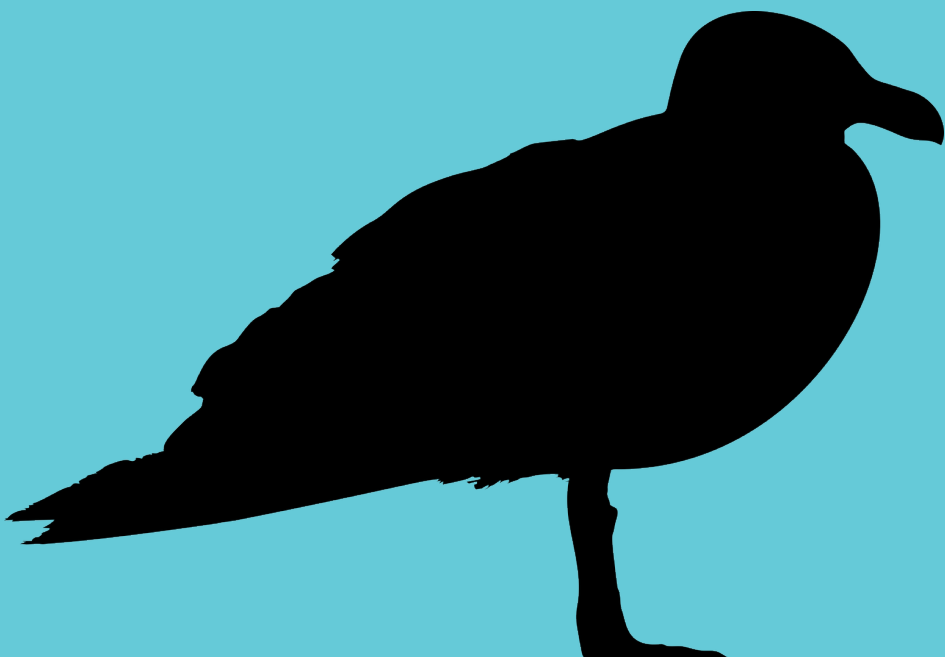


Figure S1 | Relationship between body condition index (BCI) and whole-body log[THg] (ng/g dw) in *C. auratus* individuals at 0d, 30d, 60d after experimental setup. The regression line depicts the positive relationships observed between BCI and [THg] in fish exposed to rice-fields at 30 days and 60 days. Data points for different agricultural practices are shown as follows: CW (open circles), EW (open triangles), ED (filled circles).

04 CHAPTER 4

Following up mercury pollution in the Ebro Delta (NE Spain): Audouin's gull fledglings as model organisms to elucidate anthropogenic impacts on the environment



04

CHAPTER 4

**Following up mercury pollution in the Ebro Delta (NE Spain):
Audouin's gull fledglings as model organisms to elucidate
anthropogenic impacts on the environment**

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Seguiment de la contaminació per mercuri en el Delta de l'Ebre (NE Península Ibèrica): els volantons de gavina corsa com a organisme model per dilucidar els impactes antropogènics sobre el medi

Resum

Els ocells marins, degut a la seva posició com a predadors apicals en els ecosistemes marins son considerats bioindicadors apropiats per una gran varietat de contaminants. El mercuri (Hg) es un contaminant global, que pateix biomagnificació a les xarxes tròfiques marines i d'aigües dolces. Es per això que els nivells de mercuri en ocells marins, com les gavines, son capaços d'integrar informació sobre els contaminants al medi. Al Delta de l'Ebre (NE Península Ibèrica), el llegat de contaminació per mercuri procedent d'una planta clor-àlcali localitzada 100 km aigües amunt del riu ha arribat fins al seu delta. En aquest treball hem analitzat el Hg en una sèrie temporal de 15 anys (2004-2019) en la colònia reproductora de gavina corsa (*Ichthyetus audouinii*) en el Delta de l'Ebre per veure si les fluctuacions en els nivells de mercuri en aquesta espècie estan associats a les activitats humanes de caire industrial que han tingut lloc en les aigües amunt del riu. D'altra banda, hem caracteritzat l'ecologia tròfica de l'espècie mitjançant les signatures isotòpiques de C i N ($\delta^{13}\text{C}_{\text{bulk}}$ i $\delta^{15}\text{N}_{\text{bulk}}$). Vàrem trobar que $\delta^{13}\text{C}_{\text{bulk}}$ mostrava una associació amb els nivells de THg, però $\delta^{15}\text{N}_{\text{bulk}}$ no.

Paraules clau: mercuri, gavina corsa, *Ichthyetus audouinii*, Delta de l'Ebre, CSIA-AA

Abstract

As top-predators in marine ecosystems, seabirds are regarded as appropriate bioindicator species for a variety of contaminants. Mercury (Hg) is a global pollutant, which can biomagnify along marine and freshwater food webs. Therefore, mercury body burden in seabirds, such as gulls, will integrate information about pollution in the environment. In the Ebro Delta (NE Spain), legacy mercury pollution from a chlor-alkali industry located ca. 100 km upstream of the Ebro river mouth has been affecting the delta environment. We have analyzed a 15-year temporal series (2004 to 2019) of Hg in birds from a breeding colony of Audouin's gull (*Ichthyaetus audouinii*) in the Ebro Delta to understand how fluctuations in Hg levels are coupled to human activities in the industrial area in the upstream region of the river. Stable isotopic signatures of C and N ($\delta^{13}\text{C}_{\text{bulk}}$ and $\delta^{15}\text{N}_{\text{bulk}}$) are determined to characterize the trophic ecology of the species. Since only $\delta^{13}\text{C}_{\text{bulk}}$ but not $\delta^{15}\text{N}_{\text{bulk}}$ was associated with THg levels, we used compound-specific stable nitrogen isotope analysis of amino acids (AA-CSIA) to evaluate the causes of variation in $\delta^{15}\text{N}_{\text{bulk}}$ to further investigate the idea of a decoupling of $\delta^{15}\text{N}_{\text{bulk}}$ and THg over time. We found Audouin's gull to be sensitive to Hg variations in the environment due to anthropogenic changes and to be a good indicator species for this contaminant in the Ebro Delta.

Keywords: mercury, Audouin's gull, *Ichthyaetus audouinii*, Ebro Delta, CSIA-AA

1. Introduction

Mercury is a non-essential, ubiquitous toxic pollutant, that in sufficient concentration can have deleterious effects both in humans and wildlife (Clarkson and Magos 2006; Eagles-Smith et al. 2018; Evers 2018). Even if mercury is naturally present in the environment, during the past two centuries the anthropogenic-derived input of this metal into the environment has increased significantly (Selin 2009; UNEP 2019). The major emission sources of mercury from human activities include several industrial emissions (e.g., cement, chlor-alkali, coal combustion) and artisanal gold mining activities (UNEP 2019).

Inorganic mercury can undergo diverse transformation processes, of special importance is its methylation in aquatic environments (Gilmour and Henry 1991; Fitzgerald et al. 2007), which transforms inorganic mercury to methylmercury (MeHg), a highly toxic form that is readily bioavailable to biota (Morel et al. 1998). Furthermore, MeHg biomagnifies in aquatic food webs and is bioaccumulated in organisms (Wiener et al. 2003; Lavoie et al. 2013; Evers 2018). Therefore, top predators are exposed to higher mercury levels, thus being more susceptible to its effects. Seabirds have often been used as bioindicators of mercury in the environment as they often occupy the highest levels in food webs and because they are long-lived organisms, resulting in the bioaccumulation of mercury in their bodies (Monteiro and Furness 1995).

The Ebro Delta (NE Iberian Peninsula; Fig. 1) has been impacted by the legacy pollution of a chlor-alkali plant located ca. 100 km upstream of the Ebro river mouth, in the locality of Flix, which has been operating since the late 19th century (ACA 2013). During the active period of the plant, 550 000 – 700 000 m³ of contaminated sediments, mainly with mercury and organochlorine compounds, were accumulated on the bottom of the river (Grimalt et al. 2003; ACA 2013). Although plant operations producing chlorine using mercury ceased in 2017 (Ercros 2019), legacy mercury pollution persists and has been impacting downstream Ebro River environments due to the transport of polluted particles along the river (Grimalt et al. 2003; Carrasco et al. 2010, 2011; Palanques et al. 2020). Moreover, during 2013-2014, a sediment remediation plan, including the dredging and disposal of polluted sediments, was implemented to remove toxicants from the riverbed in the vicinity of the factory (Ercros 2019).

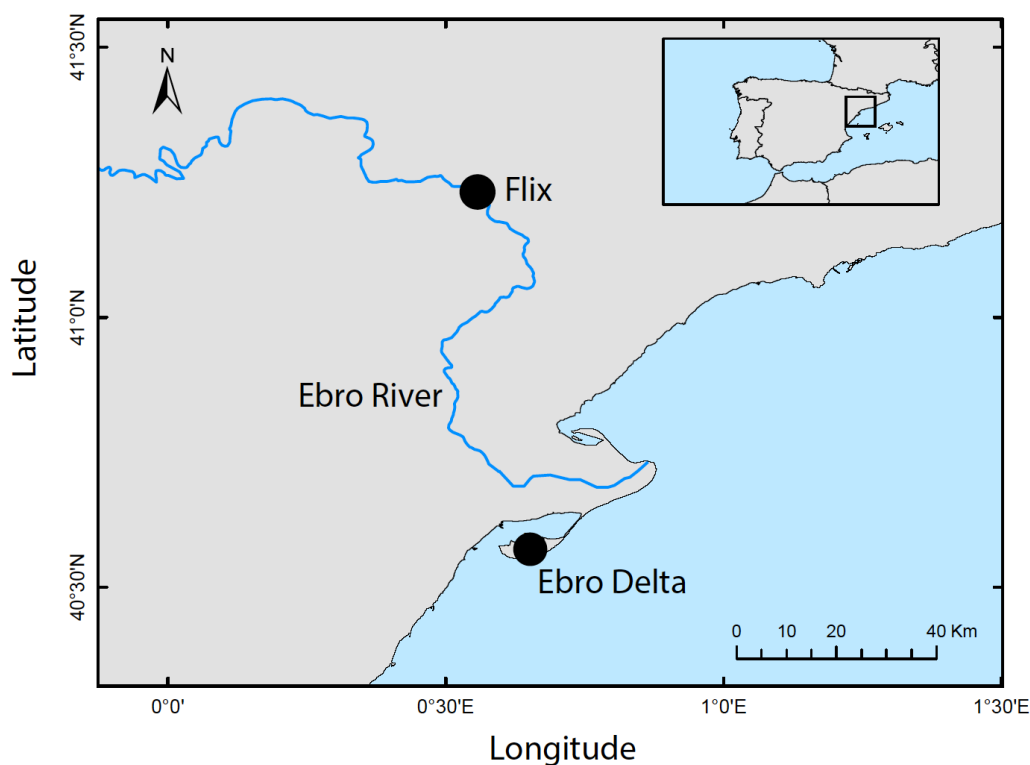


Figure 1 | Map showing the location of the Audouin's gull colony in the Ebro Delta at which samples were collected. Also shown is the location of the chlor-alkali industry upstream at Flix.

The Ebro Delta comprises 30,000 ha of wetlands, with 75% of its area used for rice-farming, 20% protected as a natural reserve, and 5% is urbanized (Mañosa et al. 2001). It is one of the most important wetlands in the western Mediterranean for the protection of birds. It is well known that wetland areas can constitute important mercury sinks and environments with high MeHg production rates (Boening 2000). This is due to the presence of anoxic environments where sulfate- and Fe(III)-reducing bacteria biomethylate inorganic mercury into MeHg (Podar et al. 2015). Furthermore, due to its biogeochemical activity, mercury and methylmercury levels in the Mediterranean Sea are high compared to other oceanic regions (Cossa and Coquery 2005); this is reflected in mercury concentrations in fishes (Llull et al. 2017; Junqué et al. 2018). Therefore, organisms feeding on prey associated with the Ebro Delta aquatic food webs will be exposed to MeHg. This is the case in top predators such as the Audouin's gull (*Ichthyiaetus audouinii*), an emblematic species breeding in the area. Audouin's gulls exploit freshwater resources, mainly rice-field prey, marine food resources, coming from fisheries discards in the neighboring area, and to a lesser extent prey from terrestrial environments (Oro et al. 1996; Ruiz et al. 1996; Pedrochi et al. 2002).

Bulk stable isotope signatures of carbon ($\delta^{13}\text{C}_{\text{bulk}}$) and nitrogen ($\delta^{15}\text{N}_{\text{bulk}}$) in feathers are often analyzed in order to characterize the trophic ecology of birds (Forero and Hobson 2003; Jaeger et al. 2010). Because stable isotope baselines can be highly variable between years in highly dynamic systems such as river deltas (Bouillon et al. 2011), these isotopes only provide rough estimates of gull trophic ecology (mostly, on trophic position exploited). More recently, compound specific stable isotope analysis of amino acids (AA-CSIA) of nitrogen is being used to estimate the trophic position of individuals providing more accurate insights into the trophic ecology of seabirds (McMahon et al. 2015; Gagne et al. 2018; Hebert and Popp 2018). Given that, in addition to measuring total mercury levels (THg), $\delta^{13}\text{C}_{\text{bulk}}$, and $\delta^{15}\text{N}_{\text{bulk}}$, we carried out AA-CSIA of nitrogen isotopes. An advantage of AA-CSIA is that it does not require estimating $\delta^{15}\text{N}_{\text{bulk}}$ in primary producers of food webs in order to generate a $\delta^{15}\text{N}$ baseline that is necessary to estimate trophic position of consumers (Hebert et al. 2016). The rationale behind AA-CSIA is the ability to estimate the trophic position of a consumer based on the main metabolic pathways that different amino acids undergo in cells (Chikaraishi et al. 2007). Those amino acids known as “trophic” amino acids, e.g., glutamic acid (Glu), undergo deamination and transamination reactions, cleaving N-C bonds, which results in nitrogen isotope fractionation. Thus, $\delta^{15}\text{N}$ values in trophic amino acids will be greater in consumers compared to their diet. Conversely, “source” amino acids, e.g., phenylalanine (Phe), only undergo deamination/transamination reactions to a very limited degree, thus, “source” AA will reflect $\delta^{15}\text{N}$ values at the base of the food web (Chikaraishi et al. 2007; Popp et al. 2007; Hebert et al. 2016).

Our work evaluates temporal trends in mercury levels in Audouin’s gulls from the Ebro Delta colony from 2004 to 2019. Fledglings were used as biomonitors since they are mainly fed prey from the area around the colony (Ruiz et al. 1996). Mercury concentrations were analyzed in mantle feathers as feathers are one of the main pathways of methylmercury excretion in birds (Goede and De Bruin 1986; Bond and Diamond 2009). Specifically, we (i) describe temporal trends of mercury in Audouin’s gulls from the Ebro Delta breeding colony during two periods: 2004-2013 (during which the chlor-alkali plant reduced its activity) and 2014-2019 (the period following sediment remediation which could have affected the bioavailability of legacy Hg in the environment), and (ii) evaluate the contribution of both trophic position and foraging area to the observed Hg levels in gulls from the Ebro Delta colony.

2. Materials and Methods

2.1. Sample collection

The present study was conducted in the Ebro Delta Natural Park (NE Iberian Peninsula: 40°33' N, 00°39' E) during the breeding seasons of 2004, and from 2008 to 2019 (from 2005-2007 no field work was carried out at this colony). For each of the years, we sampled 10-30 chicks at the fledging stage (approx. 3 weeks old). We sampled one chick per brood to avoid pseudoreplication. From each individual we took 10-15 mantle feathers and stored them in polyethylene bags until analysis. Upon sample collection, we ringed each individual with a metal and plastic ring to aid identification and measured weight, right tarsus length and bill-head length.

2.2. Total mercury determination

Feathers were washed using 0.25 M NaOH and rinsed with distilled water (following Bearhop et al. 2002). After cleaning, samples were dried to constant mass at 50°C and then ground to a homogenous powder with an impactor mill (Freezer Mill 6850, SpexCertiPrepH Inc., Metuchen, NJ, USA) operating at liquid nitrogen temperature.

Total mercury (THg) concentration in feathers (ng/g dry weight) was determined by ICP-MS (samples from 2004, 2008-2014) or by Direct Mercury Analyzer (Milestone® DMA-80; samples from 2015-2019). For ICP-MS mercury determinations, approximately 30 mg of feather sample were acid digested in a microwave oven using HNO₃ (1-2 mL) and H₂O₂ (0.5-1 mL) in Teflon containers. For DMA-80 mercury determinations, feather samples (approx. 20-30 mg) were directly analyzed by combustion. To check the accuracy of measurements using both methodologies, we analyzed a human hair certified reference material (NIES No. 13 CRM), every 10-15 samples. Mean recoveries for ICP-MS and DMA-80, ranged from 98 – 101% and 90 – 94%, respectively. Therefore, no corrections were applied to the THg values of the samples prior to statistical analyses.

2.3. C and N bulk stable isotope signature determination

Bulk stable isotopic analyses for carbon and nitrogen and AA-CSIA for N, were carried out at the Scientific and Technical Centers of the Universitat de Barcelona (Spain). For the bulk C and N analyses, 0.30-0.35 mg of feather powder were placed into tin capsules

and analyzed using a Thermo-Finnigan Flash 1112 elemental analyser (CE Elantech, Lakewood, NJ, USA) coupled to a Delta-C isotope ratio mass spectrometer via a CONFLO III interface (Thermo Finnigan MAT, Bremen, Germany). Stable isotope signatures are expressed using the standard delta notation relative to Vienna Pee Dee Belemnite ($\delta^{13}\text{C}_{\text{bulk}}$) and atmospheric nitrogen ($\delta^{15}\text{N}_{\text{bulk}}$). IAEA standards were applied every 12 samples to calibrate the system. Replicate assays of standards indicated analytical measurement errors of $\pm 0.1\text{‰}$ and $\pm 0.2\text{‰}$ for $\delta^{13}\text{C}_{\text{bulk}}$ and $\delta^{15}\text{N}_{\text{bulk}}$, respectively.

2.4. Compound Specific Stable Isotopic Analysis of Amino Acids (AA-CSIA)

In a subset of $N = 10$ samples for each of the years 2010, 2013, 2014, 2017 we performed nitrogen AA-CSIA. Briefly, 3 mg of sample were acid-digested in 1 mL of HCl 6 M for 20 h at 110 °C. Samples were then evaporated until completely dry (approximately 2h) under a stream of N_2 . Next, AA derivatization was performed with the addition of 50 μL of pyridine and 50 μL of N-tert-Butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA) to the samples and allowed to react at 70°C for 1h. Finally, derivatized AA were injected into a gas chromatography column to separate them and $\delta^{15}\text{N}$ for each of the AA was determined by means of a Trace GC ULTRA coupled to a Delta V Advantage isotope ratio mass spectrometer via a GC-Isolink interface (ThermoFisher Scientific). For each sample, we determined $\delta^{15}\text{N}$ values in 11 AA (Ala, Gly, Val, Leu, Ile, Pro, Ser, Thr, Phe, Asp, Glu) adjusted by an internal standard (norleucine, Nor) with known isotopic composition (Nor: $\delta^{15}\text{N}$ (mean $\pm$ SD): 14.60 ± 0.94) co-injected with each sample.

From the AA-CSIA determinations, the trophic position (TP) of the individuals was estimated using a multi-TDF (trophic discrimination factor) approach (Hebert et al. 2016; see the Supplementary Material, Table S1 for a comparison between TP calculation approaches), based on glutamic acid (Glu) as “trophic” amino acid, and phenylalanine (Phe) as “source” amino acid:

$$\text{TP}_{\text{AA-CSIA}} = 1 + \left[\frac{\Delta\delta^{15}\text{N}_{\text{GluPhe}} + f_{(\text{aq})} \times \beta_{(\text{aq})} + (1 - f_{(\text{aq})}) \times \beta_{(\text{terr})}}{\text{TDF}_{(\text{GluPhe})\text{avian}}} \right]$$

where $\Delta\delta^{15}\text{N}_{\text{GluPhe}}$ is the difference in feather $\delta^{15}\text{N}$ values of Glu and Phe (i.e., $\delta^{15}\text{N}_{\text{Glu}} - \delta^{15}\text{N}_{\text{Phe}}$), $f_{(\text{aq})}$ is the fractional abundance of aquatic food in the diet (we set it to 0.8, according to the results by Navarro et al. (2010), although other studies have reported higher proportions of marine prey items [e.g., Pedrocchi et al. 2002; Sanpera et al. 2007; García-Tarrasón et al. 2013]). $\beta_{(\text{aq})}$ represents the difference between $\delta^{15}\text{N}$ values of Glu and Phe of

primary producers associated with aquatic food webs. It has been estimated to be -3.4‰ for primary producers in aquatic food webs (McClelland and Montoya 2002; Chikaraishi et al. 2010). $\beta(\text{terr})$ represents the difference in $\delta^{15}\text{N}$ between Glu and Phe of primary producers associated with terrestrial food webs, including those from rice-fields (+8.4‰ for terrestrial C3 plants; Chikaraishi et al. 2010). Finally, $\text{TDF}_{(\text{GluPhe})\text{avian}}$ is the trophic discrimination factor between food sources and avian-consumers in Glu relative to Phe (i.e., $\Delta^{15}\text{N}_{\text{Glu}} - \Delta^{15}\text{N}_{\text{Phe}}$) for marine food webs. In the case of seabirds, $\text{TDF}_{(\text{GluPhe})\text{avian}}$ has been estimated to be 3.5‰ for gentoo penguin (*Pygoscelis papua*; McMahon et al. 2015), which we found appropriate as a $\text{TDF}_{(\text{GluPhe})\text{avian}}$ proxy for the species in our study.

2.5. Statistical analyses

T-tests were used to compare THg concentrations, $\delta^{15}\text{N}_{\text{bulk}}$ between the two periods studied: before the dredging of sediments (from 2004-2013, hereafter “before management”) and after the dredging of sediments (from 2014-2019, hereafter “after management”). Regarding $\delta^{13}\text{C}_{\text{bulk}}$ values, due to highly skewed data distribution and the presence of influential values, we tested the difference in the medians between the two periods of study (before and after the management) using a permutation test that was run for 1000 iterations.

We used generalized least squares (GLS) to assess the association between THg levels in feathers and the two periods studied: before management and after management. The model included the following covariates: time (years), management period (before-management and after-management) and the interaction between these variables, while accounting for variance among years. A GLS framework was used to control for residual heteroscedasticity of THg among years (Fligner-Killeen test: $\chi^2 = 26.991$, $p = 0.007$), when estimating the parameters in the model (Zuur et al. 2009). A second model was fitted by updating the previous model adding the explanatory variables $\delta^{15}\text{N}_{\text{bulk}}$, $\delta^{13}\text{C}_{\text{bulk}}$ and their respective interactions with the factor management period ($\delta^{15}\text{N}_{\text{bulk}} \times \text{Management}$, $\delta^{13}\text{C}_{\text{bulk}} \times \text{Management}$). Relationships between $\delta^{15}\text{N}_{\text{bulk}}$ with THg and $\delta^{13}\text{C}_{\text{bulk}}$ with THg are given as partial correlation coefficients (r_p) and 95% CI. The significance of the effects was tested by t-tests.

In addition, GLS models were fitted separately for $\delta^{15}\text{N}_{\text{bulk}}$ and $\delta^{13}\text{C}_{\text{bulk}}$ to evaluate the association between these response variables, and time (years), management period and the interaction between time $\times$ management period. All GLS models are fitted using the function `gls()` from the *nlme* R package (Pinheiro et al. 2019).

On the subset of samples analyzed using AA-CSIA methodology, separate multiple regression models were fitted to evaluate the effects of (i) time (years) and (ii) management period on the estimated trophic position (TP) of the individuals ($TP_{AA-CSIA}$) or $\delta^{15}N_{bulk}$. Partial correlation tests were performed to evaluate the relationship between THg or $\delta^{15}N_{bulk}$ versus $TP_{AA-CSIA}$ in the subset of samples analyzed using AA-CSIA. All statistical analyses used a significance level of $\alpha = 0.05$ unless otherwise specified. All statistical analyses were performed using R v. 3.5.1 (R Core Team, 2018).

3. Results

3.1. Total mercury temporal trends, relationship with $\delta^{15}N_{bulk}$ and $\delta^{13}C_{bulk}$

We analyzed feather samples from 237 individuals sampled in 2004 and 2008-2019. Mean total mercury (THg) concentration in fledgling feathers ranged from 2222.6 to 8448.5 ng/g (Table 1). Concentrations of THg in feathers were higher after sediment management than before ($t = 8.51$, $p < 0.0001$; Fig. 2). $\delta^{15}N_{bulk}$ values were significantly higher after management than before ($t = 15.95$, $p < 0.0001$). The permutation test for the difference of medians did not detect significant differences regarding the $\delta^{13}C_{bulk}$ before and after the management ($p = 0.54$; Fig. 2).

Table 1 | Descriptive statistics of annual total mercury concentrations, bulk $\delta^{13}C$, bulk $\delta^{15}N$, and amino acid-specific $\delta^{15}N$ values for Glu and Phe in feathers from fledgling Audouin's gulls.

Year	<i>N</i>	Feather [THg] (ng/g dw)		$\delta^{13}C_{bulk}$ (‰)		$\delta^{15}N_{bulk}$ (‰)		$\delta^{15}N_{AA}$ (‰)					
								$\delta^{15}N_{Glu}$		$\delta^{15}N_{Phe}$			
		Mean	SD	Mean	SD	Mean	SD	N	Mean	SD	Mean	SD	
2004	39	5250.9	1268.9	-17.09	0.38	12.30	0.35						
2008	15	7031.4	2782.6	-17.25	0.40	13.61	0.57						
2009	14	3166.2	1020.4	-17.69	0.71	13.03	0.32						
2010	16	4762.0	1408.6	-18.58	0.91	11.99	0.82	9	12.79	2.13	3.49	1.20	
2011	12	2418.3	635.6	-18.50	0.46	12.80	0.41						
2012	15	3172.9	745.5	-17.60	0.30	12.98	0.40						
2013	15	2222.6	474.6	-20.44	2.40	12.60	0.29	9	13.33	0.86	4.40	1.07	
2014	19	7884.4	2354.9	-17.14	0.87	13.71	0.41	10	13.89	1.57	4.01	1.10	
2015	15	5444.2	1596.5	-17.34	0.32	13.96	0.41						
2016	19	4246.8	1286.6	-17.55	0.59	13.80	0.48						
2017	29	8448.5	1472.7	-17.88	0.78	13.85	0.39	10	13.86	1.05	4.80	0.97	
2018	14	5257.2	1384.4	-17.50	0.59	13.52	0.30						
2019	15	7501.9	1392.2	-17.78	0.70	13.80	0.28						

From the GLS model evaluating the effect of time and management period on THg concentrations in feathers we found a significant interaction between management period and time ($t = -5.11$, $p < 0.0001$). From 2004-2013 (before management period), feather THg concentrations decreased significantly ($t = -12.99$, $p < 0.0001$). In 2014, feather THg levels increase and from 2014-2019 there is an increase in feather THg levels through time ($t = 2.34$, $p = 0.020$; Fig. 3).

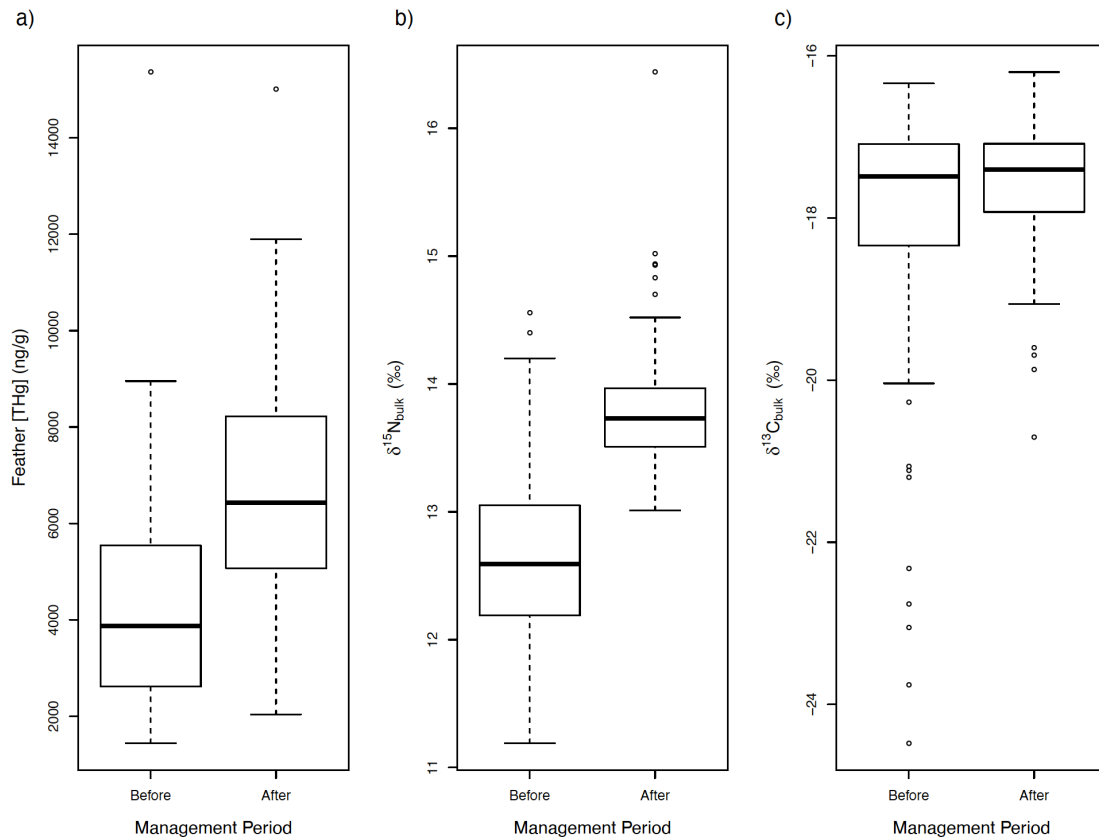


Figure 2 | Boxplots of (a) [THg] (ng/g), (b) $\delta^{15}\text{N}_{\text{bulk}}$ (‰) and (c) $\delta^{13}\text{C}_{\text{bulk}}$ (‰) measured in mantle feathers of fledgling Audouin's gulls collected from the breeding colony in the Ebro Delta. Management periods are before (2004-2013) and after (2014-2019) sediment management activities associated with remediating the chlor-alkali plant site at Flix in 2013-2014 (see text for details). Black bars represent median values, boxes represent the interquartile range, and circles represent data outliers.

The model for THg as response variable, with the addition of $\delta^{15}\text{N}_{\text{bulk}}$ and $\delta^{13}\text{C}_{\text{bulk}}$ as their interactions with management period, showed a significant association between THg and $\delta^{13}\text{C}_{\text{bulk}}$ ($r_p = 0.36$, 95%CI: 0.24 – 0.47, $p < 0.0001$). However, we did not find any statistically significant association between THg levels and $\delta^{15}\text{N}_{\text{bulk}}$ ($r_p = 0.06$, 95%CI: -0.07 – 0.18, $p = 0.41$). Interactions of $\delta^{15}\text{N}_{\text{bulk}}$ and $\delta^{13}\text{C}_{\text{bulk}}$ with management period were not significant ($\delta^{15}\text{N}_{\text{bulk}} \times \text{Management}$: $t = -0.97$, $p = 0.33$; $\delta^{13}\text{C}_{\text{bulk}} \times \text{Management}$: $t = -0.01$, $p = 0.99$).

Regarding $\delta^{13}\text{C}_{\text{bulk}}$, we detected an association with time ($t = -3.43$, $p = 0.0007$) and management period ($t = -3.90$, $p = 0.0001$; Fig. 3), but no significant interaction between these two variables was observed ($t = 1.44$, $p = 0.15$). $\delta^{13}\text{C}_{\text{bulk}}$ decreased from 2004 to 2013 and from 2014 to 2019. Compared to the rest of the years, $\delta^{13}\text{C}_{\text{bulk}}$ values were anomalously low in 2013 ($t = -4.49$, $p < 0.0001$; Table 1).

We found significant differences between $\delta^{15}\text{N}_{\text{bulk}}$ and management period ($t = -5.67$, $p < 0.0001$) with higher values after management (Fig. 3). There was a significant interaction between time and management for $\delta^{15}\text{N}_{\text{bulk}}$ ($t = 2.91$, $p = 0.004$). $\delta^{15}\text{N}_{\text{bulk}}$ values increased slightly during the period before management ($t = 5.61$, $p < 0.0001$), while after management there was no significant trend ($t = -0.50$, $p = 0.62$); however, $\delta^{15}\text{N}_{\text{bulk}}$ values remained higher than before management. Higher $\delta^{15}\text{N}_{\text{bulk}}$ values could be explained by gulls feeding at a higher trophic position (Blight et al. 2015) or a change in baseline $\delta^{15}\text{N}_{\text{bulk}}$ values (Cole et al. 2004) resulting in an overall change in food web $\delta^{15}\text{N}_{\text{bulk}}$ values. Therefore, to evaluate these two scenarios we estimated gull trophic position using CSIA-AA ($\text{TP}_{\text{CSIA-AA}}$) in a subset of samples.

All the GLS models were fitted again excluding 2013 data, however, there was no interpretation difference in the results obtained and therefore, the discussion of the results is based on the models including the data from 2013.

3.2. Compound-specific stable isotope analysis of amino acids (AA-CSIA)

We analyzed $N = 10$ samples of each of the following years: 2010, 2013, 2014 and 2017. For all, but one sample from 2010 and one sample from 2013 (both samples excluded from the analyses since we could not measure AA $\delta^{15}\text{N}$ values for Glu), we determined $\text{TP}_{\text{AA-CSIA}}$ (TP estimated using $\delta^{15}\text{N}$ values in Phe and Glu; Table 1; Table S1). In the subset of samples selected for AA-CSIA of nitrogen, there were no significant differences in $\text{TP}_{\text{AA-CSIA}}$ among years ($F_{(3,34)} = 0.59$, $p = 0.62$), nor considering before management and after management years as a factor ($F_{(1,36)} = 0.42$, $p = 0.52$; Fig. 4). We found significant differences among years ($F_{(3,34)} = 19.24$, $p < 0.0001$) and management periods ($F_{(1,36)} = 60.69$, $p < 0.0001$) on $\delta^{15}\text{N}_{\text{bulk}}$ in this subset of samples. $\text{TP}_{\text{AA-CSIA}}$ and $\delta^{15}\text{N}_{\text{bulk}}$ were not significantly correlated ($r_p = -0.20$, $p = 0.23$; Fig. 4). Furthermore, $\text{TP}_{\text{AA-CSIA}}$ was not correlated with THg in feathers ($r_p = 0.20$, $p = 0.24$; Fig. 5).

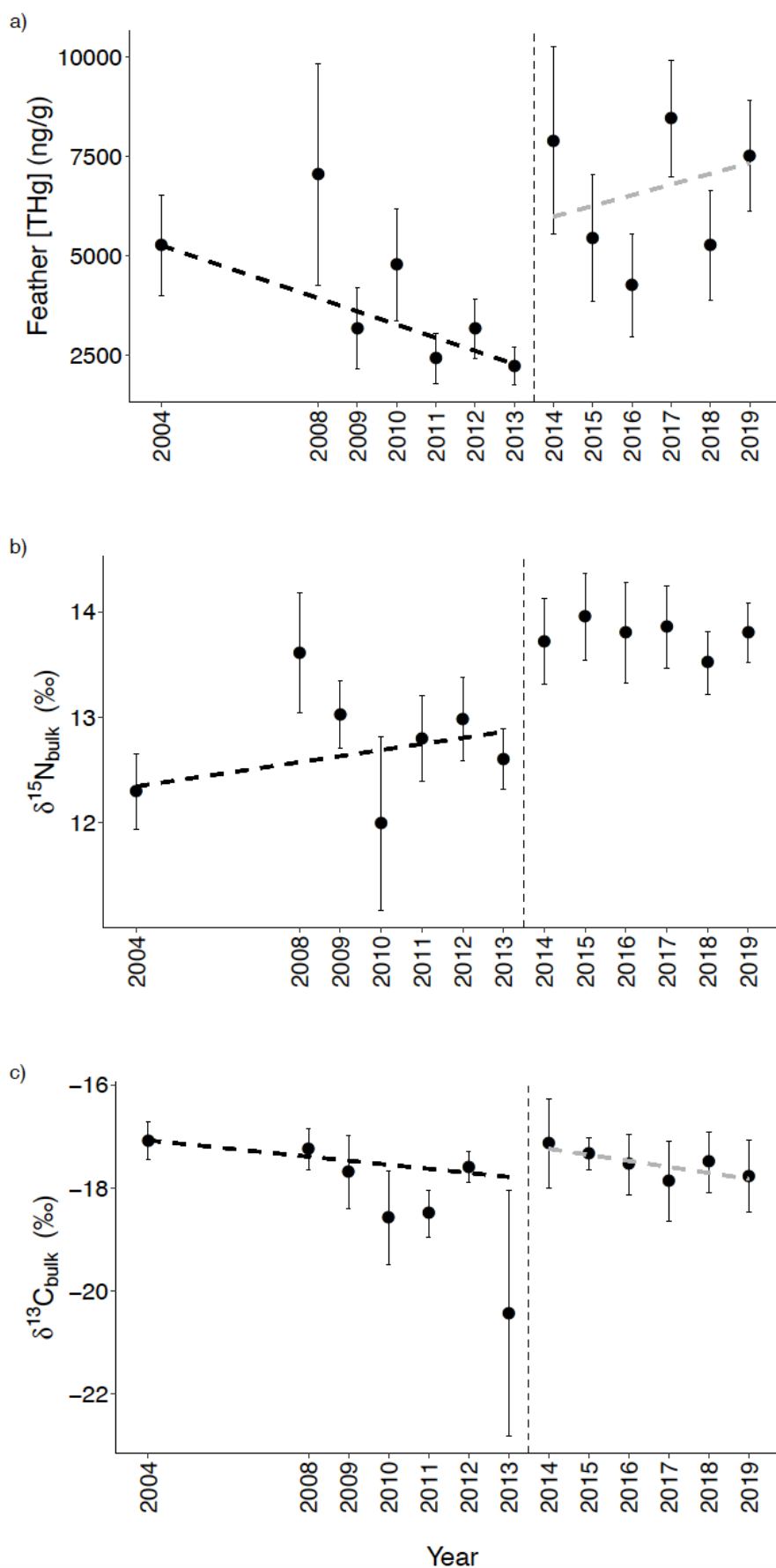


Figure 3 | Temporal trends of (a) [THg] (ng/g), (b) $\delta^{15}\text{N}_{\text{bulk}}$ (‰) and (c) $\delta^{13}\text{C}_{\text{bulk}}$ (‰) measured in mantle feathers of Audouin's gull fledglings taken from the breeding colony in the Ebro Delta. Points are means $\pm$ SD (solid lines). When statistically significant, re-gression trends are shown (black and grey dashed lines). Vertical dashed-line divides periods before and after sediment remediation at the chlor-alkali site (see text for details).

Figure 4 | $\delta^{15}\text{N}_{\text{bulk}}$ (‰) versus trophic position estimated from AA-CSIA ($\text{TP}_{\text{AA-CSIA}}$). Different symbols represent years analyzed (2010: $N=9$; 2013: $N=9$; 2014: $N=10$; 2017: $N=10$). Filled symbols represent the years before sediment remediation at the chlor-alkali site and empty symbols the years after sediment management. No significant correlation was found between $\delta^{15}\text{N}_{\text{bulk}}$ (‰) and $\text{TP}_{\text{AA-CSIA}}$ values.

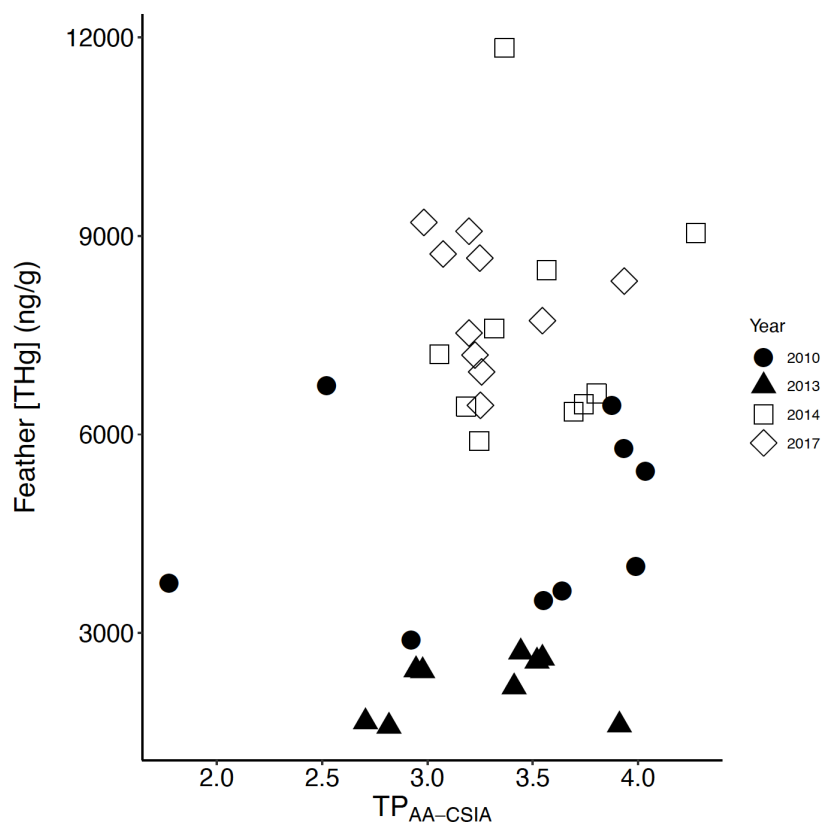
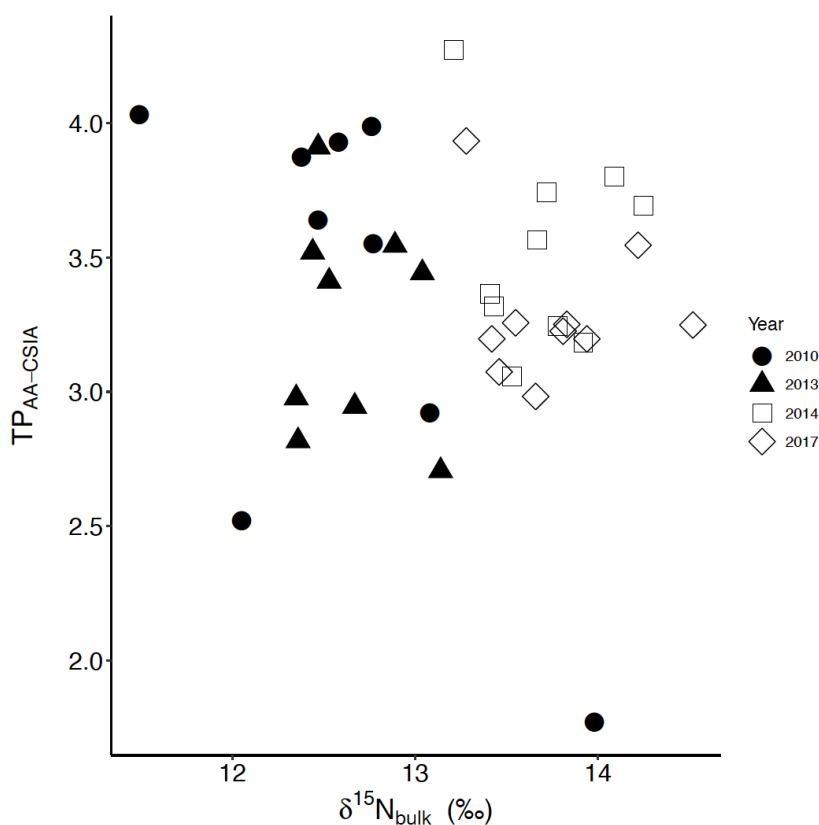


Figure 5 | Scatterplot of feather [THg] versus trophic position estimated by AA-CSIA ($\text{TP}_{\text{AA-CSIA}}$). Different symbols represent years analyzed (2010: $N=9$; 2013: $N=9$; 2014: $N=10$; 2017: $N=10$). Filled symbols represent the years before sediment remediation at the chlor-alkali site and empty symbols the years after sediment management. No significant correlation between $\text{TP}_{\text{AA-CSIA}}$ values and [THg] in feathers was found.

4. Discussion

Effects of mercury on birds have been extensively studied elsewhere (Seewagen 2010; Whitney and Cristol 2017; Evers 2018). In 54% of the fledglings studied, THg levels in feathers were above those considered to have adverse effects on birds (5000 ng/g for feathers; Burger and Gochfeld 1997), although no experimental studies have been performed with this species to confirm these thresholds. Furthermore, we acknowledge that such thresholds could vary, not only because they could be species-specific, but also in relation to particular environmental conditions, both biotic and abiotic (Goutte et al. 2014).

In the Ebro Delta, Audouin's gull is the species in which highest total mercury (THg) levels have been found, both in adults (Arcos et al. 2002) and in eggs (Morera et al. 1997). As far as we know, THg levels at the fledgling stage in Audouin's gulls from the Ebro Delta, might be among the highest levels found in seabird species inhabiting that area (Cotín et al. 2011). The reason could be the differential dietary resources Audouin's gulls exploit, feeding mostly on fisheries discards but also on prey originating from rice-fields and terrestrial sites (Oro et al. 1996; Ruiz et al. 1996; Sanpera et al. 2007; García-Tarrasón et al. 2013). Fisheries discards are mainly comprised of large proportions of mesopelagic and benthic fish species (Pedrocchi et al. 2002) which occupy higher trophic levels than epipelagic fishes (Arcos et al. 2002), and inhabit environments in the water column which enhance mercury methylation (Monteiro et al. 1996; Chouvelon et al. 2012), thus potentially accumulating and transferring more MeHg to consumers. Rice-field prey include the red crayfish (*Procambarus clarkii*; Navarro et al. 2010), which is known to bioaccumulate Hg in polluted environments (Suárez-Serrano et al. 2010) transferring it to higher trophic levels of food webs (Geiger et al. 2005). Furthermore, compared to other Audouin gull breeding colonies along the Iberian Peninsula, available data suggest that fledglings from the Ebro Delta colony have the highest levels of THg, this has been attributed to a higher Hg pollution in the Ebro Delta area (Sanpera et al. 2007; García-Tarrasón 2013).

THg in Audouin's gulls from the Ebro Delta breeding colony increased from 2004 to present. However, mercury trends look markedly different when considering the years before and after management of the chlor-alkali site. First, we found a significant decrease in feather mercury levels from 2004 to 2013, possibly reflecting the reduction of activities associated with the chlor-alkali plant in 2004 (Ercros 2019) and a consequent decrease in Hg emissions (Pujadas 2016) with the possibility of ecosystem recovery (Kopec et al. 2019). From 2014 onwards, although the plant operations were decreasing (Pujadas 2016) until the complete

cessation in 2017 (Ercros 2019), there was a slight increase in feather THg levels over time. Thus, an effect of the 2013 management actions that took place near the chlor-alkali plant at Flix on the environmental availability of Hg cannot be discounted. Dredging of sediments could have remobilized legacy mercury (Foxgrover et al. 2019) leading to an increase in THg levels in gull fledglings from 2014 onwards. Therefore, as observed in other studies with lower trophic level fauna (fishes: Carrasco et al. 2011, arthropods: George and Batzer 2008, mussels: Carrasco et al. 2008), foodwebs in mercury-affected areas integrate information on mercury levels in the environment and fluctuations in Hg availability.

Regarding the relationship of THg levels and trophic ecology isotopic markers (C and N), we found a positive correlation between THg and $\delta^{13}\text{C}_{\text{bulk}}$, and significant variation in $\delta^{13}\text{C}_{\text{bulk}}$ values across years. In particular, $\delta^{13}\text{C}_{\text{bulk}}$ values in 2013 were lower than in the rest of the years, suggesting that fledglings were fed a higher proportion of freshwater or terrestrial prey in that year (Bearhop et al. 1999). This is consistent with the fact that $\delta^{13}\text{C}_{\text{bulk}}$ values observed in 2013 ($\delta^{13}\text{C}_{\text{bulk}}$ [mean $\pm$ SD]: -20.44 ± 2.40) were closer to those observed in other *Laridae* species breeding in the area and which forage mainly on freshwater aquatic prey (reported in Cotín et al. 2011: Whiskered terns (*Chlidonias hybridus*; $\delta^{13}\text{C}_{\text{bulk}}$ [mean $\pm$ SD]: -24.6 ± 0.7) and Gull-billed terns (*Sterna nilotica*; $\delta^{13}\text{C}_{\text{bulk}}$ [mean $\pm$ SD]: -24.8 ± 0.5)). Although the lower $\delta^{13}\text{C}_{\text{bulk}}$ signatures in 2013 might be partly related to the lower levels of THg in that year, no firm conclusion can be made unless we reconstruct the diet to estimate the proportion of freshwater prey in chick diets in 2013 compared to other years. Furthermore, recently developed methodologies to estimate the contribution of different sources of Hg to the total mercury burden in organisms such as Hg stable isotope analyses (Renedo et al. 2018; Feng et al. 2019) seem a promising tool to that aim.

In the case of $\delta^{15}\text{N}_{\text{bulk}}$ values, the expected correlation with THg (Coehlo et al. 2013), as $\delta^{15}\text{N}_{\text{bulk}}$ is usually regarded as a proxy for trophic position and THg biomagnifies along food webs, was not encountered in our study. However, $\delta^{15}\text{N}_{\text{bulk}}$ values were significantly higher in the years after sediment management than before. Remarkably, $\delta^{15}\text{N}_{\text{bulk}}$ increased significantly between 2013 and 2014, and afterwards, $\delta^{15}\text{N}_{\text{bulk}}$ values remained higher than during the 2004-2013 period. Even if both $\delta^{15}\text{N}_{\text{bulk}}$ values and THg concentrations increased in the years after sediment management the models fitted did not find any relationship between $\delta^{15}\text{N}_{\text{bulk}}$ and THg in our study system, indicating that both processes were uncoupled and that THg levels were not related to changes in trophic position across years but rather to concurrent higher levels of both ^{15}N and Hg in the environment (Rumbold et al. 2018). To

further explore the possible causes of $\delta^{15}\text{N}_{\text{bulk}}$ variation we conducted AA-CSIA analyses (Hebert et al. 2016).

In this study, AA-CSIA was used to estimate the trophic position of individual birds during periods before and after sediment remediation. Gulls, as tertiary consumers, are expected to occupy a trophic position above 3.0 (Chikaraishi et al. 2009). In our study this is the case of most individuals when using a multi-TDF approach to estimate the trophic position. Otherwise, using a single-TDF approach (Chikaraishi et al. 2009) on the same dataset did provide underestimated trophic position values ($\text{TP} < 3.0$) for most of the gulls (Table S1). Thus, in the case of top-predators feeding from both terrestrial and marine resources, it is advisable to use a multi-TDF approach (Hebert et al. 2016). Even if using a multi-TDF approach, the trophic position of some individuals in our study is underestimated ($\text{TP} < 3.0$). This could be due to the $\text{TDF}_{\text{GluPhe}}$ value applied to compute $\text{TP}_{\text{AA-CSIA}}$. A recent meta-analysis on $\text{TDF}_{\text{GluPhe}}$ among different wildlife groups has determined that there is high variability on this value among different animal taxa (Nielsen et al. 2015) and ideally, a specific $\text{TDF}_{\text{GluPhe}}$ value for the model species is needed to improve $\text{TP}_{\text{AA-CSIA}}$. Estimating specific $\text{TDF}_{\text{GluPhe}}$ needs to be done in experimental conditions, which are often difficult to attain for a wild species. Nevertheless, even of this underestimation of TP, since we are dealing with the same species and applying the same formula to calculate the TP for all the individuals, this must not be an issue regarding the results obtained. Interestingly, we did not find any significant difference between the pre-management and the post-management period regarding the $\text{TP}_{\text{CSIA-AA}}$ of the gulls, and therefore, the increase in $\delta^{15}\text{N}_{\text{bulk}}$ is likely due to causes other than a trophic shift of the species. For instance, due to the eutrophication of the environment (Cole et al. 2004; Cotín et al. 2011). Both past and ongoing eutrophication could have led to an increase on the $\delta^{15}\text{N}_{\text{bulk}}$ at the base of the food web translating to an overall $\delta^{15}\text{N}_{\text{bulk}}$ increase through the food web. Wastewaters from industries or fertilizer applications could have been one of the multiple causes leading to the eutrophication observed (McClelland and Valiela 1998; Hebert and Wassenaar 2001; Cole et al. 2004; Anderson and Cabana 2006). These results point out that after 2013 the bioavailability of THg in the environment increased along with an eutrophication episode, which, in parallel, lead to an increase in $\delta^{15}\text{N}_{\text{bulk}}$ in the food webs of the Ebro Delta area. Although limited in sample size, we demonstrate how AA-CSIA can be used to assess possible changes in TP over time and explore relationships with mercury bioavailability or mercury sources.

Correlations between $\delta^{15}\text{N}_{\text{bulk}}$ and biomagnifying pollutants such as mercury in biota inhabiting systems where changes in baseline $\delta^{15}\text{N}_{\text{bulk}}$ values may be taking place should be

interpreted with caution. Possible shifts in baseline $\delta^{15}\text{N}_{\text{bulk}}$ values need to be considered when assessing possible TP changes (Post 2002; Dolgova et al. 2018; Hebert and Popp 2018). The assumed relationship between $\delta^{15}\text{N}_{\text{bulk}}$ and biomagnifying pollutants in systems where possible eutrophication could have taken place in conjunction with pollution episodes needs to be explored more in depth in order to avoid confounding interpretations of $\delta^{15}\text{N}_{\text{bulk}}$ increase. To that aim, compound specific stable nitrogen isotope analysis of amino acids can be a reliable tool to assess potential shifts in the trophic position of individual organisms.

In summary, we found that Audouin's gull fledglings were good bioindicators of Hg contamination in the Ebro Delta. Long-term monitoring studies focused on this high trophic level integrator species could aid in the evaluation of ecosystem Hg loads as well as other pollutants.

Acknowledgements

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Supplementary Material

Following up mercury pollution in the Ebro Delta (NE Spain): Audouin's gull fledglings as model organisms to elucidate anthropogenic impacts on the environment

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Comparison of $TP_{AA-CSIA}$ estimations using different formulas available in the literature.

Different approaches to trophic position estimation have been suggested using AA-CSIA analyses, such as the single-TDF approach (Chikaraishi et al. 2009) and the multi-TDF approach (Hebert et al. 2016). To compare the $TP_{AA-CSIA}$ estimates to assess which formula is more appropriate for the gulls in our study, we have estimated the TP using both formulas on our study dataset.

To calculate the trophic position (TP) using the two methods (single- and multi-TDF approaches) we used the following formulas, based on glutamic acid (Glu) as “trophic” amino acid, and phenylalanine (Phe) as “source” amino acid:

(a) a single-TDF (trophic discrimination factor) approach (Chikaraishi et al. 2009):

$$TP_{AA-CSIA} = 1 + \left[\frac{\delta^{15}N_{Glu} - \delta^{15}N_{Phe} + \beta}{TDF_{(GluPhe)avian}} \right]$$

where $\delta^{15}N_{Glu}$ is the N isotopic signature for glutamate, $\delta^{15}N_{Phe}$ is the N isotopic signature for phenylalanine. $TDF_{(GluPhe)avian}$ is the trophic discrimination factor between food sources and avian-consumers in Glu relative to Phe (i.e., $\Delta^{15}N_{Glu} - \Delta^{15}N_{Phe}$) for marine food webs. In the case of seabirds, $TDF_{(GluPhe)avian}$ has been estimated to be 3.5‰ for gentoo penguin (*Pygoscelis papua*; McMahon et al. 2015), which we found appropriate as a $TDF_{(GluPhe)avian}$ proxy for the species in our study. Finally, β is the difference between $\delta^{15}N$ values of Glu and Phe in primary producers. It has been estimated to be -3.4‰ for primary producers in aquatic food webs (McClelland and Montoya 2002; Chikaraishi et al. 2010).

(b) a multi-TDF (trophic discrimination factor) approach (Hebert et al. 2016):

$$TP_{AA-CSIA} = 1 + \left[\frac{\Delta\delta^{15}N_{GluPhe} + f_{(aq)} \times \beta_{(aq)} + (1 - f_{(aq)}) \times \beta_{(terr)}}{TDF_{(GluPhe)avian}} \right]$$

where $\Delta\delta^{15}N_{GluPhe}$ is the difference in feather $\delta^{15}N$ values of Glu and Phe (i.e., $\delta^{15}N_{Glu} - \delta^{15}N_{Phe}$), $f_{(aq)}$ is the fractional abundance of aquatic food in the diet (we set it to 0.8, according to the results by Navarro et al. (2010), although other studies have reported higher proportions of marine prey items [e.g., Pedrocchi et al. 2002; Sanpera et al. 2007; García-Tarrasón et al. 2013]). $\beta_{(aq)}$ represents the difference between $\delta^{15}N$ values of Glu and Phe of primary producers associated with aquatic food webs (-3.4‰). $\beta_{(terr)}$ represents the difference in $\delta^{15}N$ between Glu and Phe of primary producers associated with terrestrial food webs, including those from rice-fields (+8.4‰ for terrestrial C3 plants; Chikaraishi et al. 2010). Finally, $TDF_{(GluPhe)avian}$ is the trophic discrimination factor between food sources and avian consumers in Glu relative to Phe for marine food webs (3.5‰ for gentoo penguin; McMahon et al. 2015).

The results are shown in Table S1. The multi-TDF approach provided better estimates of $TP_{AA-CSIA}$ for Audouin's gull ($TP > 3.0$, for tertiary consumers) as it allowed to account for different food resources' proportions and thus, we applied these estimations throughout the study.

Table S1 | Descriptive statistics (mean and SD) of annual trophic position based on amino acid-specific $\delta^{15}N$ values for Glu and Phe in feathers from fledgling Audouin's gulls ($TP_{AA-CSIA}$) using (a) single-TDF and (b) multi-TDF approaches.

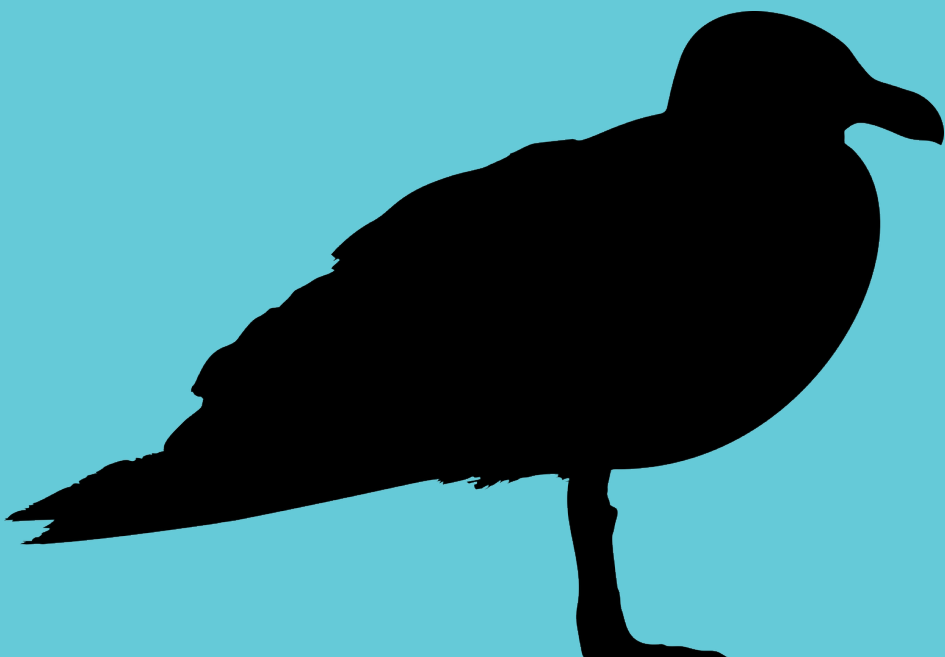
		$TP_{AA-CSIA}$			
		Single-TDF		Multi-TDF	
Year	N	Mean	SD	Mean	SD
2010	9	2.69	0.79	3.36	0.79
2013	9	2.58	0.40	3.25	0.40
2014	10	2.85	0.37	3.52	0.36
2017	10	2.62	0.27	3.29	0.27

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05 CHAPTER 5

Mercury stable isotopes in seabirds in the Ebro Delta (NE Iberian Peninsula): inter-specific and temporal differences



05

CHAPTER 5

Mercury stable isotopes in seabirds in the Ebro Delta (NE Iberian Peninsula): inter-specific and temporal differences

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Isòtops estables de mercuri en aus marines del Delta de l'Ebre (NE Península Ibèrica): diferències inter-específiques i temporals

Resum

El mercuri (Hg) és un contaminant global, que afecta especialment els ecosistemes aquàtics, els marins i els d'aigua dolça. Per això, els predadors apicals que depenen d'aquests ambients, com les aus marines, sovint són considerades com a indicadors fiables de la contaminació per mercuri. En el Delta de l'Ebre (NE Península Ibèrica), el llegat de contaminació per mercuri precedent de la fàbrica de clor-àlcali que operava a la localitat de Flix, aproximadament 100 km aigües amunt de la desembocadura del riu Ebre ha impactat en els ambients del delta i les zones costaneres més properes. A més a més, els nivells de mercuri que ocorren de manera natural en el Mar Mediterrani són anormalment elevats, comparats amb altres àrees marines. En aquest treball hem utilitzat una aproximació mitjançant isòtops estables de Hg per esclarir els processos que afecten i les fonts responsables de les diferents concentracions de mercuri en tres espècies de *Laridae* que crien en simpatria al Delta de l'Ebre (gavina corsa [*Ichtyaethus audouinii*], gavina riallera [*Chroicocephalus ridibundus*], xatrac comú [*Sterna hirundo*]). Aquestes espècies presenten diverses ecologies tròfiques, fent un ús diferencial de recursos de procedència marina (peixos mesopelàgics i epipelàgics) i de preses procedents dels arrossars. Addicionalment, per *I. audouinii* per la qual en el Delta de l'Ebre s'han documentat diferències temporals en els nivells de Hg, hem investigat els canvis en la contribució de diferents fonts de mercuri utilitzant la mateixa aproximació. Els isòtops estables de mercuri diferencien les tres espècies atenent a les seves ecologies tròfiques. A més, en la gavina corsa hem observant variacions en les signatures isotòpiques de Hg a nivell temporal possiblement associades a polució d'origen antropogènic en l'àrea d'estudi. Finalment, destacar que aquest estudi representa la primera vegada que s'obtenen dades d'isòtops estables de Hg en aus marines del NO del Mediterrani.

Keywords: gulls, terns, rice-fields, Mediterranean, Audouin's gull

Abstract

Mercury (Hg) is a global pollutant, which specially affects aquatic ecosystems, both marine and freshwater. Thus, top-predators depending on these environments, such as seabirds, are regarded as suitable bioindicator species of mercury pollution. In the Ebro Delta (NE Iberian Peninsula), legacy mercury pollution from a chlor-alkali industry operating in Flix located ca. 100 km upstream of the Ebro River mouth has been impacting the delta environment and the neighboring coastal area. Furthermore, natural occurring levels of Hg in the Mediterranean Sea are known to be high compared to other marine areas. In this work we used a Hg stable isotopic approach to disentangle the mercury sources and processes leading to different Hg concentrations in three *Laridae* species breeding in sympatry in the area (Audouin's gull [*Ichtyaethus audouinii*], black-headed gull [*Chroicocephalus ridibundus*], common tern [*Sterna hirundo*]). These species have distinct trophic ecologies and make a different use of marine resources (mesopelagic and epipelagic fishes) and rice paddies prey. Moreover, for *I. audouinii* in which in the Ebro Delta colony temporal differences in Hg levels had been documented previously, we investigated the change in the contribution of different Hg sources using the same approach. Mercury stable isotopes differentiated the three different species according to their trophic ecologies. Furthermore, in *I. audouinii* we observed temporal variations in Hg isotopic signatures possibly owing to anthropogenic-derived pollution in the Ebro Delta. In addition, to the best of our knowledge this is the first time Hg stable isotopes have been reported in seabirds from the NW Mediterranean.

Keywords: gulls, terns, rice-fields, Mediterranean, Audouin's gull

1. Introduction

Mercury (Hg) is considered a global pollutant, it is present in all Earth compartments, and exerts detrimental effects on both humans and wildlife (Clarkson and Magos 2006; Driscoll et al. 2013; Evers 2018). Although mercury naturally occurs in the environment, it can also be found as result of human activities (Driscoll et al. 2013). Among anthropogenic activities introducing mercury into the environment are artisanal gold mining or industrial processes (e.g., coal combustion, chlor-alkali plants) (UNEP 2019). Anthropogenic activities have significantly increased mercury levels in the environment since the pre-industrial era (Amos et al. 2013; UNEP 2019), which poses an urge to monitor environmental loads of this pollutant and be able to distinguish between anthropogenic and natural sources in order to help environmental-related decisions (Wiener et al. 2003).

Particularities of this element include a complex biogeochemical cycle resulting in chemical speciation (Selin 2009; Gustin et al. 2020), which depends on both the biotic and abiotic factors in the environment where mercury is found (Ullrich et al. 2001; Lyman et al. 2020; Bowman et al. 2020). For instance, in aquatic environments, inorganic mercury (Hg^{2+}) is known to be methylated by mainly sulfate- and iron-reducing bacteria (Podar et al. 2015), but also by abiotic processes (Celo et al. 2006), transforming Hg^{2+} into methylmercury (MeHg). MeHg is a readily bioavailable and highly toxic form of mercury which can enter to the base of the food webs (Morel et al. 1998), bioaccumulate in organisms and get biomagnified along food webs (Lavoie et al. 2013; Evers 2018).

In this context, top-predator and long-lived species, such as seabirds, are more exposed to Hg and its negative effects including physiological, reproductive, neurological and teratogenic effects (Whitney and Cristol 2017). In fact, seabirds have been extensively used as bioindicator species of environmental pollution for an array of contaminants, including mercury (Furness and Camphuysen 1997; Becker et al. 2002; Burger and Gochfeld 2004; Sanpera et al. 2007; Blévin et al. 2013; Albert et al. 2019; Bond and Lavers 2020).

The Ebro Delta (NE Iberian Peninsula) comprises 30,000 ha of wetlands, with 75% of its area used for rice-farming, 20% protected as a natural reserve, and 5% is urbanized (Mañosa et al. 2001). Since the late 19th century, a chlor-alkali plant has been operating ca. 100 km upstream the Ebro River mouth. During the active period of the plant, 550 000 – 700 000 m³ of contaminated sediments, mainly with mercury and organochlorine compounds, were accumulated on the bottom of the river dam close to the factory (Grimalt et al. 2003; ACA 2013). Although the production of chlorine using mercury ceased in 2017, legacy

mercury pollution persists and has been impacting all the environments in the Ebro River delta, as a result of the transport of contaminated particles along the river (Carrasco et al. 2010, 2011; Cotín et al. 2012; Palanques et al. 2020). Moreover, during 2013-2014, a sediment remediation plan, including the dredging and disposal of polluted sediments, was implemented to remove toxicants from the riverbed in the vicinity of the factory. In a previous study, Sánchez-Fortún et al. (2020) reported the possible influence of the remediation tasks which took place in 2013-2014 on the increase of mercury levels observed in the Audouin's gull breeding colony in the Ebro Delta area. Furthermore, due to its biogeochemical activity, methylmercury levels in the Mediterranean Sea are high compared to other oceanic regions (Cossa and Coquery 2005). Thus, seabird species depending on food webs from the Ebro Delta area might be also exposed to naturally high levels of mercury (Arcos et al. 2002; Sanpera et al. 2007; Cotín et al. 2011; Sánchez-Fortún et al. 2020) besides legacy mercury coming from the chlor-alkali plant activity. However, the relative importance of mercury sources impacting aquatic habitats from the area (freshwater or coastal areas) has never been analyzed in depth.

In systems with acknowledged anthropogenic sources of Hg pollution, Hg stable isotopes have proven as a useful tool to unravel origins of Hg burdens in an array of environmental compartments (e.g., sediments [Gehrke et al. 2011; Wiederhold et al. 2015; Reinfelder and Janssen 2019], atmosphere [Estrade et al. 2010]), and in humans (Laffont et al. 2009; 2011) and wildlife (Day et al. 2012; Kwon et al. 2014; Bonsignore et al. 2015).

Mercury has seven stable isotopes with atomic masses 196, 198, 199, 200, 201, 202, 204 amu, which can undergo both mass-dependent fractionation (MDF) and mass-independent fractionation (MIF). MDF occurs as a consequence of an array of physical, chemical and biological processes including mercury biological methylation and demethylation (Kritee et al. 2009; Rodríguez-González et al. 2009; Janssen et al. 2016), photoreduction (Bergquist and Blum 2007), trophic transfer (Laffont et al. 2009), metabolic reactions (Laffont et al. 2009; Feng et al. 2015; Renedo et al. 2020; 2021). MIF occurs mainly as consequence of photochemical reactions in the water column prior to bioaccumulation of Hg (Bergquist and Blum 2007). Although MIF processes have been described for isotopes ^{200}Hg and ^{204}Hg (i.e., even-MIF), and for ^{199}Hg and ^{201}Hg (i.e., odd-MIF; Kwon et al. 2020), odd-MIF has been observed commonly in biological samples (Tsui et al. 2020). Thus, MDF and MIF signatures offer a framework to trace Hg pollution and may provide information on Hg sources in organisms. This information coupled with carbon and nitrogen stable isotopes values, which have been used extensively as proxies of the trophic ecology of

organisms (Post 2002; Boecklen et al. 2011), might provide valuable information on the causes leading to high Hg levels in wildlife.

The main aim of this study is to relate the trophic ecology of different *Laridae* species inhabiting in the Ebro Delta area to the total mercury concentration present in their bodies and to the mercury sources leading to the mercury body burdens observed. Thus, on one hand, we have analyzed Hg stable isotopes in feathers of fledglings individuals of three different species breeding in the Ebro Delta, that present contrasted dietary characteristics: the common tern (*Sterna hirundo*; SH), an epipelagic marine forager (Cotín et al. 2011), the black-headed gull (*Chroicocephalus ridibundus*; CR), which mostly forages in rice-fields (Antón-Tello et al. 2021), and the Audouin's gull (*Ichthyophaga audouinii*, IA), which forages in both marine environments (fisheries discards) and in rice-fields (Sanpera et al. 2007; García-Tarrasón et al. 2015). On the other hand, we analyzed the stable isotopes of Hg to unravel the differential contribution of various Hg sources over years on the Audouin's gull breeding colony in the Ebro Delta, to comprehend if differences of this heavy metal observed from before and after 2013 (Sánchez-Fortún et al. 2020), are related to changes in Hg sources.

2. Materials and methods

2.1. Fieldwork and sample collection

As part of a continued monitoring program of the Audouin's breeding colony in the Ebro Delta (NE Iberian Peninsula: 40°33' N, 00°39' E), 10-15 mantle feathers of juvenile individuals (approx. 3 weeks old) were sampled on years 2010, 2013, 2014, 2017 (N = 10 individuals each year; see Sánchez-Fortún et al. 2020 for further details on sampling). Additionally, during the breeding season of 2017, 10-15 mantle feathers of juvenile individuals from the following species breeding in the Ebro Delta area were sampled (N = 10 individuals for each species): common tern (*Sterna hirundo*; approx. 3 weeks old individuals), black-headed gull (*Chroicocephalus ridibundus*; approx. 3 weeks old individuals). Feathers were stored in zip-lock bags until analysis.

2.2. Total mercury determination

Total mercury (THg) determination in our samples was performed at the Scientific and Technological Centers of the University of Barcelona (Spain). Feather samples were washed using 0.25 M NaOH and rinsed with distilled water to remove external Hg contamination (Bearhop et al. 2000). After cleaning, samples were oven dried to constant

mass at 50°C and then ground to a homogenous powder with an impactor mill (Freezer Mill 6850, SpexCertiPrepH Inc., Metuchen, NJ, USA) operating at liquid nitrogen temperature. Total mercury concentration in feathers ([THg] ng/g dry weight) was determined by combustion using a Direct Mercury Analyzer (Milestone® DMA-80) for all the species samples from 2017, or by Induction Coupled Plasma-Mass Spectrometer (ICP-MS) for Audouin's gull samples from 2010, 2013 and 2014. DMA-80 analyses were directly analyzed by combustion using 0.20-0.30 mg of feather sample. For the ICP-MS mercury determinations, ~30 mg of feather sample were acid digested in a microwave oven using 1-2 mL of nitric acid (HNO₃ 70%) and 0.5-1 mL of hydrogen peroxide (H₂O₂ 30%) in Teflon containers. To check for the accuracy of measurements for both methods, we analyzed a certified reference material in duplicate (NIES-13 Human Hair CRM) every 10 to 15 samples with recoveries ranging from 91% to 94% for DMA-80 analyses and 97 to 103% in ICP-MS analyses. Thus, no corrections were applied to the THg values of the samples prior to statistical analyses.

2.3. Analysis of Hg stable isotopes

Stable isotopes of mercury were analyzed following method and protocol reported by Renedo et al. 2018, feather powder samples (aprox. 0.2 g) were digested with 5 mL of HNO₃ (65%, INSTRA quality) and 1/3 of the total volume of H₂O₂ (30%, ULTREX quality), and extracted in Hotblock at 75 °C during 8 h (6 h in HNO₃, plus 2 h after the addition of H₂O₂). Hg isotopic composition for each of the 6 most abundant stable isotopes of Hg (¹⁹⁸Hg, ¹⁹⁹Hg, ²⁰⁰Hg, ²⁰¹Hg, ²⁰²Hg and ²⁰⁴Hg) was determined by means of a cold-vapor generator (CVG)-multicollector (MC)-ICPMS (Nu Instruments). Hg isotopic values were reported using the delta notation relative to the bracketing standard NIST-3133 certified reference material. Instrumental mass-bias was corrected using the internal Tl standard, NIST 997 CRM. A secondary standard NIST RM-8160 (UM-Almadén standard) was used for validation of the analytical session. Furthermore, NIES-13 Human Hair CRM and an internal reference material composed of king penguin feathers (F-KP) were used for the Hg stable isotopes analyses. Results on mercury stable isotopes analyses are reported in detail in Table S1, Table S2 and Table S3 from the Supplementary Materials.

Mass-dependent fractionation (MDF) is reported as $\delta^{202}\text{Hg}$ values (‰) calculated as follows (Blum and Bergquist 2007):

$$\delta^{202}\text{Hg} = [({}^{202}\text{Hg}/{}^{198}\text{Hg})_{\text{sample}}/({}^{202}\text{Hg}/{}^{198}\text{Hg})_{\text{NIST3133}} - 1] \times 1000$$

Mass-independent Hg isotopic fractionation (MIF) is reported as $\Delta^{199}\text{Hg}$, $\Delta^{200}\text{Hg}$, $\Delta^{201}\text{Hg}$, $\Delta^{204}\text{Hg}$ (‰), calculated using the difference by the $\delta^{\text{xxx}}\text{Hg}$ value and a value predicted based on MDF (Bergquist and Blum 2007):

$$\Delta^{204}\text{Hg} \approx \delta^{204}\text{Hg} - (\delta^{202}\text{Hg} \times 1.493)$$

$$\Delta^{201}\text{Hg} \approx \delta^{201}\text{Hg} - (\delta^{202}\text{Hg} \times 0.752)$$

$$\Delta^{200}\text{Hg} \approx \delta^{200}\text{Hg} - (\delta^{202}\text{Hg} \times 0.502)$$

$$\Delta^{199}\text{Hg} \approx \delta^{199}\text{Hg} - (\delta^{202}\text{Hg} \times 0.252)$$

Where $\Delta^{199}\text{Hg}$ and $\Delta^{201}\text{Hg}$ are known as odd-MIF, and $\Delta^{200}\text{Hg}$ and $\Delta^{204}\text{Hg}$ as even-MIF. Additionally, we calculated the ratio $\Delta^{199}\text{Hg}/\Delta^{201}\text{Hg}$, which has proven to be indicative of different photochemical transformation processes and speciation of Hg (i.e., photoreduction of Hg(II) vs. photodemethylation of MeHg) in experimental settings (Bergquist and Blum 2007). $\Delta^{199}\text{Hg}/\Delta^{201}\text{Hg} > 1$ has been determined on samples containing predominantly MeHg, for instance, biota samples, whereas $\Delta^{199}\text{Hg}/\Delta^{201}\text{Hg} \approx 1$ has been observed in environmental samples (e.g., sediments, water) containing predominantly Hg(II) (Tsui et al. 2020).

2.4. Stable isotopes of carbon and nitrogen analysis

Stable isotopic analyses for carbon and nitrogen were carried out at the Scientific and Technical Centers of the Universitat de Barcelona (Spain). 0.30-0.35 mg of feather powder were placed into tin capsules and analyzed using a Thermo-Finnigan Flash 1112 elemental analyser (CE Elantech, Lakewood, NJ, USA) coupled to a Delta-C isotope ratio mass spectrometer via a CONFLO III interface (Thermo Finnigan MAT, Bremen, Germany). Stable isotope signatures are expressed using the standard delta notation relative to Vienna Pee Dee Belemnite ($\delta^{13}\text{C}$) and atmospheric nitrogen ($\delta^{15}\text{N}$). IAEA standards (IAEA CH₇, IAEA CH₆, USGS 24 for ^{13}C , and IAEA N₁, IAEA N₂, IAEA NO₃ for ^{15}N) were applied every 12 samples to calibrate the system. Replicate assays of standards indicated analytical measurement errors of $\pm 0.1\text{‰}$ and $\pm 0.2\text{‰}$ for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, respectively.

2.5. Statistical analyses

THg values and stable isotopes signatures ($\delta^{202}\text{Hg}$, $\Delta^{199}\text{Hg}$, $\Delta^{201}\text{Hg}$, $\Delta^{200}\text{Hg}$, $\Delta^{204}\text{Hg}$, $\delta^{13}\text{C}$, $\delta^{15}\text{N}$) were visually inspected to assess normality. THg values were log-transformed to

approximate residual normality. Homogeneity of variances was tested using the Breusch-Pagan test.

2.5.1. THg and stable isotopes signatures in *Laridae* species

ANOVA tests were performed to test differences among the three species studied (Audouin's gull, black headed gull and common tern) in [THg], $\delta^{202}\text{Hg}$, $\Delta^{199}\text{Hg}$, $\Delta^{201}\text{Hg}$, $\Delta^{200}\text{Hg}$ and $\Delta^{204}\text{Hg}$, $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ observed in feathers. Further pairwise comparisons were evaluated using Tukey tests. A linear model was fitted in order to evaluate the association between log [THg] (response variable) and $\delta^{13}\text{C}$, $\delta^{15}\text{N}$ and Species and the interactions $\delta^{13}\text{C} \times \text{Species}$ and $\delta^{15}\text{N} \times \text{Species}$ as covariates.

Intraspecific correlation tests were performed for each species for each of the mercury isotopic tracers which fractionations are commonly observed in biological samples ($\delta^{202}\text{Hg}$, $\Delta^{199}\text{Hg}$, $\Delta^{201}\text{Hg}$) and [THg] in feathers, each of the mercury isotopic tracers and $\delta^{13}\text{C}$, each of the mercury isotopic tracers and $\delta^{15}\text{N}$. For the correlations analyses we omitted a data point from Audouin's gull that was an outlier in terms of $\delta^{13}\text{C}$ and affected the significance of the correlations test.

Linear models were fitted to estimate the slopes of $\Delta^{199}\text{Hg}/\delta^{202}\text{Hg}$, $\Delta^{199}\text{Hg}/\Delta^{201}\text{Hg}$ and $\Delta^{200}\text{Hg}/\Delta^{204}\text{Hg}$ for all the species studied.

2.5.2. Temporal changes in THg and stable isotope signatures in Audouin's gull fledglings

ANOVA tests were performed to test the differences in [THg], $\delta^{202}\text{Hg}$, $\Delta^{199}\text{Hg}$, $\Delta^{201}\text{Hg}$ and $\delta^{15}\text{N}$ observed in feathers among years studied (2010, 2013, 2014, 2017). Further pairwise comparisons were evaluated using Tukey tests. Since assumptions of normality and homogeneity of variances were not met for $\delta^{13}\text{C}$ residuals, Kruskal Wallis test was used to examine the among years differences for $\delta^{13}\text{C}$ values and Wilcoxon Rank Sum Test for pairwise comparisons.

A linear mixed effects model was fitted in order to evaluate the association between log [THg] (response variable) and $\delta^{13}\text{C}$, $\delta^{15}\text{N}$ (covariates), with year as a random effect. Correlation tests were used to assess correlations between each of the mercury isotopic

tracers and [THg] in feathers, $\delta^{13}\text{C}$, and $\delta^{15}\text{N}$ for each of the years studied and for all the years studied.

Besides, a linear model was fitted to test the association between $\Delta^{199}\text{Hg}$ and $\delta^{202}\text{Hg}$, and $\Delta^{200}\text{Hg}/\Delta^{204}\text{Hg}$. Finally, a linear model of $\Delta^{199}\text{Hg}$ as a response variable and $\Delta^{201}\text{Hg}$ as covariate was fitted to estimate the slope $\Delta^{199}\text{Hg}/\Delta^{201}\text{Hg}$ for the years considered in the study. In this linear model one extreme value was omitted from the dataset.

Correlation tests for each year and for the whole dataset were performed for each of the mercury isotopic tracers with greater fractionation observed in biological samples ($\delta^{202}\text{Hg}$, $\Delta^{199}\text{Hg}$, $\Delta^{201}\text{Hg}$) and [THg] in feathers, each of the mercury isotopic tracers and $\delta^{13}\text{C}$, each of the mercury isotopic tracers and $\delta^{15}\text{N}$. For the correlations analyses we omitted a data point from Audouin's gull that was an outlier in terms of $\delta^{13}\text{C}$ and affected the significance of the correlations test.

All statistical analyses used a significance level of $\alpha = 0.05$ unless otherwise specified. Summary statistics are reported as mean $\pm$ SD, unless otherwise stated. All statistical analyses were performed using R v. 4.0.3 (R Core Team 2020).

3. Results

3.1. Species differences in THg concentrations and isotopic composition

3.1.1. Total mercury (THg) concentrations

We analyzed feather samples from 10 individuals sampled in 2017 breeding season for each of the species of study (Audouin's gull (IA), black-headed gull (CR), common tern (SH)). Table 1 shows the mean $\pm$ SD for each of the variables studied. Significant differences in total mercury (THg) concentrations in fledglings feathers were observed among species ($F_{2,27} = 167.64$, $p < 0.0001$; Fig. 1). Post-hoc tests showed significant differences among the three species considered (Fig. 1). Mean total mercury (THg) concentrations in feathers observed was greater for Audouin's gull, followed by common terns, and black-headed gull fledglings (Table 1).

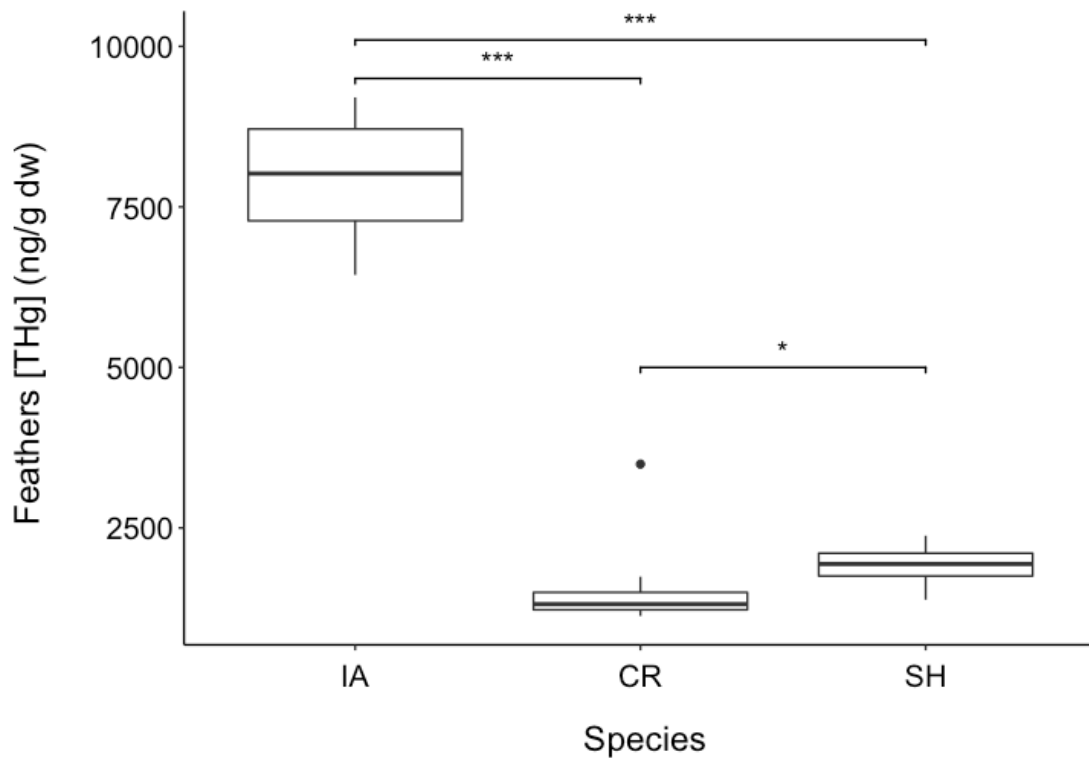


Figure 1 | Boxplots for [THg] (ng/g dw) in feathers of Audouin's gulls (IA), black-headed gull (CR) and common tern (SH) fledglings from the Ebro Delta in 2017. Pairwise significant differences between species are represented above the boxplots.

3.1.2. Carbon and nitrogen stable isotopes and THg

Significant differences in both $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ were observed among species ($\delta^{13}\text{C}$: $F_{2,27} = 68.64$, $p < 0.0001$ and $\delta^{15}\text{N}$: $F_{2,27} = 29.02$, $p < 0.0001$). Post-hoc pairwise comparisons showed significant differences in $\delta^{13}\text{C}$ between IA-CR ($p < 0.0001$) and CR-SH ($p < 0.0001$), but not between IA-SH ($p = 0.29$) with $\delta^{13}\text{C}$ values being (IA $\approx$ SH) $>$ CR (Fig. 2). Regarding $\delta^{15}\text{N}$ values, we detected differences between IA-SH ($p < 0.0001$) and CR-SH ($p < 0.0001$). However, $\delta^{15}\text{N}$ did not differ significantly between IA-CR ($p = 0.48$). $\delta^{15}\text{N}$ values observed were higher in IA and CR than SH (Fig. 2).

The linear model did not show a significant association between $\delta^{15}\text{N}$ or $\delta^{13}\text{C}$ and the observed THg levels in feathers ($\delta^{15}\text{N}$: $F_{1,21} = 0.025$, $p = 0.88$; $\delta^{13}\text{C}$: $F_{1,21} = 0.004$, $p = 0.95$). The interaction terms in the model had no effect on THg levels ($\delta^{15}\text{N} \times \text{Species}$: $F_{2,21} = 0.52$, $p = 0.60$; $\delta^{13}\text{C} \times \text{Species}$: $F_{2,21} = 1.22$, $p = 0.32$).

Table 1 | Summary statistics (mean, SD) for each of the parameters ($\delta^{15}\text{N}$, $\delta^{13}\text{C}$, [THg], $\Delta^{199}\text{Hg}$, $\Delta^{201}\text{Hg}$) in fledglings feathers for each of the three species studied during the 2017 breeding season in the Ebro Delta. IA: *I. audouinii* (Audouin’s gull), CR: *C. ridibundus* (black-headed gull), SH: *S. hirundo* (common tern). For $\Delta^{200}\text{Hg}$ and $\Delta^{204}\text{Hg}$ see Table S4.

Species	$\delta^{15}\text{N}$ (‰)				$\delta^{13}\text{C}$ (‰)				[THg] (ng/g dw)				$\Delta^{199}\text{Hg}$ (‰)				$\Delta^{201}\text{Hg}$ (‰)				$\delta^{202}\text{Hg}$ (‰)			
	Mean	SD	Max	Min	Mean	SD	Max	Min	Mean	SD	Max	Min	Mean	SD	Max	Min	Mean	SD	Max	Min	Mean	SD	Max	Min
IA	13.77	0.38	14.52	13.28	-17.65	0.82	-16.83	-19.69	7982.97	951.94	9205.55	6439.79	0.89	0.16	1.11	0.60	0.68	0.14	0.85	0.38	0.26	0.10	0.43	0.11
CR	13.50	0.66	14.80	12.82	-21.33	1.15	-19.32	-22.50	1555.94	704.45	3494.11	1128.31	1.00	0.20	1.34	0.77	0.78	0.19	1.13	0.54	0.57	0.17	0.84	0.37
SH	12.16	0.43	13.15	11.64	-17.05	0.59	-16.57	-18.13	1916.14	284.04	2379.45	1381.47	2.70	0.30	3.13	1.98	2.23	0.26	2.61	1.62	1.05	0.09	1.20	0.94

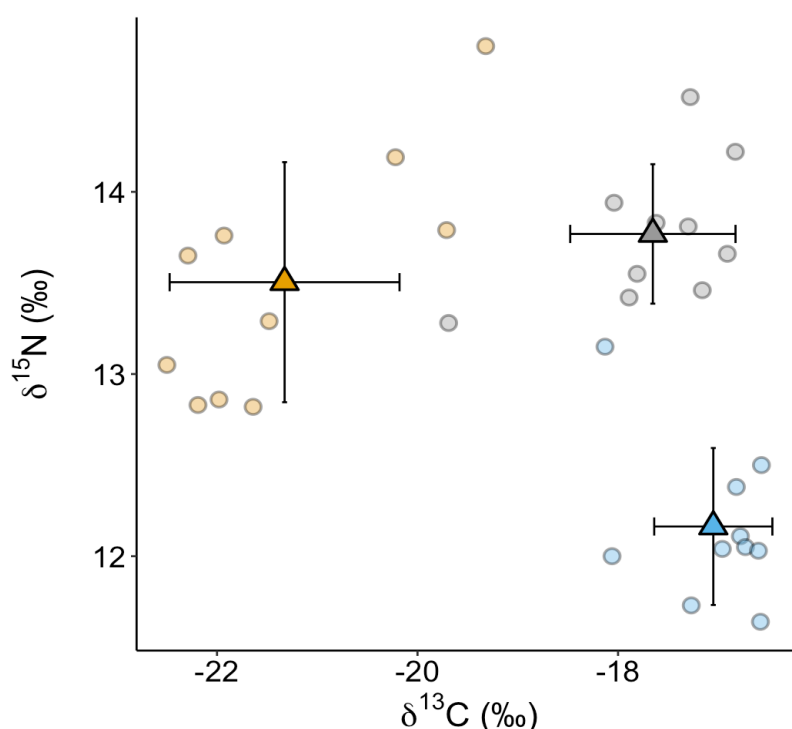


Figure 2 | Biplot of $\delta^{15}\text{N}$ (‰) and $\delta^{13}\text{C}$ (‰) in feathers of Audouin's gulls (IA, in gray), black-headed gull (CR, in orange) and common tern (SH, in blue) fledglings from the Ebro Delta in 2017. Individual data are plotted as dots. Triangles and error bars represent mean $\pm$ SD.

3.1.3. Mercury stable isotope composition

Regarding mercury stable isotopes ($\delta^{202}\text{Hg}$, $\Delta^{199}\text{Hg}$, $\Delta^{201}\text{Hg}$, $\Delta^{200}\text{Hg}$ and $\Delta^{204}\text{Hg}$) we found significant differences among species for $\delta^{202}\text{Hg}$ values ($F_{2,27} = 101.50$, $p < 0.0001$), $\Delta^{199}\text{Hg}$ ($F_{2,27} = 198.41$, $p < 0.0001$) and $\Delta^{201}\text{Hg}$ ($F_{2,27} = 181.49$, $p < 0.0001$). Post-hoc pairwise comparisons showed significant differences among the three species for $\delta^{202}\text{Hg}$ (all comparisons: $p < 0.0001$; Fig. 3) and between common tern and black-headed gull, and common tern and Audouin's gull, but not between Audouin's gull and black-headed gull for both $\Delta^{199}\text{Hg}$ (IA-SH: $p < 0.0001$; CR-SH: $p < 0.0001$; IA-CR: $p = 0.53$; Fig. 3) and $\Delta^{201}\text{Hg}$ (IA-SH: $p < 0.0001$; CR-SH: $p < 0.0001$; IA-CR: $p = 0.50$). Finally, regarding $\Delta^{200}\text{Hg}$ and $\Delta^{204}\text{Hg}$ we did not find significant differences among species for $\Delta^{200}\text{Hg}$ ($F_{2,27} = 1.99$, $p = 0.15$) or $\Delta^{204}\text{Hg}$ ($F_{2,26} = 1.12$, $p = 0.34$; Table S4).

Regression of $\Delta^{199}\text{Hg}$ against $\delta^{202}\text{Hg}$ values showed a significant positive relationship ($F_{1,24} = 144.37$, $p < 0.0001$; Fig. 3), with a regression slope of 2.21. The ratio obtained from the regression between odd-MIF values $\Delta^{199}\text{Hg}/\Delta^{201}\text{Hg}$ for the three species studied was 1.16 (Fig.S1). The ratio between $\Delta^{200}\text{Hg}/\Delta^{204}\text{Hg}$ was not significantly different from 0 (Fig. S2).

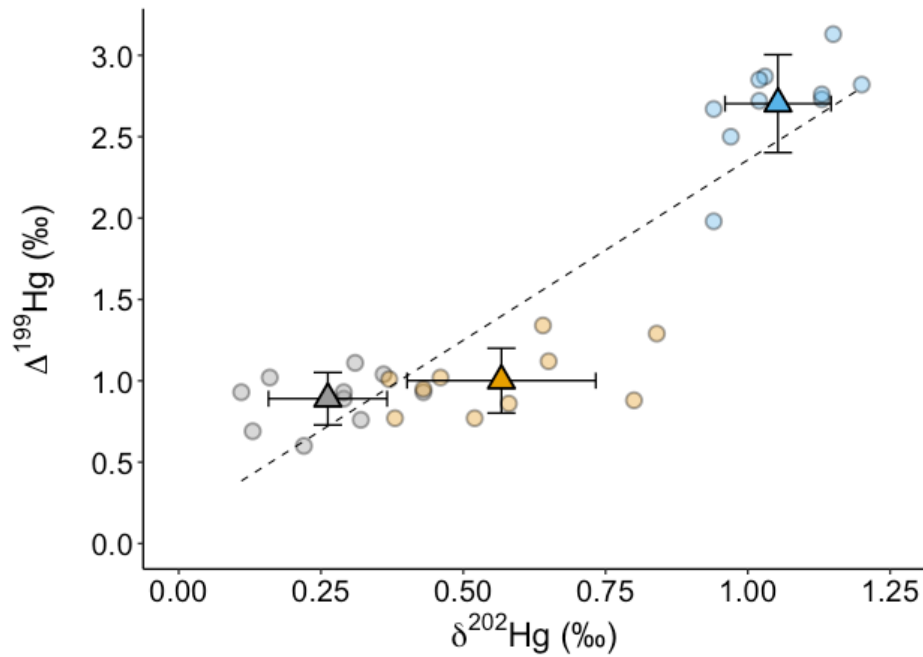


Figure 3 | Biplot of MDF $\delta^{202}\text{Hg}$ (‰) and odd-MIF $\Delta^{199}\text{Hg}$ (‰) in feathers of Audouin's gulls (IA, in gray), black-headed gull (CR, in orange) and common tern (SH, in blue) fledglings from the Ebro Delta in 2017. Individual data are plotted as dots. Triangles and error bars represent mean $\pm$ SD. Dashed line represents linear regression between these variables for the three species: $y = 0.1407 + 2.2167x$; $R^2 = 0.7963$; $p < 0.0001$.

Correlations among mercury stable isotopes ($\delta^{202}\text{Hg}$, $\Delta^{199}\text{Hg}$ and $\Delta^{201}\text{Hg}$) and [THg], $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$, for each of the species are shown in Table 2.

Table 2 | Pearson's correlation tests between each of the mercury isotopic tracers ($\delta^{202}\text{Hg}$, $\Delta^{199}\text{Hg}$ and $\Delta^{201}\text{Hg}$) and [THg], $\delta^{13}\text{C}$, and $\delta^{15}\text{N}$ in fledglings feathers for each of the three species studied during the 2017 breeding season in the Ebro Delta. IA: *I. audouinii* (Audouin's gull), CR: *C. ridibundus* (black-headed gull), SH: *S. hirundo* (common tern). Significant correlations are highlighted in bold.

		Audouin's gull (IA)			Black-headed gull (CR)			Common tern (SH)		
		$\delta^{202}\text{Hg}$	$\Delta^{199}\text{Hg}$	$\Delta^{201}\text{Hg}$	$\delta^{202}\text{Hg}$	$\Delta^{199}\text{Hg}$	$\Delta^{201}\text{Hg}$	$\delta^{202}\text{Hg}$	$\Delta^{199}\text{Hg}$	$\Delta^{201}\text{Hg}$
[THg]	r	-0.21	-0.41	-0.34	0.05	0.19	0.17	-0.51	-0.85	-0.83
	p-value	0.56	0.23	0.34	0.88	0.60	0.63	0.13	0.002	0.002
$\delta^{13}\text{C}$	r	-0.14	0.66	0.72	-0.03	0.56	0.65	0.07	0.44	0.44
	p-value	0.70	0.04	0.02	0.94	0.09	0.04	0.86	0.21	0.20
$\delta^{15}\text{N}$	r	-0.50	-0.09	-0.03	0.03	0.48	0.52	0.18	-0.09	-0.16
	p-value	0.14	0.80	0.92	0.93	0.16	0.12	0.63	0.80	0.65

3.2. Temporal variation in stable isotopic composition in Audouin's gull fledglings

3.2.1. Total mercury (THg) concentrations

Along the Audouin's gull samples ($N = 10$ individuals) analyzed in 2017, we also determined the [THg] in feathers from 10 Audouin's gull fledglings in 2010, 2013, 2014, for a total of 40 feather samples. Table 3 shows the mean $\pm$ SD of the variables studied. We found significant differences among years ($F_{3,36} = 55.24$, $p < 0.0001$). Post-hoc pairwise comparisons detected significant differences among all year's pairs except 2014-2017 (Fig. 4). Mean mercury concentrations in Audouin's gull fledgling feathers were higher in 2017 $\approx$ 2014 > 2010 > 2013 (Table 3).

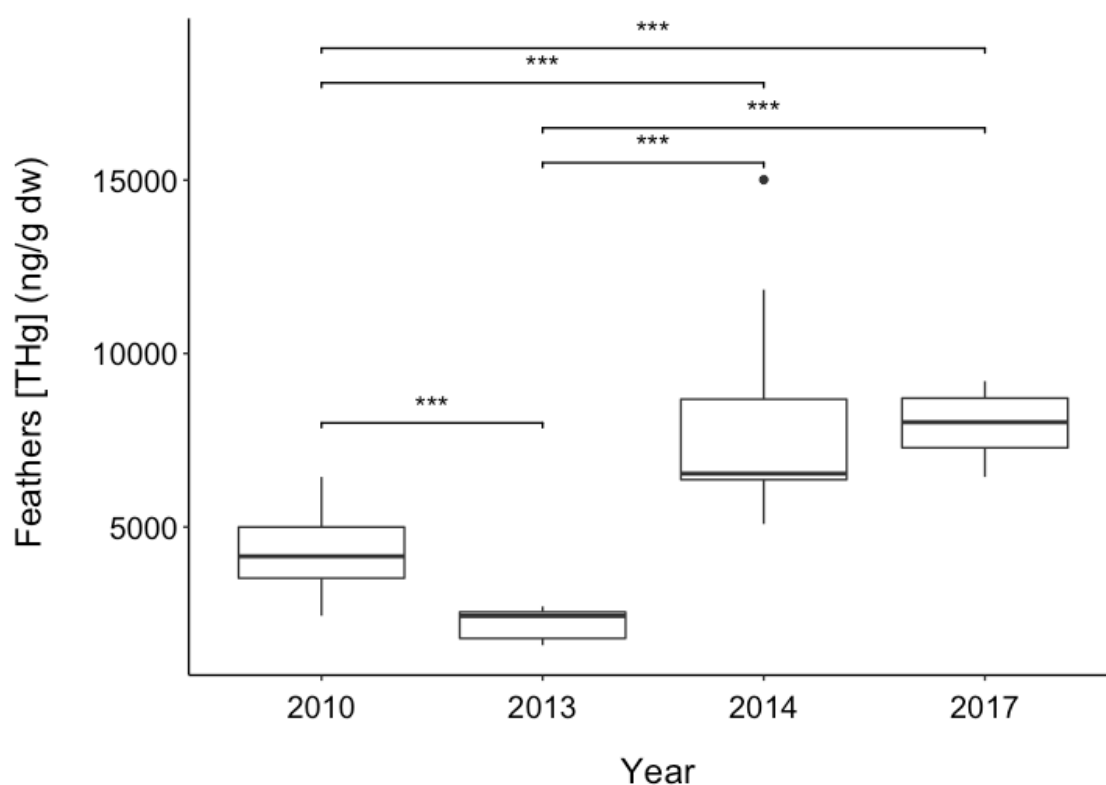


Figure 4 | Boxplots for [THg] (ng/g dw) in feathers of fledglings Audouin's gulls (*I. audouinii*) from breeding seasons of 2010, 2013, 2014, 2017 ($N = 10$ individuals for each year) in the Ebro Delta breeding colony. Pairwise significant differences between years are represented above the boxplots.

3.2.2. Carbon and nitrogen stable isotopes and THg

Significant differences were observed for $\delta^{13}\text{C}$ values ($H = 10.16$, $p = 0.017$) and $\delta^{15}\text{N}$ values ($F_{3,36} = 32.90$, $p < 0.0001$) among years. Multiple pairwise comparisons for $\delta^{13}\text{C}$ values in feathers showed significant differences between 2013-2014 ($p = 0.011$) and 2013-2017 ($p = 0.019$), but not for the rest of pairwise comparisons (2010-2013: $p = 0.22$; 2010-2014: $p = 0.12$; 2010-2017: $p = 0.052$; 2014-2017: $p = 0.50$; Fig. 5). Pairwise comparisons for $\delta^{15}\text{N}$ signatures showed significant differences among all years' pairs ($p < 0.0001$ in all cases), except 2010-2013 ($p = 1.00$) and 2014-2017 ($p = 0.96$; Fig. 5), with $\delta^{15}\text{N}$ values higher in 2014 and 2017 than in 2010 and 2013 (Table 3).

The linear mixed effects model did show a significant association between $\delta^{13}\text{C}$ and the observed THg levels in feathers ($F_{1,34} = 5.20$, $p = 0.029$). Nonetheless, no significant association was observed between $\delta^{15}\text{N}$ values and THg levels in feathers ($F_{1,34} = 1.29$, $p = 0.26$).

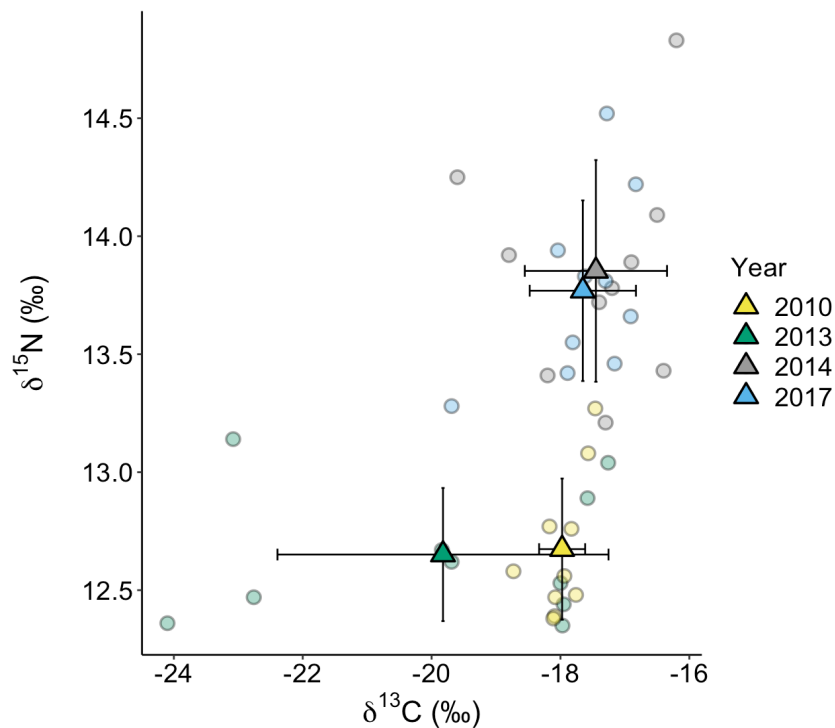


Figure 5 | Biplot of $\delta^{15}\text{N}$ (‰) and $\delta^{13}\text{C}$ (‰) in feathers of Audouin's gulls (*I. audouinii*) fledglings from the Ebro Delta breeding colony in 2010, 2013, 2014 and 2017 ($N = 10$ individuals for each year). Individual data are plotted as dots. Triangles and error bars represent mean $\pm$ SD.

3.2.3. Mercury stable isotopes composition

We found significant differences among years for $\delta^{202}\text{Hg}$ values ($F_{3,36} = 25.30$, $p < 0.0001$), $\Delta^{199}\text{Hg}$ ($F_{3,36} = 11.68$, $p < 0.0001$) and $\Delta^{201}\text{Hg}$ ($F_{3,36} = 8.25$, $p = 0.0002$) in gulls' feathers. Post-hoc pairwise comparisons showed significant differences for $\delta^{202}\text{Hg}$ signatures among all years' pairs ($p < 0.0001$) except the pairs 2013-2017 ($p = 0.30$) and 2010-2014 ($p = 1.00$). Regarding $\Delta^{199}\text{Hg}$ pairwise comparisons showed significant differences between 2010-2013 ($p = 0.02$), 2010-2017 ($p < 0.0001$) and 2014-2017 ($p = 0.001$), although no significant differences were detected among the other years' pairs (2010-2014: $p = 0.47$; 2013-2014: $p = 0.32$; 2013-2017: $p = 0.091$). For $\Delta^{201}\text{Hg}$ values pairwise comparisons showed significant differences between years 2010-2017 ($p = 0.0003$) and 2014-2017 ($p = 0.003$), but not among the other years' pairs (2010-2013: $p = 0.11$; 2010-2014: $p = 0.85$; 2013-2014: $p = 0.44$; 2013-2017: $p = 0.12$).

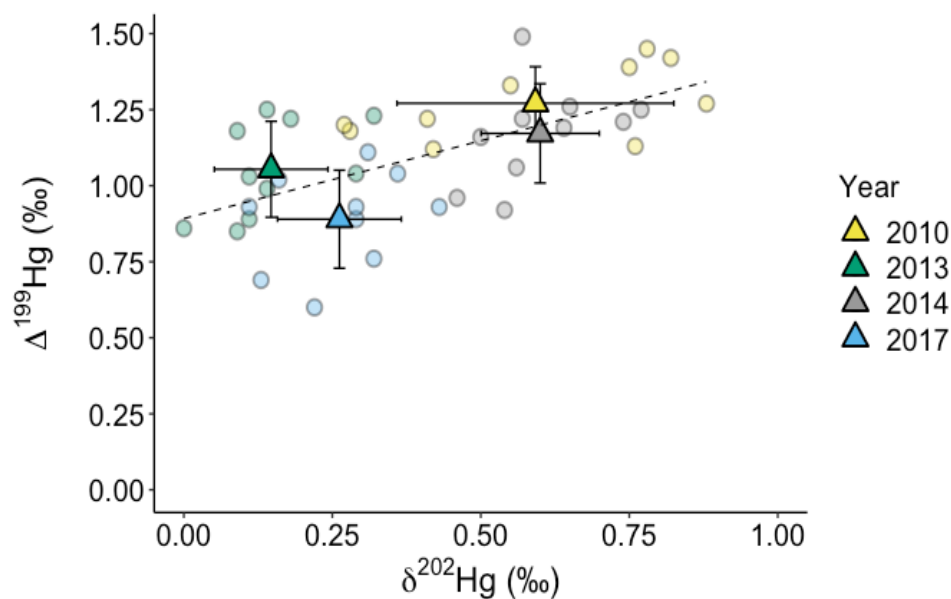


Figure 6 | Biplot of MDF $\delta^{202}\text{Hg}$ (‰) and odd-MIF $\Delta^{199}\text{Hg}$ (‰) in feathers of Audouin's gulls (IA) fledglings from the Ebro Delta breeding colony in 2010, 2013, 2014 and 2017 ($N = 10$ individuals for each year). Individual data are plotted as dots. Triangles and error bars represent mean $\pm$ SD. Dashed line represents linear regression between these variables for all the years studied: $y = 0.8920 + 0.5115x$; $R^2 = 0.3611$; $p < 0.0001$.

Regarding $\Delta^{200}\text{Hg}$ and $\Delta^{204}\text{Hg}$ we did find significant differences among species for $\Delta^{200}\text{Hg}$ ($F_{3,36} = 3.45$, $p = 0.026$) but not for $\Delta^{204}\text{Hg}$ ($F_{3,36} = 0.86$, $p = 0.47$). Post-hoc comparisons showed significant differences in $\Delta^{200}\text{Hg}$ values for 2010-2013 ($p = 0.037$) but not among the other years' pairs (in all cases, $p < 0.05$).

Regression of $\Delta^{199}\text{Hg}$ against $\delta^{202}\text{Hg}$ values showed a significant positive relationship ($F_{1,38} = 23.05$, $p < 0.0001$; Fig. 6). The ratio between odd-MIF values $\Delta^{199}\text{Hg}/\Delta^{201}\text{Hg}$ for all the years was 1.09 (Fig. S3). The ratio between $\Delta^{200}\text{Hg}/\Delta^{204}\text{Hg}$ was not significantly different from 0 (Fig. S4).

Correlations among mercury stable isotopes and [THg], $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$, for each of the years and all the years pooled, are shown in Table 4.

4. Discussion

4.1. Species differences in THg concentrations and isotopic composition

4.1.1. Total mercury (THg) concentrations

Aquatic environments, both marine and freshwater are prone to mercury methylation (Ullrich et al. 2001; Regnell and Watras 2019) and, therefore are regarded as hotspots for mercury bioavailability, bioaccumulation and biomagnification through the food webs (Lavoie et al. 2013; Evers et al. 2018). Previous results in the Ebro Delta area demonstrated the influence of legacy mercury from the chlor-alkali plant upstream the Ebro River and later on, the restoring of the polluted site in 2013 suggest its impact on the environments receiving their waters (Cotín et al. 2011; Sánchez-Fortún et al. 2020), including rice-fields (Suárez-Serrano et al. 2010), and the coastal marine area affected by the river discharge (Campillo et al. 2019). Besides, the Mediterranean Sea due to its biogeochemical specificity is regarded as a methylmercury hotspot for marine food webs (Cossa and Coquery 2005). Thus, we expect that species feeding in these areas will be subject to high mercury levels.

Fledglings from the three species studied (Audouin's gull, black-headed gull and common tern) presented accused differences in THg levels in feathers. Audouin's gulls fledglings feathers had higher mercury concentration than those from common terns or black-headed gulls. This fact would be related to the different trophic resources exploited by each species, as mercury is mainly acquired through diet and deposited in feathers during their growth as an excretion mechanism (Bond and Diamond 2009). Although the three species coexist in the same area and their breeding colonies are few km apart, they have distinct trophic ecologies: Audouin's gulls and common terns depend mainly on marine resources, whereas black-headed gulls forage mainly on rice-fields. Moreover, Audouin's gulls and common terns exploit different marine resources. Essentially, common terns are

epipelagic foragers (Cotín et al. 2011), while Audouin's gulls are opportunistic foragers on fisheries discards (Sanpera et al. 2007; García-Tarrasón et al. 2015). Besides, Audouin's gulls also forage in terrestrial and freshwater environments (e.g., rice-fields; Oro et al. 1996; Ruiz et al. 1996; Pedrocchi et al. 2002; Sanpera et al. 2007; Christel et al. 2012).

4.1.2. Mercury, carbon and nitrogen stable isotopes

Regarding the mercury isotopic tracers, differences among the three species were found for $\delta^{202}\text{Hg}$, whereas $\Delta^{199}\text{Hg}$ and $\Delta^{201}\text{Hg}$ only differed between common terns and Audouin's gulls or black-headed gulls, having both gull species similar values. The differences observed for the odd-MIF and MDF signatures might reflect differences in sources of mercury and methylmercury, the distinct trophic ecologies among species, and differences among processes linked to Hg speciation among foraging environments. Differences in $\Delta^{200}\text{Hg}$ and $\Delta^{204}\text{Hg}$ signatures were not observed among the three species.

$\Delta^{199}\text{Hg}$ values reflect differences in MeHg sources owing to photodemethylation in the water column (Bergquist and Blum 2007). Since odd-MIF signatures are maintained along the food webs (Kwon et al. 2012), they reflect the foraging habitats from which fledglings of the different species are fed. Common terns (SH) consumed epipelagic resources, where the incidence of sunlight and photodemethylation processes predominate and, therefore, higher values of odd-MIF are expected (Blum et al. 2013; Bonsignore et al. 2015; Renedo et al. 2018). Moreover, in marine pelagic food webs, MeHg derives mainly from wet precipitation or deposition of elemental Hg, with $\Delta^{199}\text{Hg}$ from -1.00 – 1.00 ‰ and 0.5 – 1.00 ‰, respectively (Kwon et al. 2020), in accordance with the high MIF values observed in SH feathers. Conversely, Audouin's gull (AI) are fed mainly by benthic and mesopelagic species from fisheries discards (Sanpera et al. 2007; García-Tarrasón et al. 2013), for which the incidence of sunlight and thus photodemethylation rates are not as high (Blum et al. 2013), so odd-MIF signatures observed in AI fledglings' feathers will be lower. Analogously, black-headed gulls (CR) feed juveniles with rice-field preys associated to the paddy soils (e.g. crayfish) and possibly due to rice canopy and turbidity of waters photochemical reactions are inhibited in these environments (Gantner et al. 2009; Senn et al. 2010; Sherman and Blum 2013), resulting in low odd-MIF values in CR fledglings' feathers.

Table 3 | Summary statistics (mean, SD, max and min) for each of the parameters ($\delta^{15}\text{N}$, $\delta^{13}\text{C}$, [THg], $\Delta^{199}\text{Hg}$, $\Delta^{201}\text{Hg}$ and $\delta^{202}\text{Hg}$) in fledglings' feathers for each of the years studied (2010, 2013, 2014 and 2017) on the Audouin's gull (*I. audouinii*) breeding colony in the Ebro Delta. For $\Delta^{200}\text{Hg}$ and $\Delta^{204}\text{Hg}$ see Table S5.

Year	$\delta^{15}\text{N}$ (‰)				$\delta^{13}\text{C}$ (‰)				[THg] (ng/g dw)				$\Delta^{199}\text{Hg}$ (‰)				$\Delta^{201}\text{Hg}$ (‰)				$\delta^{202}\text{Hg}$ (‰)			
	Mean	SD	Max	Min	Mean	SD	Max	Min	Mean	SD	Max	Min	Mean	SD	Max	Min	Mean	SD	Max	Min	Mean	SD	Max	Min
2010	12.67	0.30	13.27	12.38	-17.97	0.36	-17.46	-18.73	4288.55	1260.41	6441.93	2438.68	1.27	0.12	1.45	1.12	1.02	0.11	1.16	0.89	0.59	0.23	0.88	0.27
2013	12.65	0.28	13.14	12.35	-19.82	2.57	-17.26	-24.10	2227.59	443.56	2710.85	1588.99	1.05	0.16	1.25	0.85	0.85	0.12	1.00	0.67	0.15	0.10	0.32	0.00
2014	13.85	0.47	14.83	13.21	-17.45	1.10	-16.20	-19.60	8032.03	3119.42	15009.73	5084.04	1.17	0.16	1.49	0.92	0.96	0.25	1.57	0.63	0.60	0.10	0.77	0.46
2017	13.77	0.38	14.52	13.28	-17.65	0.82	-16.83	-19.69	7982.97	951.94	9205.55	6439.79	0.89	0.16	1.11	0.60	0.68	0.14	0.85	0.38	0.26	0.10	0.43	0.11

Table 4 | Pearson's correlation tests between each of the mercury isotopic tracers ($\delta^{202}\text{Hg}$, $\Delta^{199}\text{Hg}$ and $\Delta^{201}\text{Hg}$) and [THg], $\delta^{13}\text{C}$, and $\delta^{15}\text{N}$ in fledglings' feathers for each of the years studied (2010, 2013, 2014 and 2017) and for all the years together on the Audouin's gull (*I. audouinii*) breeding colony in the Ebro Delta. Significant correlations are highlighted in bold.

		2010			2013			2014			2017			All Years		
		$\delta^{202}\text{Hg}$	$\Delta^{199}\text{Hg}$	$\Delta^{201}\text{Hg}$	$\delta^{202}\text{Hg}$	$\Delta^{199}\text{Hg}$	$\Delta^{201}\text{Hg}$	$\delta^{202}\text{Hg}$	$\Delta^{199}\text{Hg}$	$\Delta^{201}\text{Hg}$	$\delta^{202}\text{Hg}$	$\Delta^{199}\text{Hg}$	$\Delta^{201}\text{Hg}$	$\delta^{202}\text{Hg}$	$\Delta^{199}\text{Hg}$	$\Delta^{201}\text{Hg}$
[THg]	r	0.49	0.38	0.28	-0.32	-0.52	-0.47	-0.07	0.46	0.49	-0.18	-0.41	-0.33	0.29	-0.09	-0.02
	p-value	0.15	0.28	0.43	0.37	0.13	0.17	0.84	0.19	0.15	0.61	0.24	0.33	0.07	0.57	0.91
$\delta^{13}\text{C}$	r	-0.07	-0.38	-0.44	-0.35	-0.39	-0.37	0.46	0.47	0.22	-0.14	0.66	0.72	0.29	0.04	0.03
	p-value	0.84	0.28	0.20	0.32	0.26	0.29	0.18	0.17	0.54	0.70	0.04	0.02	0.07	0.83	0.85
$\delta^{15}\text{N}$	r	0.11	-0.29	-0.49	0.17	-0.41	-0.37	0.45	-0.14	-0.38	-0.50	-0.09	-0.03	0.15	-0.32	-0.34
	p-value	0.77	0.42	0.15	0.63	0.24	0.29	0.20	0.71	0.28	0.14	0.80	0.92	0.33	0.04	0.031

The information obtained from the Hg stable isotopic signatures regarding the trophic ecology for each species is consistent with the information obtained from the trophic markers ($\delta^{15}\text{N}$ and $\delta^{13}\text{C}$). $\delta^{13}\text{C}$ values are regarded as a proxy of carbon sources in diet and $\delta^{15}\text{N}$ is a proxy of trophic position (Post et al. 2002). Audouin's gulls (IA) fledglings are fed mainly from benthic and mesopelagic discarded fish which have higher trophic levels (Pedrocchi et al. 2002) resulting in higher $\delta^{15}\text{N}$ than common terns (SH), which are fed mainly with epipelagic fish species of lower trophic levels (Cotín et al. 2011). $\delta^{15}\text{N}$ values in black-headed gull fledglings are similar to those observed in Audouins' gulls. Nevertheless, it is important to consider that the application of fertilizers in agricultural fields might be increasing $\delta^{15}\text{N}$ in these environments' food webs (Herbert and Wasseenar 2001; Cole et al. 2004; Cotín et al. 2011). Therefore, black-headed gull nitrogen signatures need to be interpreted with caution, since they might not reflect accurately their trophic position, when comparing species across habitats. The $\delta^{13}\text{C}$ signatures observed in each of the three species are consistent with the foraging habitats for each of the species, with IA and SH fledglings presenting higher values of $\delta^{13}\text{C}$ than CR fledglings, associated to trophic sources from marine environments (García-Tarrasón et al. 2013). Conversely, the lower $\delta^{13}\text{C}$ signatures observed in CR fledglings, are similar to those found in other avian species in the area foraging freshwater habitats (Cotín et al. 2011).

Furthermore, rice-fields are flooded with water coming from the Ebro river, that transports anthropogenic Hg downstream from the chlor-alkali site (Palanques et al. 2020). Generally, significant MIF has not been observed during river transport of Hg downstream (e.g., Washburn et al. 2017; Schudel et al. 2018; Reinfelder and Janssen 2019). Thus, we would expect CR fledglings will present odd-MIF signatures closer to those from the polluted site. And since Audouin's gulls' fledglings present odd-MIF signatures similar to those from black-headed gulls, this could point out that part of its mercury has a continental origin from the sediments transported by the river as influenced by the upstream polluted site. Indeed, although marine sedimentary Hg is often dominated by Hg of geogenic origin (e.g., volcanic activity, cinnabar deposits), surface layers of marine sediments have been shown to retain anthropogenic contributions (Ogrinc et al. 2019). Therefore, it is possible that anthropogenic mercury transported by the river has been deposited over decades in the sea sediment (Gustin et al. 2020), and it has accessed the benthic/mesopelagic food web (Perrot et al. 2010; Bonsignore et al. 2015), from which IA fledglings are fed. This observation is consistent with several studies, that have reported in sediments and biota from polluted sites, MIF signatures are close to 0 ‰ (e.g., Kwon et al. 2014; Bonsignore et al. 2015; Wiederhold et al. 2015; Feng et al. 2019, Tsui et al. 2020).

The species studied presented a $\Delta^{199}\text{Hg}/\Delta^{201}\text{Hg}$ slope of 1.17 (Fig. S1). $\Delta^{199}\text{Hg}/\Delta^{201}\text{Hg}$ ratios are used to identify mechanisms involving MIF variations. Also, they are assumed to be preserved along food webs biomagnification after MeHg assimilation by primary producers (Bergquist and Blum 2007). Experimentally theoretical $\Delta^{199}\text{Hg}/\Delta^{201}\text{Hg}$ slopes have been determined for aquatic systems, being for MeHg photodemethylation $1.36 (\pm 0.02, 2 \text{ SE})$ and $1.00 (\pm 0.02, 2 \text{ SE})$ for inorganic Hg photoreduction (Bergquist and Blum 2007). Several studies reported in marine and freshwater biota $\Delta^{199}\text{Hg}/\Delta^{201}\text{Hg}$ slopes higher than 1.00 and slightly lower than 1.30 (e.g., Senn et al. 2010; Gehrke et al. 2011; Point et al. 2011; Day et al. 2012; Renedo et al. 2018). Studies comparing $\Delta^{199}\text{Hg}/\Delta^{201}\text{Hg}$ slopes in coastal marine fish and lake fish have reported $\Delta^{199}\text{Hg}/\Delta^{201}\text{Hg}$ values of ~ 1.18 and ~ 1.26 respectively (Blum et al. 2013; Kwon et al. 2020), consistent with our findings ($\Delta^{199}\text{Hg}/\Delta^{201}\text{Hg}$ for the three species: 1.17). This deviation from the experimentally determined slopes is probably due to the organic ligands binding of Hg in the freshwater or marine environments (Bergquist and Blum 2007; Zheng and Hintelmann 2009).

In the case of MDF ($\delta^{202}\text{Hg}$) values, both CR and IA were significantly lower (IA: 0.26 ± 0.10 ; CR: 0.57 ± 0.17) than those from SH (1.05 ± 0.09) and are closer to 0 ‰. Although odd-MIF is much more sensible to photochemical reactions, the higher positive MDF values observed in SH feathers might reflect photochemical demethylation of MeHg in the photic zone of the marine water column, leaving a residual MeHg enriched in $\delta^{202}\text{Hg}$ (Blum et al. 2013; Bonsignore et al. 2015; Renedo et al. 2018) compared to CR and IA. Also, MDF signatures in fish from estuarine freshwater ecosystems compared to marine environments are shown to be more negative (Li et al. 2016). Thus, this reflects the trophic ecologies of CR and IA, and the influence of Hg derived from terrestrial/freshwater environments in the diet of IA fledglings. We also detected significant differences in MDF signatures between CR and IA, with IA $\delta^{202}\text{Hg}$ values being lower than CR values (Fig. 3). These differences possible arise from the impact of Hg legacy pollution from the chlor-alkali industry. Indeed, Cransveld et al. (2017) compared sea bass (*Dicentrarchus labrax*) populations among coastal polluted areas, and those subject to local chlor-alkali derived Hg pollution, presented higher $\delta^{202}\text{Hg}$ signatures than those in which background atmospherically Hg-contamination was assumed to constitute the main driver of $\delta^{202}\text{Hg}$ values. Chlor-alkali processes induce MDF, which help to trace Hg coming from industrial waste waters (Wiederhold et al. 2015). Although this possibly explains the higher MDF values of CR fledglings compared to those from IA fledglings we cannot rule out other factors that could be modifying MDF values and that would need further studying (e.g., trophic transfer

(Laffont et al. 2009; Perrot et al. 2010, but see Kwon et al. 2012), metabolic reactions or physiological processes from the individual (Renedo et al. 2021).

The regression between odd-MIF $\Delta^{199}\text{Hg}$ and MDF ($\delta^{202}\text{Hg}$) values, showed a significant positive relationship with a slope of 2.21. This value is similar to those reported in previous studies, for lake and marine organisms (Berquist and Blum 2007; Motta et al. 2019; Kwon et al. 2020). These increasing trends are consistent with experimental studies, reporting increasing $\delta^{202}\text{Hg}$ and $\Delta^{199}\text{Hg}$ in remaining MeHg during photodegradation (Bergquist and Blum 2007), thus, implying a benthic-epipelagic gradient.

Finally, $\Delta^{200}\text{Hg}$ values are consistent with those reported in previous studies using seabirds as model organisms (Point et al. 2011; Day et al. 2012) and in Mediterranean biota (Jiskra et al. 2021). These signatures have been used to trace atmospheric inputs of Hg via precipitation. In precipitation samples, $\Delta^{200}\text{Hg}/\Delta^{204}\text{Hg}$ ratio has been observed to be of -0.2 (Kwon et al. 2020). In our study, although it was negative, it was not significantly different from 0. Possibly, even-MIF values might be influenced by other sources of contamination, trophic transfers or other environmental factors that might be modifying the slope between these two isotopic tracers observed in precipitation samples (Kwon et al. 2020).

Correlation analyses did not show significant correlations for any of the species studied between MDF ($\delta^{202}\text{Hg}$) and [THg], $\delta^{13}\text{C}$ or $\delta^{15}\text{N}$ in feathers. This reflects the fact that MDF mercury isotopic signatures are not only affected by the trophic ecology of the individuals and biomagnification processes along food webs (Laffont et al. 2009; Perrot et al. 2010) but also intrinsic metabolism and physiological processes, especially in high trophic level organisms (Laffont et al. 2009; Jackson 2018; Rua-Ibarz et al. 2019; Tsui et al. 2020; Renedo et al. 2020; 2021) and other environmental factors, including differences in Hg dynamics and fractionation on rice-paddies (e.g., Yin et al. 2013; Qin et al. 2020) vs. marine environments (e.g., Archer and Blum 2018), which need further study and are beyond the scope of the present work. However, Hg levels in feathers of common terns did correlate negatively with odd-MIF values ($\Delta^{199}\text{Hg}$ and $\Delta^{201}\text{Hg}$), but not for Audouin's gull or black-headed gull's fledglings. The negative correlation observed might be due to the fact that, even if major diet items foraged by common terns in the colony studied to feed their chicks are epipelagic fish species (Arcos et al. 2002), some of the individuals might also rely on mesopelagic/demersal resources obtained from fisheries discards (Guitart et al. 2003; Cotín et al. 2011). This is consistent with a higher variability in odd-MIF signatures among individuals in this species (Fig. 3). Conversely, both gulls species present low odd-MIF

variability (Fig. 3). Finally, we found significant positive correlations between $\delta^{13}\text{C}$ and odd-MIF values in black-headed gulls and Audouin's gulls (Table 2). These positive significant correlations are in accordance to those reported in guillemots (*Uria aalge* and *U. lomvia*) breeding in the Alaskan Arctic, which was attributed to a $\delta^{13}\text{C}$ gradient from terrestrial to oceanic systems' sources and a lower photochemical demethylation in coastal systems due to higher turbidity (Day et al. 2012). In our study, these positive correlations might reflect individual variability within species in dietary sources with different proportions of continental and coastal marine resources, especially in IA (Sanpera et al. 2007; García-Tarrasón et al. 2015).

Thus, mercury stable isotopes confirm the trophic ecologies for the species under study and point out a possible influence of Hg from anthropogenic origin in IA fledglings, as signatures are similar to those in CR fledglings. Furthermore, they distinguish the foraging ecologies in a vertical gradient of IA and SH, with IA consuming mainly benthic/mesopelagic species and SH consuming epipelagic fish.

4.2. Temporal variation in stable isotopic signatures in Audouin's gull fledglings

4.2.1. Total mercury (THg) concentrations

Total mercury concentrations in the Audouin's gull colony varied among years (Fig. 4). Previous studies in this colony (Sánchez-Fortún et al. 2020), have suggested this could be related to the sediment dragging that took place in 2013-2014 in the site next to the chlor-alkali plant, which remobilized legacy mercury. In the present study, Hg levels were $2017 \approx 2014 > 2010 > 2013$. This is consistent with the results obtained by Sánchez-Fortún et al. (2020) for a longer time series from the same breeding colony using larger sample sizes. Accordingly, we would expect that the mercury found in 2014 is predominantly mercury sourced from the chlor-alkali plant surroundings that has been transported through the river (see below). The peak in [THg] in feathers of Audouin's gull fledglings in 2017 could be attributed to the deposition over time of mercury transported by the river on the coastal seabed (Palanques et al. 2020).

4.2.2. Mercury, carbon and nitrogen stable isotopes

We found differences in both $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ stable isotopes signatures in feathers among years. The $\delta^{13}\text{C}$ signatures from 2010, 2014 and 2017 are consistent with carbon stable

isotopes signatures from marine environments (Fig. 5; García-Tarrasón et al. 2013; Sánchez-Fortún et al. 2020). Individuals from 2013 presented the largest range of $\delta^{13}\text{C}$ values, indicating significant differences in carbon sources among the individuals sampled. According to the $\delta^{13}\text{C}$ observed in 2013 at least 5 individuals were being fed predominantly with freshwater (i.e., rice-fields) sources (Fig. 5), with their $\delta^{13}\text{C}$ ranging from -19.7 to -24.1 ‰. This is consistent with this and other studies in the area using aquatic birds feeding from freshwater resources as model organisms (e.g., black-headed gull [Table 1, this study], Cotín et al. 2011). $\delta^{13}\text{C}$ values were positively associated with THg values in feathers, what might be related to the different dietary proportions from rice-fields or marine environments, with individuals fed with higher proportions of rice-field preys (i.e., lower $\delta^{13}\text{C}$), having lower THg levels in feathers (García-Tarrasón et al. 2013).

Regarding $\delta^{15}\text{N}$ signatures, there were clear differences between 2010 and 2013, and 2014 and 2017, although we did not detect any association with THg levels in feathers. Besides, in areas where contamination and possible sources of eutrophication exist (e.g., agricultural environments), variation in baseline $\delta^{15}\text{N}$ over time might be influencing the consumer's $\delta^{15}\text{N}$ (Cole et al. 2004), thus not reflecting the actual trophic position of the individuals if comparing the values of this proxy among years. In this colony, it has been observed, that besides a possible pollution episode derived from the cleansing of the chlor-alkali plant site in 2013-2014, a parallel eutrophication process could have been occurred, as suggested by Sánchez-Fortún et al. (2020) in a longitudinal study using compound specific stable isotopes of nitrogen.

Mercury stable isotopes showed differences among years for $\delta^{202}\text{Hg}$, $\Delta^{199}\text{Hg}$ and $\Delta^{201}\text{Hg}$ values. Regarding odd-MIF values ($\Delta^{199}\text{Hg}$), the year 2017 presented the lowest values among the years studied, followed by 2013, 2014 which presented similar $\Delta^{199}\text{Hg}$ values, and 2010 with the highest odd-MIF values, although statistically significant differences were not always detected due to intra-annual variability in individual signatures. $\Delta^{199}\text{Hg}$ in 2017 ranged from 0.60 – 1.11, whereas in 2010 from 1.12 – 1.45, in 2013 from 0.85 – 1.25 and in 2014 from 0.92 – 1.49. $\Delta^{199}\text{Hg}$ (and $\Delta^{201}\text{Hg}$) did show a significant negative correlation with THg when pooling the data for all the years (Table 4). As stated previously, odd-MIF signatures vary with photochemical reactions in the water column, namely photoreduction of Hg(II) and photodemethylation of MeHg (Bergquist and Blum 2007). Thus, since individuals being fed with more proportion of fisheries discards (mesopelagic and benthic species) rather than epipelagic or freshwater/terrestrial sources will likely present higher levels of Hg in their bodies (García-Tarrasón et al. 2013) and lower odd-MIF isotopic

composition (Blum et al. 2013) this might explain the negative correlation encountered. The $\Delta^{199}\text{Hg}/\Delta^{201}\text{Hg}$ slope obtained from the regression on this dataset was 1.09, which lays in between the experimentally derived slopes for Hg(II) photoreduction (~ 1.00) and MeHg photodemethylation (~ 1.30). The odd-MIF slope found for all the years in this study is consistent with the one found in common murre's eggs by Day et al. (2012), that was 1.09. However, 1.09 is inconclusive in terms of the process leading to $\Delta^{199}\text{Hg}$ and $\Delta^{201}\text{Hg}$ signatures prior to the incorporation in the food web (Day et al. 2012). $\Delta^{199}\text{Hg}/\Delta^{201}\text{Hg}$ slopes signatures depend on a variety of factors such as DOC levels relative to Hg concentrations in the environment, type of DOC present, irradiance (Bergquist and Blum 2007; Zheng and Hintelmann 2009). In natural conditions, these factors might develop an important role in determining $\Delta^{199}\text{Hg}/\Delta^{201}\text{Hg}$ slope, and for our samples, we assume they will vary widely among years and due to the complexity of the ecosystems under study (i.e., agricultural wetlands and marine coastal environments). As Day et al. (2012) pointed out, with the existing knowledge on Hg fractionation in the environment, it is not feasible to interpret which of the species of Hg is mainly fractionating using the available data in our study.

No significant correlation was found between odd-MIF signatures and $\delta^{13}\text{C}$, possibly due to the low ranges observed in $\delta^{13}\text{C}$ values in the years studied, except for $N = 3$ individuals in 2013 which presented lower $\delta^{13}\text{C}$ values (Fig. 5). We found significant negative correlation between MIF signatures and $\delta^{15}\text{N}$ values when considering the data for all the years (Table 4). However, due to $\delta^{15}\text{N}$ variations in food webs baselines over years, interpretations on this correlation in terms of trophic levels might be misleading (Sánchez-Fortún et al. 2020). Nonetheless, even if data on Hg stable isotopes from the polluted site at Flix is not available, for all the years studied, odd-MIF values range from 0.60 – 1.49. These values seem to indicate that overall, there might be an influence of Hg proceeding from the polluted site in the Audouin's gull colony in the Ebro Delta over the years in a varying degree.

Regarding even-MIF values, for the years considered in this study, the $\Delta^{200}\text{Hg}/\Delta^{204}\text{Hg}$ ratio in IA fledglings feathers was not significantly different from 0. This might be indicative of other sources of contamination, trophic transfers or other environmental factors that might be modifying the slope between these two isotopic tracers found in global precipitation samples (-0.2; Kwon et al. 2020).

Interestingly, MDF ($\delta^{202}\text{Hg}$) in Audouin's gull fledgling feathers presented similar values between 2013 and 2017 (mean $\pm$ SD: 0.20 ± 0.11) and between 2010 and 2014 (mean

$\pm$ SD: 0.60 ± 0.18), being MDF values in 2010 and 2014 higher than those from 2013 and 2017. Since $\delta^{202}\text{Hg}$ values were not correlated with $\delta^{15}\text{N}$ nor $\delta^{13}\text{C}$, this $\delta^{202}\text{Hg}$ difference among years was not due to an altered trophic ecology (i.e., MDF modified as a result of trophic level exploited [Point et al. 2011; Tsui et al. 2020]). Furthermore, MDF values for 2010 and 2014 are closer to those obtained for CR in this study (mean $\pm$ SD: 0.57 ± 0.17), a species more impacted by anthropogenic derived mercury.

Several studies in the Ebro river and the Ebro Delta area have pointed out the possible influence of the presence of the Flix chlor-alkali plant on Hg levels in biota (Sanpera et al. 2007; Faria et al. 2010; Cotín et al. 2011; Carrasco et al. 2011; Sánchez-Fortún et al. 2020). The phase-down of activities using Hg in the plant started in 2004 with a consequent decrease in Hg emissions (Pujadas 2016), until the complete cessation of them in 2017. Furthermore, in 2013 a management plan to remediate the contaminated soils nearby the chlor-alkali site was performed, which could have had an impact on biota downstream the river, due to mobilization of legacy mercury. From 2004 to 2013, in the Audouin's gull colony in the Ebro Delta a progressive decrease in Hg levels in feathers of fledglings has been described, followed by a slightly increase from 2014 onwards (Sánchez-Fortún et al. 2020). Thus, we expected the Audouin's gull colony in the years 2010 and 2014 to be affected by a major extent with Hg transported from the chlor-alkali plant site, while in 2013 and 2017 would be less impacted by anthropogenic pollution due to ecosystem recovery (Kopeck et al. 2019). The results of the present study regarding MDF values on these years seem to be consistent with this idea, with individuals presenting higher MDF values in 2010 and 2014, associated to higher influence of chlor-alkali derived Hg (Cransveld et al. 2017; Feng et al. 2019).

Nonetheless, although we did find a weak positive correlation between $\delta^{202}\text{Hg}$ and THg levels in feathers, which would reinforce the idea of an increase of $\delta^{202}\text{Hg}$ coupled to the additional pollution from the chlor-alkali site, it was not significant ($r = 0.29$; $p = 0.07$). Unfortunately, with the available data we are not able to discern among the possible factors and sources leading to the different concentrations in total mercury in feathers from the different years. Possibly, river dynamics over decades has had an influence in transporting Hg from the polluted site towards the coastal seabed sediments surrounding the colony (Palanques et al. 2020), which would lead to an increase in Hg on the marine food web (e.g., Sánchez-Fortún et al. 2020), although other factors not taken into account in this study (e.g., physiology of species; Renedo et al. 2020, 2021) and the complexity of estuarine

environments could be influencing THg levels or MDF signatures, and thus, modifying the correlation between MDF and THg values.

5. Conclusion

Stable isotopes of mercury have been shown to be a promising tool in investigating processes and sources leading to Hg body burdens in biota. In this study we applied this methodology to understand differences in Hg levels observed in three species of seabirds breeding in the Ebro Delta area (NE Iberian Peninsula), which has been long impacted by the legacy mercury pollution from a chlor-alkali plant. Furthermore, we have also investigated how temporal changes in anthropogenic activities leading to Hg release or remobilization in the environment can be traced by Hg stable isotopes measured in a bioindicator species such as the Audouin's gull. Nonetheless, due to complexity of ecosystems, trophic ecology, physiology of species and other processes affecting mercury isotopic signatures, particularly MDF, more studies in this direction should be performed in order to be able to discern processes related to Hg accumulation in top-predators foraging in complex ecosystems such as wetlands and marine environments.

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Supplementary Material

Sources of Hg in aquatic top-predators in the Ebro Delta (NE Iberian Peninsula) by means of mercury stable isotopes: inter-specific and temporal differences

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Hg isotopic composition in feather samples of *I. audouinii*, *C. ridibundus* and *S. hirundo* fledglings

Results for Hg isotopic compositions are reported using the standard delta notation (in ‰) relative to the standard NIST 3133, according to the following equation (Bergquist and Blum 2007):

$$\delta^{xxx}\text{Hg} = [({}^{xxx}\text{Hg}/{}^{198}\text{Hg})_{\text{sample}}/({}^{xxx}\text{Hg}/{}^{198}\text{Hg})_{\text{NIST3133}} - 1] \times 1000$$

where, ‘xxx’ represents the mass number of Hg (199, 200, 201, 202, 204), $({}^{xxx}\text{Hg}/{}^{198}\text{Hg})_{\text{sample}}$ is the isotope ratio measured for the sample and $({}^{xxx}\text{Hg}/{}^{198}\text{Hg})_{\text{NIST3133}}$ is the mean isotope ratio for the standard NIST 3133. In this study, MDF was represented by $\delta^{202}\text{Hg}$ signatures.

MIF are reported using the capital delta notation, using the difference by the $\delta^{xxx}\text{Hg}$ value and a value predicted based on MDF according to Bergquist and Blum (2007):

$$\Delta^{204}\text{Hg} \approx \delta^{204}\text{Hg} - (\delta^{202}\text{Hg} \times 1.493)$$

$$\Delta^{201}\text{Hg} \approx \delta^{201}\text{Hg} - (\delta^{202}\text{Hg} \times 0.752)$$

$$\Delta^{200}\text{Hg} \approx \delta^{200}\text{Hg} - (\delta^{202}\text{Hg} \times 0.502)$$

$$\Delta^{199}\text{Hg} \approx \delta^{199}\text{Hg} - (\delta^{202}\text{Hg} \times 0.252)$$

Table S1 | Measurements of Hg concentrations ([THg] ng/g dw), C, N and Hg stable isotopes signatures (‰) in feathers of fledglings from three Laridae species breeding in the Ebro Delta during the 2017 breeding season. Species codes: LA (*I. audouinii*, Audouin's gull), LR (*C. ridibundus*; black-headed gull), SH (*S. hirundo*; common tern).

Sample ID	Species	Year	$\delta^{204}\text{Hg}$	$\delta^{202}\text{Hg}$	$\delta^{201}\text{Hg}$	$\delta^{200}\text{Hg}$	$\delta^{199}\text{Hg}$	$\Delta^{204}\text{Hg}$	$\Delta^{201}\text{Hg}$	$\Delta^{200}\text{Hg}$	$\Delta^{199}\text{Hg}$	$\delta^{13}\text{C}$	$\delta^{15}\text{N}$	[THg]
LA-EDB17-12	LA	2017	0.35	0.16	0.86	0.03	1.06	0.12	0.74	-0.05	1.02	-16.83	14.22	7720.26
LA-EDB17-13	LA	2017	0.45	0.31	1.09	0.13	1.19	-0.02	0.85	-0.03	1.11	-17.16	13.46	8728.11
LA-EDB17-16	LA	2017	0.33	0.22	0.54	0.12	0.65	0.00	0.38	0.01	0.60	-19.69	13.28	8318.27
LA-EDB17-17	LA	2017	0.34	0.29	0.92	0.11	0.96	-0.08	0.70	-0.03	0.89	-16.91	13.66	9205.55
LA-EDB17-21	LA	2017	0.51	0.36	1.02	0.28	1.13	-0.03	0.75	0.1	1.04	-17.62	13.83	6439.79
LA-EDB17-23	LA	2017	0.57	0.43	1.12	0.27	1.04	-0.07	0.8	0.05	0.93	-17.81	13.55	6945.61
LA-EDB17-24	LA	2017	0.23	0.11	0.81	0.11	0.96	0.07	0.73	0.05	0.93	-17.30	13.81	7200.69
LA-EDB17-26	LA	2017	0.46	0.32	0.86	0.11	0.84	-0.02	0.62	-0.05	0.76	-18.04	13.94	9072.00
LA-EDB17-29	LA	2017	0.36	0.29	0.89	0.19	1.00	-0.08	0.68	0.04	0.93	-17.89	13.42	7533.32
LA-EDB17-30	LA	2017	0.20	0.13	0.63	0.07	0.72	0.01	0.54	0.00	0.69	-17.28	14.52	8666.11
LR-EDB17-01	LR	2017	0.93	0.65	1.39	0.38	1.28	-0.05	0.90	0.05	1.12	-19.71	13.79	3494.11
LR-EDB17-03	LR	2017	0.78	0.46	1.12	0.29	1.13	0.10	0.78	0.06	1.02	-22.29	13.65	1741.96
LR-EDB17-05	LR	2017	0.74	0.52	0.94	0.30	0.90	-0.04	0.54	0.03	0.77	-22.50	13.05	1337.37
LR-EDB17-08	LR	2017	0.58	0.38	0.88	0.23	0.86	0.01	0.59	0.04	0.77	-21.98	12.86	1213.24
LR-EDB17-09	LR	2017	0.55	0.37	1.13	0.16	1.11	-0.01	0.84	-0.02	1.01	-19.32	14.80	1325.87
LR-EDB17-11	LR	2017	0.59	0.43	1.12	0.26	1.05	-0.04	0.8	0.04	0.95	-21.64	12.82	1263.41
LR-EDB17-13	LR	2017	0.91	0.64	1.61	0.31	1.50	-0.04	1.13	-0.01	1.34	-20.22	14.19	1204.44
LR-EDB17-17	LR	2017	0.79	0.58	1.05	0.31	1.00	-0.08	0.61	0.01	0.86	-22.19	12.83	1549.64
LR-EDB17-18	LR	2017	1.37	0.84	1.65	0.39	1.50	0.11	1.01	-0.03	1.29	-21.48	13.29	1301.05
LR-EDB17-20	LR	2017	1.17	0.80	1.24	0.39	1.08	-0.02	0.63	-0.02	0.88	-21.93	13.76	1128.31
SH-EDB17-01	SH	2017	1.70	1.15	3.48	0.60	3.42	-0.02	2.61	0.03	3.13	-16.96	12.04	1381.47
SH-EDB17-05	SH	2017	1.37	1.02	3.05	0.54	2.98	-0.15	2.28	0.03	2.72	-16.82	12.38	2108.95
SH-EDB17-09	SH	2017	1.67	1.13	3.08	0.57	3.01	-0.01	2.23	0.00	2.73	-16.78	12.11	2100.57
SH-EDB17-12	SH	2017	1.55	1.03	3.22	0.62	3.13	0.01	2.44	0.10	2.87	-17.27	11.73	1793.80

SH-EDB17-16	SH	2017	1.47	1.02	3.12	0.55	3.11	-0.05	2.35	0.03	2.85	-16.58	11.64	1737.98
SH-EDB17-17	SH	2017	1.38	0.94	2.94	0.50	2.90	-0.03	2.23	0.03	2.67	-16.73	12.05	1933.22
SH-EDB17-20	SH	2017	1.65	1.20	3.17	0.66	3.12	-0.14	2.27	0.05	2.82	-16.60	12.03	1944.26
SH-EDB17-23	SH	2017	1.38	0.94	2.33	0.54	2.22	-0.02	1.62	0.07	1.98	-18.06	12.00	2379.45
SH-EDB17-24	SH	2017	1.60	1.13	3.07	0.60	3.05	1.54	2.22	0.03	2.76	-18.13	13.15	1661.40
SH-EDB17-28	SH	2017	1.45	0.97	2.78	0.52	2.75	0.00	2.05	0.04	2.50	-16.57	12.50	2120.32

Table S2 | Measurements of Hg concentrations ([THg] ng/g dw), C, N and Hg stable isotopes signatures (‰) in feathers of fledglings from Audouin's gull (*I. audouinii*) breeding in the Ebro Delta during the 2010, 2013, 2014, 2017 breeding seasons.

Sample ID	Year	$\delta^{204}\text{Hg}$	$\delta^{202}\text{Hg}$	$\delta^{201}\text{Hg}$	$\delta^{200}\text{Hg}$	$\delta^{199}\text{Hg}$	$\Delta^{204}\text{Hg}$	$\Delta^{201}\text{Hg}$	$\Delta^{200}\text{Hg}$	$\Delta^{199}\text{Hg}$	$\delta^{13}\text{C}$	$\delta^{15}\text{N}$	[THg]
LA-EDB10-01	2010	0.30	0.28	1.15	0.17	1.25	-0.13	0.94	0.03	1.18	-18.17	12.77	3488.29
LA-EDB10-02	2010	0.79	0.55	1.54	0.37	1.47	-0.03	1.13	0.10	1.33	-17.76	12.48	2438.68
LA-EDB10-03	2010	0.38	0.27	1.18	0.11	1.27	-0.02	0.98	-0.02	1.20	-17.94	12.56	4300.13
LA-EDB10-04	2010	0.63	0.42	1.23	0.26	1.23	0.00	0.91	0.05	1.12	-18.09	12.39	4854.90
LA-EDB10-05	2010	0.37	0.41	1.20	0.23	1.32	-0.23	0.90	0.02	1.22	-17.57	13.08	2889.75
LA-EDB10-06	2010	0.90	0.82	1.77	0.45	1.63	-0.33	1.15	0.04	1.42	-18.73	12.58	5786.12
LA-EDB10-07	2010	1.19	0.88	1.65	0.51	1.49	-0.12	0.99	0.07	1.27	-17.46	13.27	5044.57
LA-EDB10-08	2010	1.23	0.75	1.73	0.39	1.58	0.12	1.16	0.01	1.39	-18.08	12.47	3635.68
LA-EDB10-09	2010	1.21	0.76	1.46	0.41	1.32	0.07	0.89	0.03	1.13	-17.83	12.76	4005.45
LA-EDB10-10	2010	1.14	0.78	1.74	0.44	1.64	-0.03	1.15	0.05	1.45	-18.11	12.38	6441.93
LA-EDB13-01	2013	0.31	0.32	1.20	0.16	1.31	-0.17	0.96	-0.01	1.23	-24.10	12.36	1588.99
LA-EDB13-04	2013	0.11	0.11	0.94	0.07	1.06	-0.06	0.85	0.01	1.03	-19.69	12.62	2471.91
LA-EDB13-06	2013	0.32	0.14	1.09	0.03	1.29	0.12	0.99	-0.04	1.25	-22.76	12.47	1607.24
LA-EDB13-07	2013	0.17	0.14	0.93	0.12	1.03	-0.04	0.82	0.05	0.99	-23.08	13.14	1655.81
LA-EDB13-10	2013	0.09	0.11	0.79	0.02	0.92	-0.07	0.71	-0.03	0.89	-19.84	12.67	2441.67
LA-EDB13-11	2013	0.37	0.29	1.00	0.12	1.11	-0.07	0.78	-0.03	1.04	-17.26	13.04	2710.85
LA-EDB13-12	2013	0.11	0.09	1.07	0.03	1.20	-0.04	1.00	-0.02	1.18	-17.95	12.44	2572.82
LA-EDB13-13	2013	0.12	0.18	1.09	0.10	1.26	-0.15	0.95	0.01	1.22	-18.00	12.53	2183.09
LA-EDB13-14	2013	-0.05	0.00	0.67	0.02	0.86	-0.05	0.67	0.02	0.86	-17.97	12.35	2427.23
LA-EDB13-15	2013	0.09	0.09	0.83	0.04	0.87	-0.04	0.76	-0.01	0.85	-17.58	12.89	2616.27
LA-EDB14-01	2014	0.70	0.50	1.33	0.31	1.29	-0.04	0.95	0.06	1.16	-16.40	13.43	7599.76
LA-EDB14-05	2014	0.86	0.57	1.42	0.31	1.36	0.02	0.99	0.03	1.22	-16.50	14.09	6619.52
LA-EDB14-06	2014	0.64	0.46	1.05	0.25	1.08	-0.04	0.71	0.02	0.96	-18.80	13.92	6421.69
LA-EDB14-07	2014	0.87	0.57	2.00	0.29	1.63	0.02	1.57	0.01	1.49	-18.20	13.41	11842.48
LA-EDB14-09	2014	0.74	0.56	1.27	0.33	1.20	-0.09	0.85	0.05	1.06	-17.30	13.21	9043.08
LA-EDB14-10	2014	0.72	0.54	1.03	0.31	1.06	-0.08	0.63	0.04	0.92	-19.60	14.25	6343.53
LA-EDB14-12	2014	1.09	0.74	1.57	0.39	1.40	0.00	1.02	0.02	1.21	-17.40	13.72	6458.19

LA-EDB14-14	2014	0.92	0.65	1.48	0.37	1.42	-0.05	1.00	0.04	1.26	-16.90	13.89	15009.73
LA-EDB14-17	2014	0.93	0.64	1.48	0.36	1.35	-0.02	0.99	0.04	1.19	-17.20	13.78	5898.27
LA-EDB14-18	2014	1.02	0.77	1.48	0.39	1.45	-0.13	0.90	0.01	1.25	-16.20	14.83	5084.04
LA-EDB17-12	2017	0.35	0.16	0.86	0.03	1.06	0.12	0.74	-0.05	1.02	-16.83	14.22	7720.26
LA-EDB17-13	2017	0.45	0.31	1.09	0.13	1.19	-0.02	0.85	-0.03	1.11	-17.16	13.46	8728.11
LA-EDB17-16	2017	0.33	0.22	0.54	0.12	0.65	0.00	0.38	0.01	0.60	-19.69	13.28	8318.27
LA-EDB17-17	2017	0.34	0.29	0.92	0.11	0.96	-0.08	0.70	-0.03	0.89	-16.91	13.66	9205.55
LA-EDB17-21	2017	0.51	0.36	1.02	0.28	1.13	-0.03	0.75	0.10	1.04	-17.62	13.83	6439.79
LA-EDB17-23	2017	0.57	0.43	1.12	0.27	1.04	-0.07	0.80	0.05	0.93	-17.81	13.55	6945.61
LA-EDB17-24	2017	0.23	0.11	0.81	0.11	0.96	0.07	0.73	0.05	0.93	-17.30	13.81	7200.69
LA-EDB17-26	2017	0.46	0.32	0.86	0.11	0.84	-0.02	0.62	-0.05	0.76	-18.04	13.94	9072.00
LA-EDB17-29	2017	0.36	0.29	0.89	0.19	1.00	-0.08	0.68	0.04	0.93	-17.89	13.42	7533.32
LA-EDB17-30	2017	0.20	0.13	0.63	0.07	0.72	0.01	0.54	0.00	0.69	-17.28	14.52	8666.11

Table S3 | Mean $\pm$ 2SD values of Hg isotopic composition (‰) obtained for reference materials NIST RM 8610 (UM-Almadén), NIES-13 (human hair) and internal reference material F-KP (penguin feathers).

Sample	Reference	n	$\delta^{204}\text{Hg}$ (‰)	$\delta^{202}\text{Hg}$ (‰)	$\delta^{201}\text{Hg}$ (‰)	$\delta^{200}\text{Hg}$ (‰)	$\delta^{199}\text{Hg}$ (‰)	$\Delta^{204}\text{Hg}$ (‰)	$\Delta^{201}\text{Hg}$ (‰)	$\Delta^{200}\text{Hg}$ (‰)	$\Delta^{199}\text{Hg}$ (‰)
NIST RM 8610 (UM-Almadén)	This study Reference values	18	-0.82 $\pm$ 0.16	-0.53 $\pm$ 0.14	-0.44 $\pm$ 0.12	-0.26 $\pm$ 0.09	-0.16 $\pm$ 0.09	-0.04 $\pm$ 0.13	-0.05 $\pm$ 0.06	0.00 $\pm$ 0.06	-0.02 $\pm$ 0.07
			-0.82 $\pm$ 0.07	-0.56 $\pm$ 0.03	-0.46 $\pm$ 0.02	-0.27 $\pm$ 0.01	-0.17 $\pm$ 0.01	-	-0.04 $\pm$ 0.01	0.00 $\pm$ 0.01	-0.03 $\pm$ 0.02
NIES-13	This study Yamakawa et al. (2016)	4	3.14 $\pm$ 0.13	2.16 $\pm$ 0.12	3.18 $\pm$ 0.12	1.15 $\pm$ 0.07	2.39 $\pm$ 0.09	-0.09 $\pm$ 0.08	1.55 $\pm$ 0.06	0.06 $\pm$ 0.03	1.84 $\pm$ 0.07
		11	2.76 $\pm$ 0.16	1.89 $\pm$ 0.10	2.77 $\pm$ 0.10	0.98 $\pm$ 0.08	2.13 $\pm$ 0.07	-0.04 $\pm$ 0.11	1.36 $\pm$ 0.07	0.04 $\pm$ 0.04	1.65 $\pm$ 0.06
F-KP	This study Renedo et al. (2018)	4	2.32 $\pm$ 0.16	1.53 $\pm$ 0.07	2.68 $\pm$ 0.07	0.77 $\pm$ 0.05	2.18 $\pm$ 0.07	0.03 $\pm$ 0.19	1.53 $\pm$ 0.11	0.00 $\pm$ 0.03	1.79 $\pm$ 0.06
		17	2.40 $\pm$ 0.25	1.58 $\pm$ 0.22	2.67 $\pm$ 0.21	0.80 $\pm$ 0.16	2.11 $\pm$ 0.18	0.04 $\pm$ 0.24	1.48 $\pm$ 0.13	0.01 $\pm$ 0.09	1.71 $\pm$ 0.15

Table S4 | Summary statistics (mean, SD) for each of the parameters ($\Delta^{200}\text{Hg}$ and $\Delta^{204}\text{Hg}$) in fledglings feathers for each of the three species studied during the 2017 breeding season in the Ebro Delta. IA: *I. audouinii* (Audouin's gull), CR: *C. ridibundus* (black-headed gull), SH: *S. hirundo* (common tern).

Species	$\Delta^{200}\text{Hg}$				$\Delta^{204}\text{Hg}$			
	Mean	SD	Max	Min	Mean	SD	Max	Min
LA	0.01	0.05	0.10	-0.05	-0.01	0.06	0.12	-0.08
LR	0.02	0.03	0.06	-0.03	-0.01	0.06	0.11	-0.08
SH	0.04	0.03	0.10	0.00	-0.05	0.06	0.01	-0.15

Table S5 | Summary statistics (mean, SD, max and min) for each of the parameters ($\Delta^{200}\text{Hg}$ and $\Delta^{204}\text{Hg}$) in fledglings' feathers for each of the years studied (2010, 2013, 2014 and 2017) on the Audouin's gull (*I. audouinii*) breeding colony in the Ebro Delta.

Year	$\Delta^{200}\text{Hg}$				$\Delta^{204}\text{Hg}$			
	Mean	SD	Max	Min	Mean	SD	Max	Min
2010	0.04	0.03	0.10	-0.02	-0.07	0.14	0.12	-0.33
2013	-0.01	0.03	0.05	-0.04	-0.06	0.08	0.12	-0.17
2014	0.03	0.02	0.06	0.01	-0.04	0.05	0.02	-0.13
2017	0.01	0.05	0.10	-0.05	-0.01	0.06	0.12	-0.08

Fig. S1 | Odd-MIF regression between $\Delta^{199}\text{Hg}$ vs. $\Delta^{201}\text{Hg}$ in feathers of Audouin's gulls (IA, in gray), black-headed gull (CR, in orange) and common tern (SH, in blue) fledglings from the Ebro Delta in 2017. Individual data are plotted as dots. Dashed line represents linear regression between these variables for the three species: $y = 0.09558 + 1.1667x$; $R^2 = 0.9965$; $p < 0.0001$. Analytical error (2SD) for $\Delta^{199}\text{Hg}$ and $\Delta^{201}\text{Hg}$ is ± 0.07 and ± 0.06 , respectively, estimated from the 2SD of the mean Hg isotopic composition of the NIST RM 8610 (UM-Almadén) over multiple analytical sessions (see Table S3).

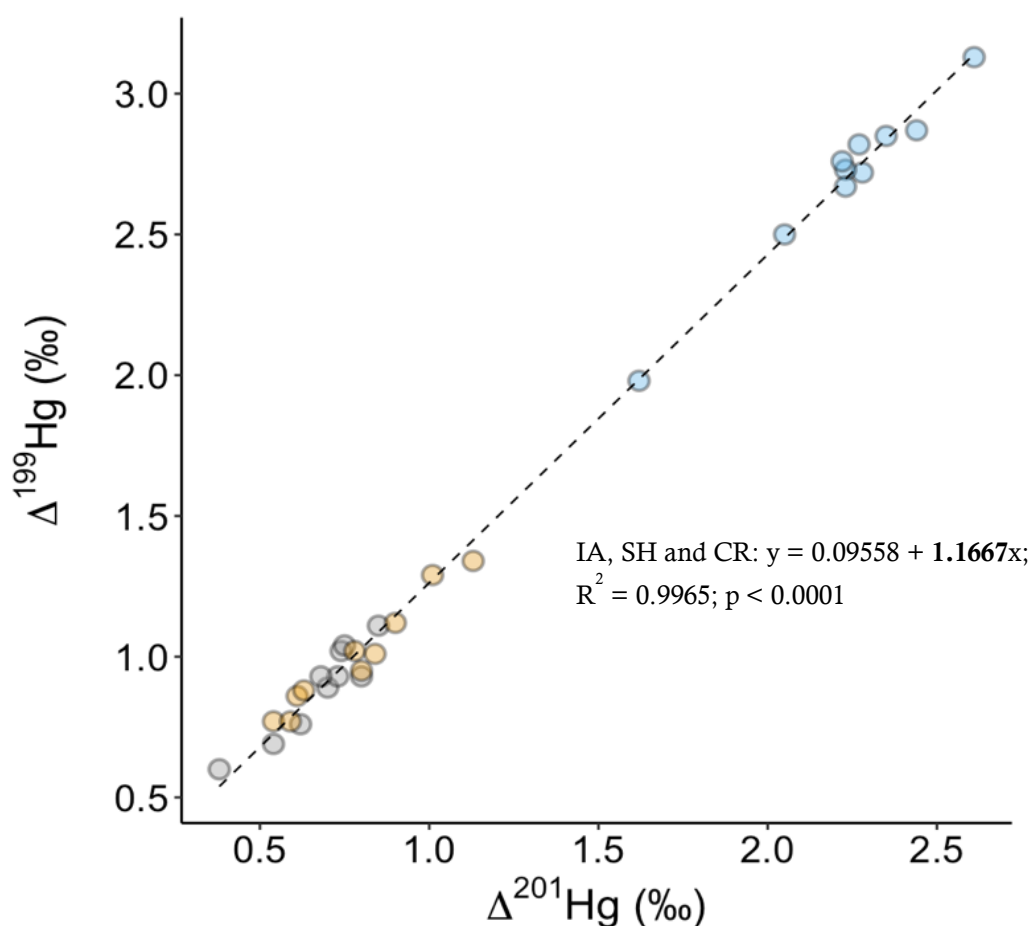


Fig. S2 | Even-MIF plot between $\Delta^{200}\text{Hg}$ vs. $\Delta^{204}\text{Hg}$ in feathers of Audouin's gulls (IA, in gray), black-headed gull (CR, in orange) and common tern (SH, in blue) fledglings from the Ebro Delta in 2017. Individual data are plotted as dots. Analytical error (2SD) for $\Delta^{200}\text{Hg}$ and $\Delta^{204}\text{Hg}$ is ± 0.06 and ± 0.13 , respectively, estimated from the 2SD of the mean Hg isotopic composition of the NIST RM 8610 (UM-Almadén) over multiple analytical sessions (see Table S3).

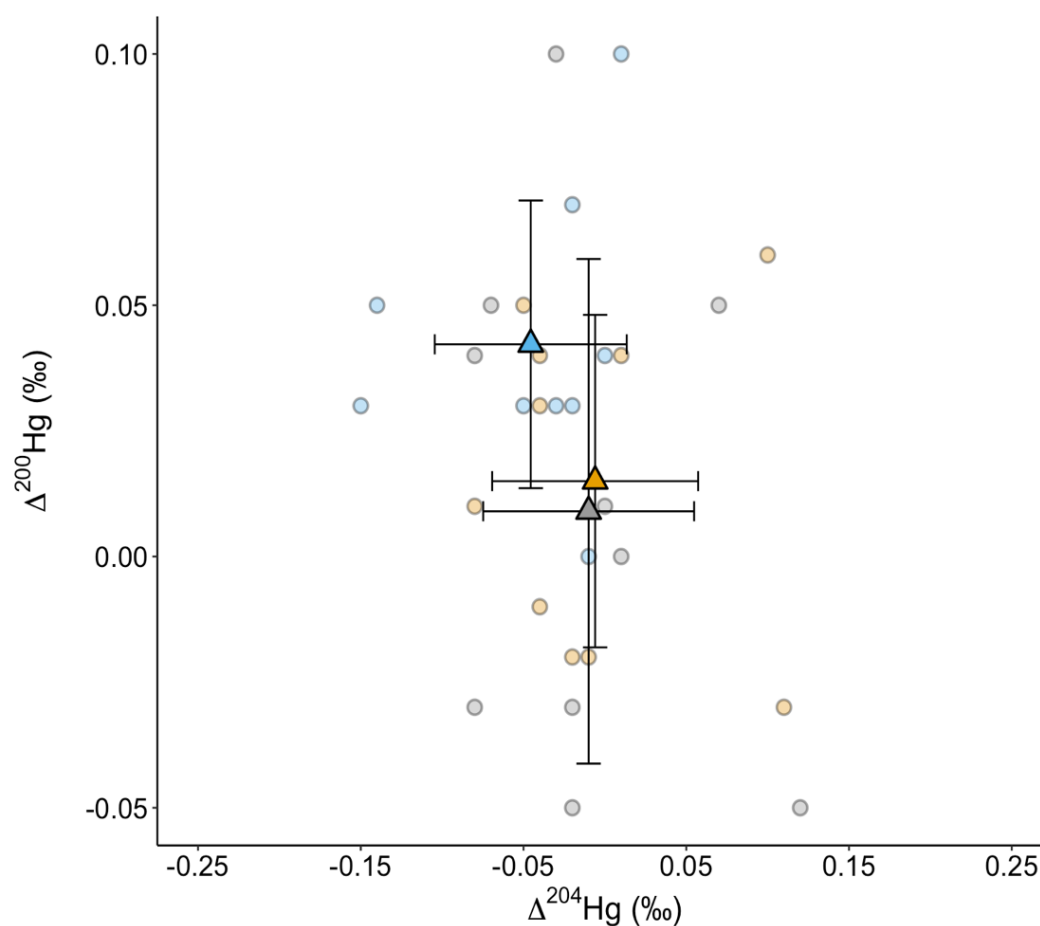


Fig. S3 | Odd-MIF regression between $\Delta^{199}\text{Hg}$ vs. $\Delta^{201}\text{Hg}$ in feathers of Audouin's gulls (IA) fledglings from the Ebro Delta in 2010 (yellow), 2013 (green), 2014 (gray) and 2017 (blue). Individual data are plotted as dots. Dashed line represents linear regression between these variables for each of the four years. Analytical error (2SD) for $\Delta^{199}\text{Hg}$ and $\Delta^{201}\text{Hg}$ is ± 0.07 and ± 0.06 , respectively, estimated from the 2SD of the mean Hg isotopic composition of the NIST RM 8610 (UM-Almadén) over multiple analytical sessions (see Table S3).

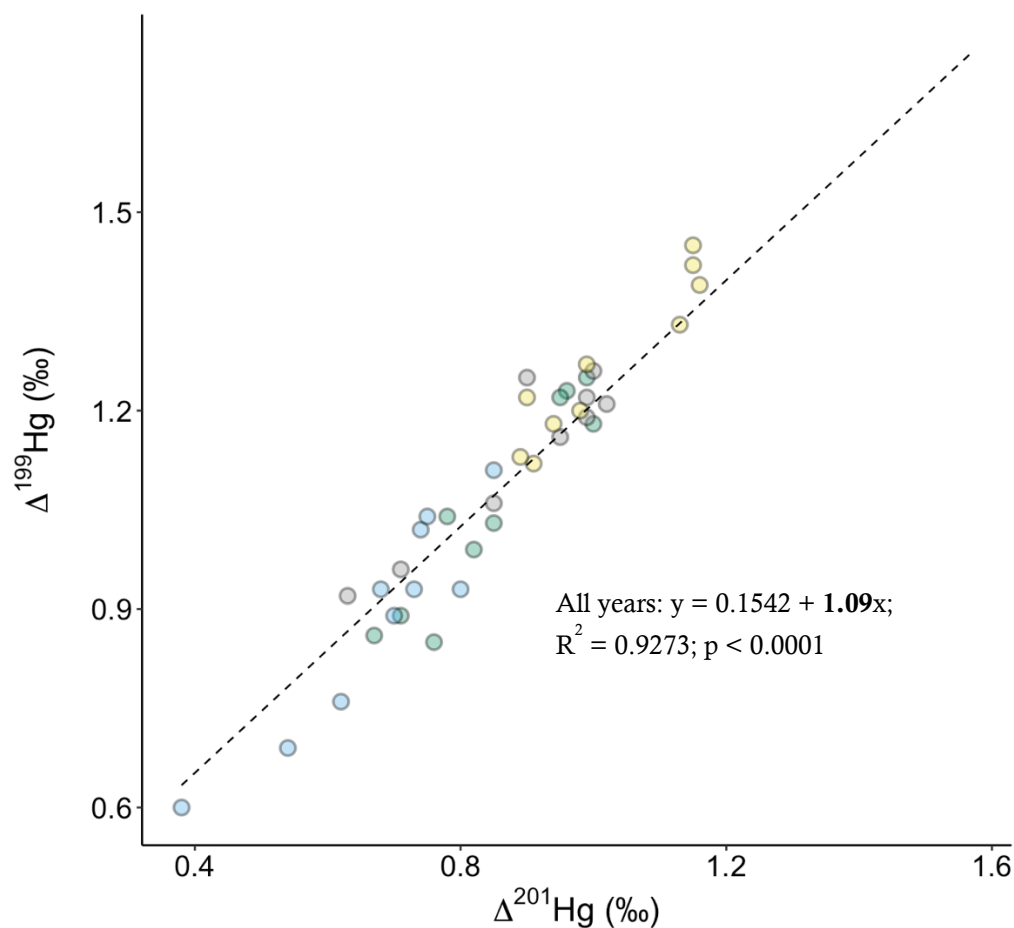
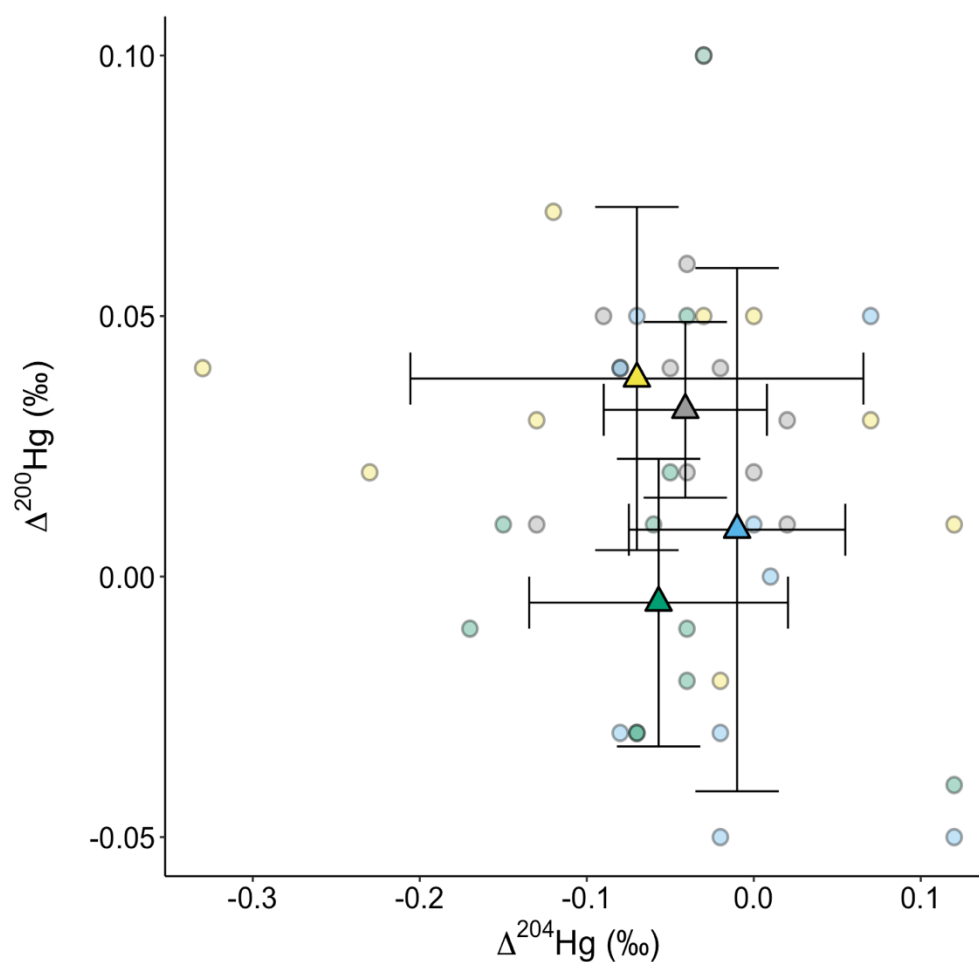


Fig. S4 | Even-MIF plot between $\Delta^{200}\text{Hg}$ vs. $\Delta^{204}\text{Hg}$ in feathers of Audouin's gulls (IA) fledglings from the Ebro Delta in 2010 (yellow), 2013 (green), 2014 (gray) and 2017 (blue). Individual data are plotted as dots. Analytical error (2SD) for $\Delta^{200}\text{Hg}$ and $\Delta^{204}\text{Hg}$ is ± 0.06 and ± 0.13 , respectively, estimated from the 2SD of the mean Hg isotopic composition of the NIST RM 8610 (UM-Almadén) over multiple analytical sessions (see Table S3).



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06

CHAPTER 6

General Discussion

06

GENERAL DISCUSSION

The Ebro Delta ecosystems are known to be impacted by mercury pollution. Although studies on mercury bioavailability for fauna have been performed previously in the area, rice paddies, being one of the most important ecosystems in the Ebro Delta, have been seldom investigated for the presence and/or transformation of mercury and its dynamics. The present thesis focuses on rice fields, which are receiving a greater attention by the scientific community as mercury hotspots. Also, a novelty of this thesis is that Audouin's gull is selected as a model organism to an in-depth comprehension on how mercury pollution distributes and affects different Ebro Delta habitats, thanks to their foraging behavior and trophic ecology (see *Introduction*). To this aim, we have used current methodologies for mercury research such as the metal stable isotopes, which have been sparingly applied to ecological problems in top predators. Altogether this thesis offers new insights on mercury pollution in the Ebro Delta and how it has been influenced by anthropogenic activities (e.g., industrial activities).

In this global discussion, first we will summarize and discuss the main findings regarding the distribution, dynamics, and availability of mercury in rice fields (1). Secondly, we will discuss the feasibility of a model organism such as Audouin's gull as a bioindicator species for mercury pollution in aquatic environments (2), and how the application of novel techniques of environmental mercury monitoring to this species can aid to further comprehend the dynamics of mercury in the environment (3). Finally, we will discuss the implications of our studies (4) and propose future research based on the questions raised in the present thesis (5).

1. Advances in the knowledge of rice fields as mercury hotspots

1.1. Ebro Delta rice fields as mercury hotspots

In many wetland environments, net mercury methylation occurs naturally (Mitchell et al. 2007; Marvin-DiPasquale et al. 2014), transforming inorganic mercury (Hg^{2+}) to methyl-mercury (MeHg), a bioavailable and bioaccumulative toxic form of mercury, which in sufficient concentrations causes health detrimental effects on humans and wildlife

(Clarkson and Magos 2006; Díez 2009; Evers 2018). Thus, monitoring mercury levels in such environments is mandatory from an ecosystem conservation point of view. Due to the worldwide human population growth, natural wetlands tend to disappear and be replaced by agricultural farming areas (Davidson 2014) such as rice paddies. Rice fields have been extensively studied as mercury hotspots (Zhao et al. 2020; Tang et al. 2020). The environmental conditions attained in rice paddies favor microbial growth and mercury methylation (Rothenberg and Feng 2012; Rothenberg et al. 2014), and this, in turn, promotes transference of mercury to rice plants and fauna depending on rice field environments (Meng et al. 2011, 2014; Wang et al. 2018).

In accordance with previous studies, our results show that rice fields in the Ebro Delta are effective sites of mercury sinkage and methylation, and thus, bioavailability to plants and fauna inhabiting the rice paddies (Rothenberg and Feng 2012). Although rice fields have also been studied regarding mercury pollution in the area (Suárez-Serrano et al. 2010), to our knowledge no studies have evaluated how mercury behaves throughout the rice cultivation season in different compartments of rice fields and/or how do agricultural practices employed for rice farming affect such dynamics.

Our results from *Chapter 2* show that despite the variability in agricultural practices employed for rice farming, significant concentrations of THg are present in the different rice field compartments studied (water, soil, plants, biota). Thus, reinforcing the idea of agricultural wetlands as mercury hotspots (Marvin-DiPasquale et al. 2014) and the urge to evaluate the presence of pollutants in such environments, both from an ecological and food safety perspective (UNEP 2019).

In rice paddies, mercury comes mainly from irrigation waters (Dai et al. 2013) and atmospheric deposition (Rodríguez et al. 2008). In the case of the Ebro Delta, the legacy mercury pollution from the chlor-alkali plant at Flix might have increased, through river transport, the levels of mercury in all the ecosystems in the area (Pujadas et al. 2016; Palanques et al. 2020). Indeed, studies in biota from the Ebro river basin have detected higher THg levels in fish downstream the chlor-alkali plant than nearby the industrial site (Carrasco et al. 2011a; 2011b), further suggesting that mercury is carried downstream by the river. Particularly, as rice paddies represent ca. 75% of the area in the Ebro Delta (Mañosa et al. 2001), the impact of mercury pollution in these ecosystems is of greatest importance.

The THg levels obtained and their trends over time in *Chapter 2*, are in accordance with previous studies reporting THg concentrations in rice plants, rice paddies' soils or water

in Hg-polluted sites (e.g., Meng et al. 2010, 2014; Rothenberg and Feng 2012). Water mercury concentrations were relatively low, however it increased significantly from the beginning of the rice farming season to the end, suggesting a continuous input of Hg into rice fields through irrigation water (Dai et al. 2013), as observed for other metals and pollutants in the area (e.g., Pb, Cr, Ni, organochlorine compounds; Mañosa et al. 2001; Calvo-Cubero et al. 2015). Although Hg entered continuously in rice fields through irrigation water, soil mercury concentrations did not present significant changes throughout the rice growing season, which reflects the multifactorial parameters that control mercury concentration in soils (e.g., inputs from atmospheric deposition, organic matter composition and concentration, transference to other compartments, methylation/demethylation rates, physicochemical conditions; Ullrich et al. 2001; Rodríguez et al. 2008; Rothenberg and Feng 2012; Zhou et al. 2015; Liu et al. 2019), and that their combination do maintain THg levels in soil throughout the rice growing period (Rothenberg and Feng 2012; Zhao et al. 2016).

Regarding the rice plants, we studied different parts, namely roots, stalks, leaves and grain, separately, since they do accumulate significant amounts of Hg, both organic (MeHg) and inorganic (Hg^{2+}) forms, but this accumulation behaves differently regarding the plant part studied (Zhang et al. 2010, Rothenberg and Feng 2012). Essentially, only roots increased THg concentrations over the plant growing period, suggesting an active accumulation of Hg within the cells (Meng et al. 2010, 2014; Zhang et al. 2010; Rothenberg et al. 2014; Turull et al. 2017), whereas leaves, stalks and grain, decreased THg levels over time, due to biodilution with biomass gain (Shu et al. 2016; Fig. 1). Both rice plant roots and grain seem to source mercury mainly from the soil compartment (Meng et al. 2010), whereas leaves and stalks mercury concentrations might be sourced from both soil and air (Meng et al. 2010; Marrugo-Negrete et al. 2016). To further understand the species of mercury accumulated by each of the tissues and organs of the plant considered, speciation analyses were performed in rice plants, with roots accumulating mainly Hg^{2+} and rice grains accumulating higher MeHg proportions (to THg) in their tissues (see Table 2 and Table 3 from *Chapter 2*).

Finally, we also evaluated THg levels in fauna from the rice fields food webs, in order to gain insight on bioaccumulation in organisms, and biomagnification along trophic levels. We found an effective biomagnification along all the food webs studied (Fig. 2; with trophic magnification factors > 1 in all cases). Rice fields in the Ebro Delta harbor an important number of vertebrates and invertebrate species (Franch et al. 2008; Clavero et al. 2015). Fish, amphibians, crayfish and insects inhabiting rice fields are important prey for many waterbird species, such as gulls, ducks or herons (Elphick 2000; Mañosa et al. 2001; Ibañez et al. 2010), which as top predators will be exposed to higher Hg levels (Lavoie et al. 2013). Thus, in this

regard, rice field food webs might function as mercury exporting agents, connecting several ecosystems through prey consumption and animal dispersion (i.e., biotransport; Schiesari et al. 2017).

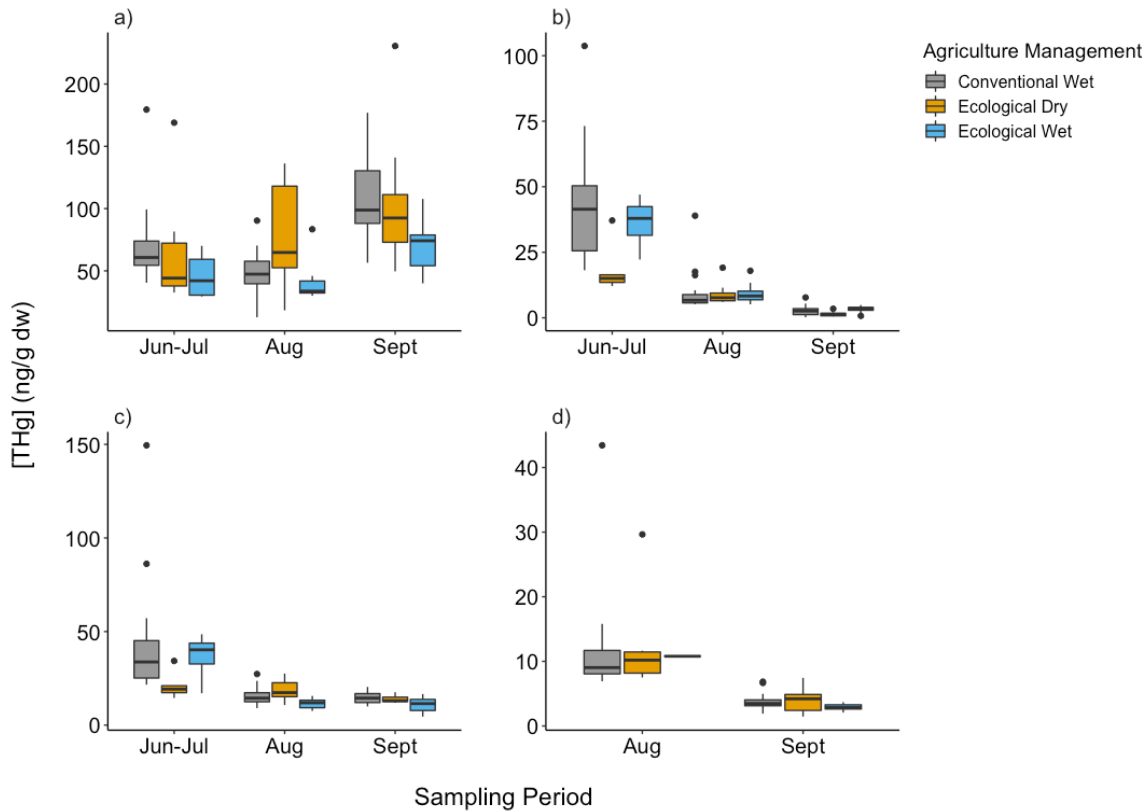


Figure 1 | THg concentrations (ng/g dw) in rice plant parts: (a) roots, (b) stalk, (c) leaves, (d) grain, in three sampling periods over the rice cultivation season from 2017 in the Ebro Delta. Samples from fields farmed using different agricultural management practices (Conventional Wet, Ecological Wet, Ecological Dry) were analyzed.

The results on Hg dynamics in rice fields (*Chapter 2*), have been complemented with an experimental *in-situ* mesocosm study (*Chapter 3*) to evaluate the bioaccumulation potential of rice paddies in fish, using *Carassius auratus* as model organism, a non-native species in the area which is already present in the rice fields' ecosystems throughout the delta (Clavero et al. 2021). The results of this experiment revealed that agricultural wetlands are a source of mercury, which is bioavailable and bioaccumulates in fauna, as suggested by similar studies by Ackerman and Eagles-Smith (2010) and Ackerman et al. (2010). Nonetheless, we further evaluated the effect of mercury dynamics within rice fields and bioaccumulation in rice fields' fauna considering the main different agricultural practices employed by rice farmers in the Ebro Delta, to assess the potentiality of these practices as drivers of Hg cycling within rice paddies.

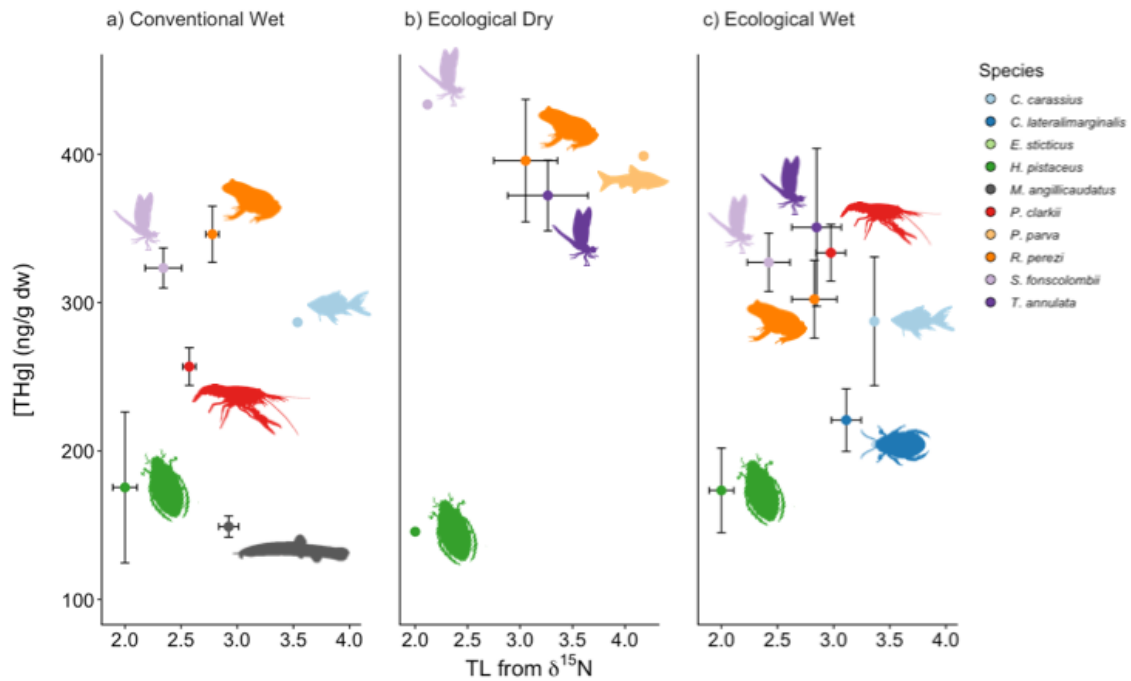


Figure 2 | THg concentrations vs. trophic level (TL) in fauna samples for the three types of agricultural management fields: (a) Conventional wet, (b) Ecological Dry and (c) Ecological wet. Trophic magnification factors (TMF) for THg were >1 in all three rice field types indicating an effective biomagnification of mercury along the foodweb.

1.2. Effect of different agricultural practices

In both *Chapters 2 and 3*, we have taken into account different rice farming practices regarding the usage of pesticides and fertilizers (conventional vs. ecological) associated to different hydroperiods (wet vs. alternating wet and dry) in Hg dynamics within rice fields. Both factors have been described as drivers of mercury dynamics in rice paddies (e.g., Rothenberg et al. 2014, 2016; Tanner et al. 2018).

For instance, fertilizers containing sulphates, which are conventionally applied to rice farming schemes (Martínez-Eixarch et al. 2017), might enhance mercury methylation in rice fields by promoting the activity of methylator microorganisms (i.e., sulphate reducing bacteria; Tang et al. 2018, 2019). Nonetheless, we did not detect overall significant differences between conventional and ecological fields in terms of THg levels or their trends over time determined for water, soil, rice plants or fauna (*Chapter 2*), or in Hg bioaccumulation levels in fish in a mesocosm experiment (*Chapter 3*). Thus, other factors than application of pesticides or fertilizers seemed to exert a greater influence in Hg dynamics and bioaccumulation in fauna.

Hydric regimes in cultivars might influence environmental physicochemical properties of rice fields which, in turn, affect the dynamics of Hg in paddies (Rothenberg et al. 2014). The standard rice farming field management implies continuously flooded fields with ca. 15-30 cm in depth throughout the rice cultivation period. However, with global climate change and the increasing water scarcity and demand, hydric regime strategies have been developed for rice farming purposes to reduce water expenses, while maintaining the crop yield (Rothenberg and Feng 2012). Among them, alternate wet and drying (AWD) methodology was developed in 1970s, being one of the water saving strategies most utilized worldwide (Bouman and Tuong 2001; Carrijo et al. 2017). It consists in alternating flooded periods with drainage periods during the rice growing season. Besides of water saving benefits, AWD has also other benefits, such as mitigating CH₄ emissions (Linguist et al. 2015) or reducing the content of some toxicants such as arsenic (As) in grains (LaHue et al., 2016). A recent study has evaluated the potentiality of AWD technique in the Ebro Delta rice paddies in terms of agronomic impact (i.e., crop yield), food safety (e.g., reduction of As content in grain) and CH₄ emissions mitigation (Martínez-Eixarch et al. 2021).

Regarding Hg dynamics, several studies have described reductions in THg and/or MeHg in rice plants and soils in fields/experimental pots managed using AWD compared to those under continuously flooded conditions (e.g., Rothenberg et al. 2014, 2016; Tanner et al. 2018). Nonetheless, reduction rates were dependent on the severity of AWD employed (Rothenberg et al. 2016). Conversely, other field studies (e.g., Marvin-DiPasquale et al. 2014) have reported that draining and reflooding might alter the redox conditions in soil-water environment and the presence of electron acceptors and bioavailable Hg²⁺, favoring mercury methylation by microorganisms upon reflooding (Rothenberg and Feng 2012; Zhao et al. 2016; Tanner et al. 2018), with consequent MeHg spikes detected just after reflooding (Marvin-DiPasquale et al. 2014). In Chapter 2, we did not detect significant differences in THg accumulation or trends in plants, soil or water owing to the different hydroperiods employed for rice farming (Fig. 1 and Fig. 3). The lack of an effect of AWD vs. continuously flooded managing on THg in these compartments might be due to the severity of AWD employed (Rothenberg et al. 2016) or to the balance among the multiple factors influencing Hg accumulation in the several compartments considered, which lead to effects in different directions regarding mercury loads in agricultural wetlands (Marvin-DiPasquale et al. 2014; Rothenberg et al. 2014; Windham-Meyers et al. 2014; Tanner et al. 2018).

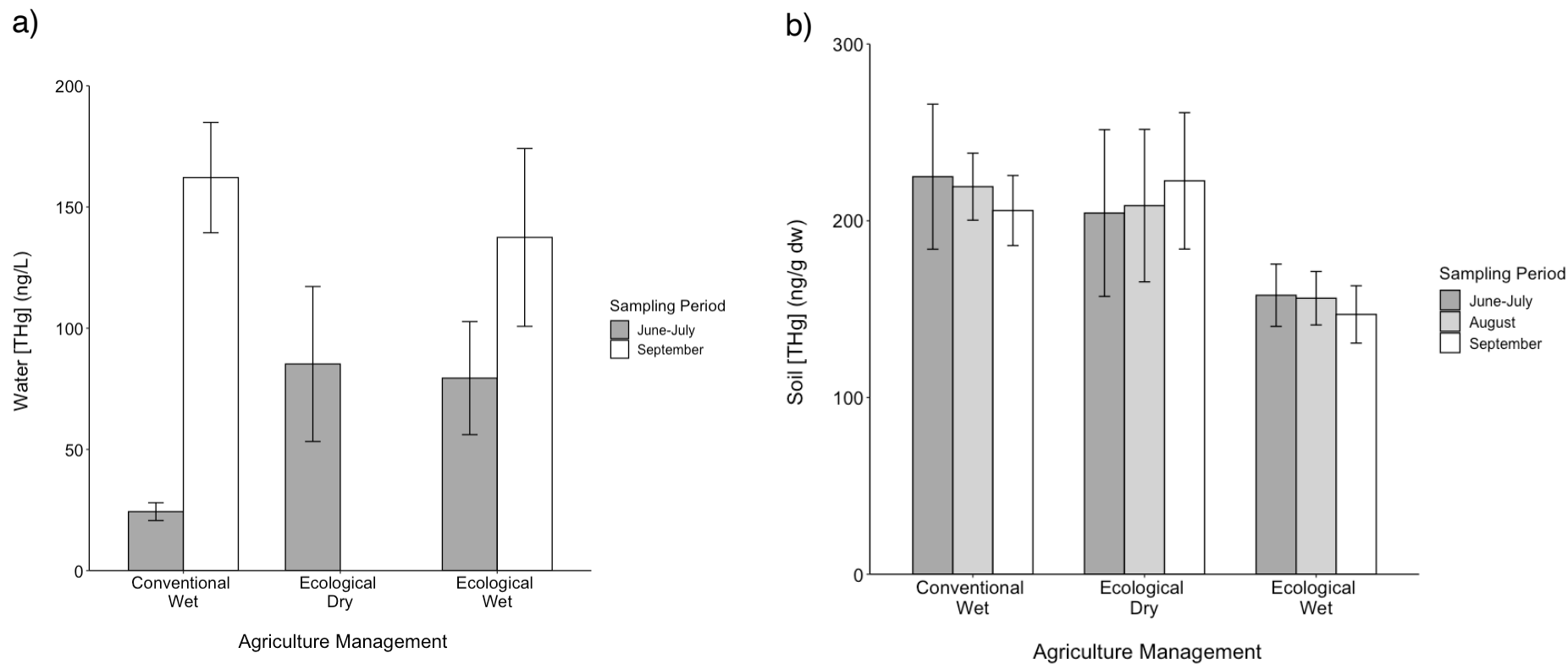


Figure 3 | (a) Water THg concentrations (ng/L) obtained by DGT technique on three sampling periods (June/July, August, September), and (b) soil THg concentrations (ng/g dw) from cores obtained on three sampling periods (June/July, August, September) during the 2017 rice cultivation season from rice fields farmed using three different agricultural management types (Conventional Wet, Ecological Wet, Ecological Dry). Error bars depict the SE of the mean. Significant differences in water THg levels were detected among CW-ED and CW-EW for the first sampling period (June/July) and between the first and third sampling periods in both CW and EW rice fields. Soil THg levels did not differ significantly among agricultural management types or sampling periods.

Although the effect of AWD was not reflected in the aforementioned compartments within rice field systems, our results for fauna samples, seem to evidence an effect of the hydroperiod on animals' THg body THg burdens. Since most of Hg in organisms corresponds to MeHg (see Table S1 in *Chapter 2*), the metal differences detected in fauna among rice fields conditions might arise from specific MeHg dynamics within fields. Organisms living in AWD treated rice fields presented slightly higher Hg concentrations and biomagnification factors (*Chapter 2*). Furthermore, results from *Chapter 3* also point to this same direction. Fish in mesocosm settings exposed to AWD rice fields accumulated higher mercury after 30 days in the experiment set up (Fig. 4). Thus, this seems to indicate that they are sensible to factors affecting mercury dynamics in rice fields, especially regarding MeHg, and indeed, species such as the red crayfish (*Procambarus clarkii*) have been used as Hg bioindicator organisms in rice paddies (Suárez-Serrano et al. 2010). Thus, from an ecosystem health point of view, AWD agricultural management seems to affect food webs by increasing mercury loads in fauna, which can be transferred to higher trophic levels via biomagnification processes.

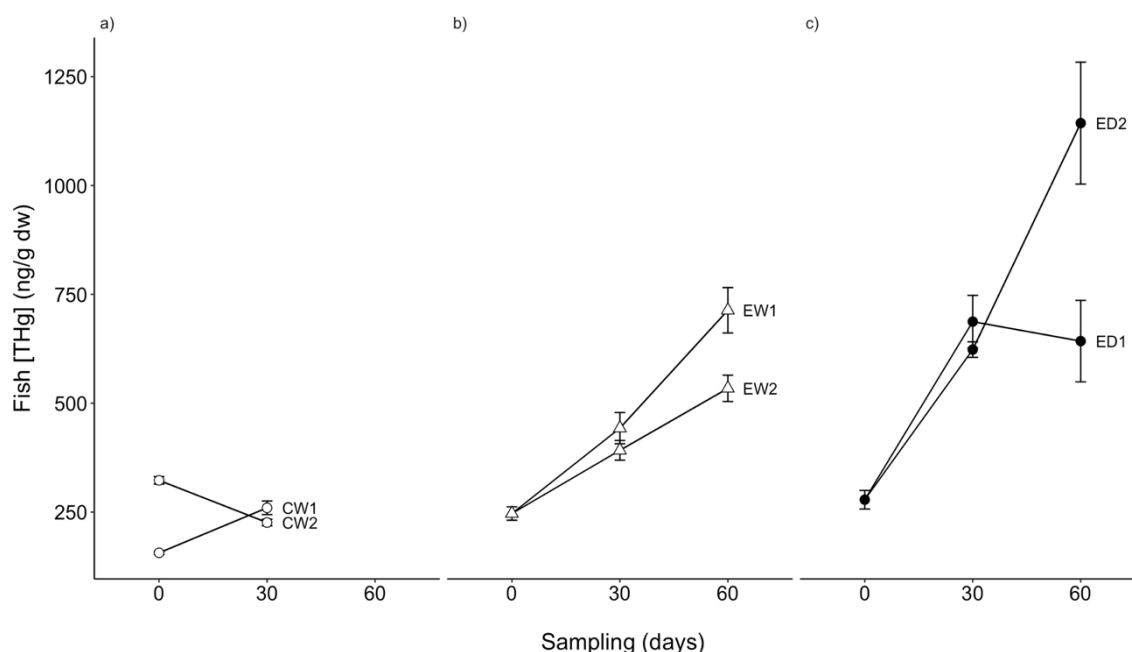


Figure 4 | Whole-body [THg] in *C. auratus* individuals under an *in situ* mesocosm experiment exposed to rice-fields under three different agricultural management practices during the rice cultivation season of 2018 in the Ebro delta area. Two rice-fields were selected for each agricultural management practice: (a) conventional wet fields (CW1 and CW2), (b) ecological wet (EW1 and EW2), (c) ecological dry (ED1 and ED2). Data are represented as mean (points) $\pm$ s.e.m (error bars). For details on samples sizes (N) for each agricultural management practice and for each sampling event see Table 1 in *Chapter 3*. See the text for details on the experimental setup.

2. Audouin's gull as a bioindicator organism for mercury pollution

The influence of legacy mercury pollution from industrial activities has been already described along the Ebro river through its delta, especially in biota (Sanpera et al. 2007; Cotín et al. 2011, 2012; Carrasco et al. 2011a; 2011b). Among different taxa used as bioindicators for mercury pollution in environments, seabirds are often considered as appropriate bioindicators to monitor Hg-pollution due to their apical position in food webs and their long-life expectancy, allowing both biomagnification and bioaccumulation of Hg in their bodies, respectively (Clatterbuck et al. 2018). Indeed, their reliability as a bioindicator group has been used in several studies across the globe and pollution situations (e.g., Monteiro and Furness 1995; Arcos et al. 2002; Provencher et al. 2014; Mills et al. 2020; Albert et al. 2021).

The present thesis further examines the feasibility of Audouin's gull as bioindicator of mercury contamination within the Ebro Delta ecosystems. Since its trophic ecology encompasses several resources from different environments, mainly marine resources (e.g., fisheries discards), rice field preys (e.g., small fish, crayfish) and other terrestrial invertebrates (Oro et al. 1996; Ruiz et al. 1996; Pedrocchi et al. 2002; García-Tarrasón et al. 2013; Bécares et al. 2015), it can be regarded as an indicator species of contamination in both habitats. However, disentangling between sources of contamination regarding mercury body burdens, has been often an elusive question due to the difficulty of analysis of mercury apportionment (i.e., anthropogenic vs natural).

Even if Audouin's gull has been previously used as bioindicator species for Hg-pollution in the Ebro Delta (Sanpera et al. 2007; García-Tarrasón et al. 2013), in this thesis this species is used to monitor Hg pollution over time and to specifically tackle anthropogenic disturbances in Hg inputs to the environment for the first time. In this regard, in *Chapter 3* we analyzed a long temporal series spanning 15 years of data (from 2004-2019), including the 2013-2014 inflection point, where a soil remediation plan was performed around the chlor-alkali plant site located at Flix (Ercros 2019). We found Audouin's gull to be a sensitive species to environmental Hg fluctuations and to be an indicator species for anthropogenic impacts regarding Hg pollution (Fig. 4). Furthermore, the results obtained indicate that legacy mercury pollution is still thriving and affecting the Ebro Delta ecosystems, as well as the coastal area, in accordance to previous research (e.g., Cotín et al. 2011; Carrasco et al. 2011a; 2011b). In addition, the usage of compound-specific stable isotopes of aminoacids (CSIA-AA) allowed us to discard that mercury levels, subject to biomagnification, resulted from a change in resource consumption and trophic position from the individuals analyzed among years studied (Hebert et al. 2016; 2018). This seemed to indicate that the soil

management and remediation tasks performed in 2013-2014 could be a factor promoting a sudden increase in Hg bioavailability, reflected in higher Hg levels observed in 2014 in individuals from the Audouin's gull breeding colony at the Ebro Delta.

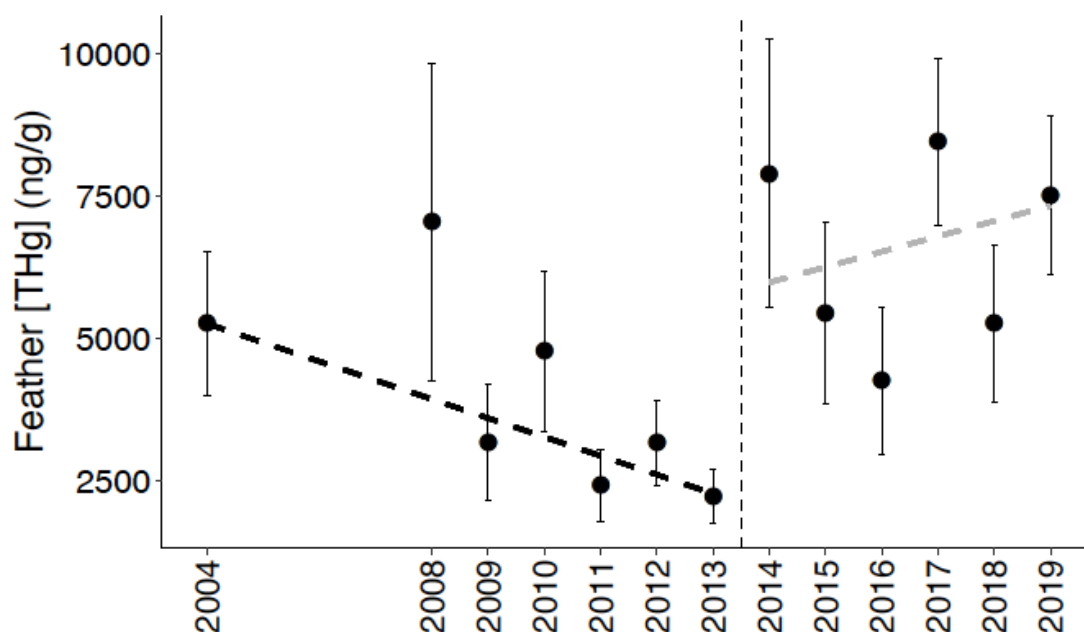


Figure 4 | Temporal trends of [THg] (ng/g) measured in mantle feathers of Audouin's gull fledglings taken from the breeding colony in the Ebro Delta. Points are means $\pm$ SD (solid lines). When statistically significant, re-gression trends are shown (black and grey dashed lines). Vertical dashed-line divides periods before and after sediment remediation at the chlor-alkali site.

To further investigate the impact of anthropogenic Hg-loads into the Ebro Delta's environments, in *Chapter 4* we conducted an interspecific study by means of mercury stable isotopes to deepen the understanding on the high mercury loads present in Audouin's gulls compared to other seabird species in the area and also their temporal fluctuations (see *Chapter 3*).

3. Advances in the usage of mercury stable isotopes to ecological pollution studies

The development of Hg stable isotopes has allowed, on one hand, advances in the knowledge of the mercury biogeochemical cycle (Blum et al. 2014; Tsui et al. 2020). On the other hand, such technique has been recently applied to the resolution of scientific questions regarding pollution topics in fauna, from individual to population level (e.g., Renedo et al. 2018, 2020; Feng et al. 2019; Pinzone et al. 2021). Among the objectives pursued by

researchers using Hg stable isotopes, describing the chemical processes leading to mercury species found in organisms and understanding the different sources of mercury contributing to mercury body levels are of special interest for toxicological studies in fauna (Tsui et al. 2020).

Mercury has seven stable isotopes, that can undergo both mass-dependent fractionation (MDF) and/or mass-independent fractionation (MIF; Berquist and Blum 2007; Blum et al. 2014). Mercury stable isotopic signatures (*Box AA in Introduction*) can serve as estimates for both MIF (odd-MIF: $\Delta^{199}\text{Hg}$ and $\Delta^{201}\text{Hg}$; even-MIF: $\Delta^{200}\text{Hg}$ and $\Delta^{204}\text{Hg}$) and MDF ($\delta^{202}\text{Hg}$) in environmental samples (Berquist and Blum 2007; Blum et al. 2014; Tsui et al. 2020), and function as proxies of sources of Hg and biogeochemical processes affecting mercury in the environment (Tsui et al. 2020).

In *Chapter 4* we aimed at disentangling by means of the mercury stable isotopes technique the habitat use and mercury bioavailability for three different seabird species breeding in the area. Also, to further deepening in the understanding of mercury level fluctuations in Audouin's gull fledglings in relationship to anthropogenic activities which can disrupt mercury levels in the environment. In first place, we analyzed total Hg, Hg stable isotopes and carbon and nitrogen isotopes in feathers from fledglings of three different species of *Laridae* with distinctive trophic ecologies breeding in the Ebro Delta area: (i) black-headed gull (*Chroicocephalus ridibundus*), foraging mainly in rice paddies (Antón-Tello et al. 2021), (ii) Audouin's gull (*Ichthyaetus audouinii*), which forages in both marine environments (i.e., fisheries discards) and in rice-fields (Sanpera et al. 2007; García-Tarrasón et al. 2015; Bécares et al. 2015; Ouled-Cheikh et al. 2020), and (iii) common tern (*Sterna hirundo*), an epipelagic marine forager (Cotín et al. 2011).

Our results showed that among the three species considered, Audouin's gull presented the highest mercury levels (Fig. 5). Previous studies already described Audouin's gull as the seabird breeding in the area with the highest Hg body levels observed, what has been related to its trophic ecology (e.g., Arcos et al. 2002; García-Tarrasón et al. 2013; Pereira et al. 2019).

Hg stable isotopic signatures efficiently traced the foraging ecologies of the three species, and are consistent with the information provided by the most common stable isotope signatures used in trophic ecology (i.e., $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$; Post 2002). Furthermore, Hg stable

isotopes allowed us to discriminate among foraging depths in the marine environment between common terns and Audouin's gulls.

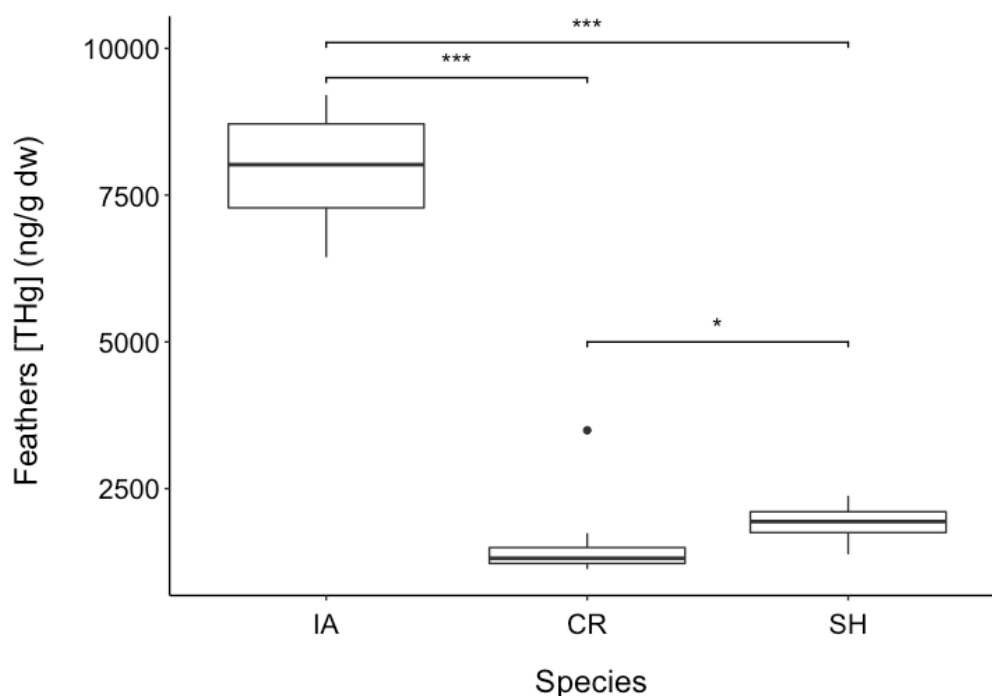


Figure 5 | Boxplots for [THg] (ng/g dw) in feathers of Audouin's gulls (IA), black-headed gull (CR) and common tern (SH) fledglings from the Ebro Delta in 2017. Pairwise significant differences between species are represented above the boxplots.

Both Audouin's gull and black-headed gull fledglings presented low odd-MIF ($\Delta^{199}\text{Hg}$) and MDF ($\delta^{202}\text{Hg}$) values compared to those of common terns (Fig. 6). $\Delta^{199}\text{Hg}$ signatures reflect differences in MeHg sources owing to photodemethylation in the water column (Berquist and Blum 2007) and are maintained along the food webs (Kwon et al. 2012). Thus, they are indicative of the foraging habitats used by adults to feed the chicks. Common terns present the highest odd-MIF values as they consume epipelagic resources, where the incidence of sunlight promotes demethylation, and consequently, MeHg enriched in odd-MIF values is incorporated in food webs (Blum et al. 2013; Bonsignore et al. 2015; Renedo et al. 2018). Conversely, Audouin's gull and black-headed gull, relying on the consumption of mesopelagic/benthic marine and rice-field resources, respectively, presented lower and closer to 0‰ $\Delta^{199}\text{Hg}$ values due to the low incidence of sunlight and, thus, lower rates of photochemical demethylation in mesopelagic/benthic marine zones or rice fields environments (Gantner et al. 2009; Senn et al. 2010; Sherman and Blum 2013; Blum et al. 2014).

$\delta^{202}\text{Hg}$ values in common terns were also higher than those found for Audouin's gull and black-headed gull individuals. This reflected higher positive MDF due to photochemical demethylation of MeHg in the photic zone of the marine water column, leaving a residual enriched in $\delta^{202}\text{Hg}$ (Blum et al. 2013; Bonsignore et al. 2015; Renedo et al. 2018). Interestingly, we observed higher MDF values in black-headed gulls than in Audouin's gulls. These signatures could indicate a higher impact of the chlor-alkali Hg legacy pollution in black-headed gulls, since chlor-alkali processes induce MDF (Wiederhold et al. 2015; Cransveld et al. 2017), and the mercury present in the irrigation water in rice fields comes mainly from the chlor-alkali site.

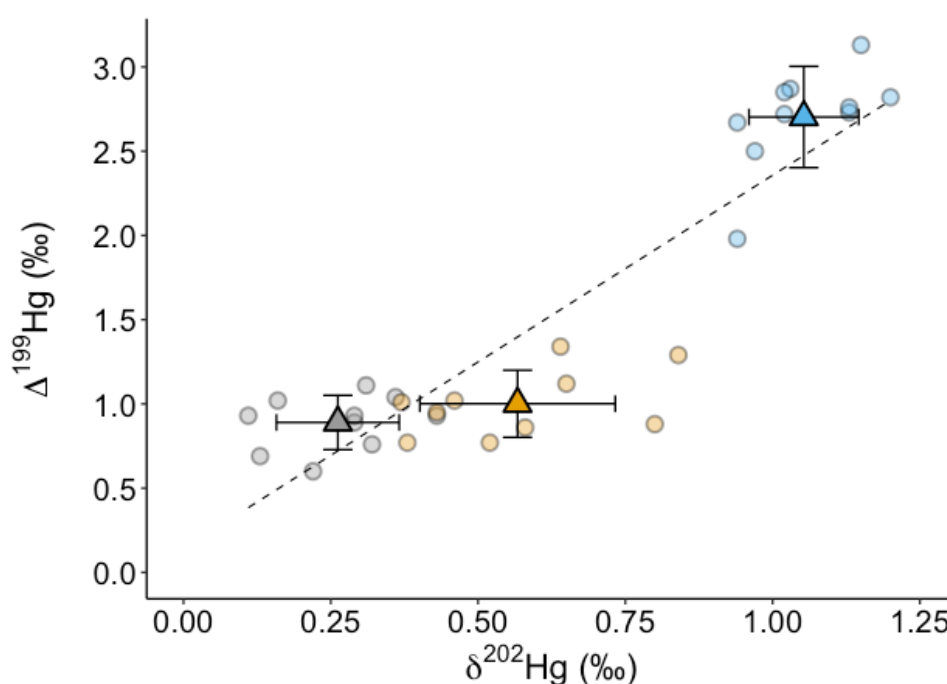


Figure 6 | Biplot of MDF $\delta^{202}\text{Hg}$ (‰) and odd-MIF $\Delta^{199}\text{Hg}$ (‰) in feathers of Audouin's gulls (IA, in gray), black-headed gull (CR, in orange) and common tern (SH, in blue) fledglings from the Ebro Delta in 2017. Individual data are plotted as dots. Triangles and error bars represent mean $\pm$ SD. Dashed line represents linear regression between these variables for the three species: $y = 0.1407 + 2.2167x$; $R^2 = 0.7963$; $p < 0.0001$.

In the second part of *Chapter 4*, we analyzed Hg isotopes in Audouin's gull fledglings from the breeding seasons of 2010, 2013, 2014 and 2017. These years correspond to the time before (2010 and 2013) and after (2014 and 2017) the sediment remediation and decontamination plan performed at the chlor-alkali plant site at Flix in 2013-2014. Interestingly, MDF values found in Audouin's gulls' feathers in 2010 and 2014 differed from

those in 2013 and 2017, being the $\delta^{202}\text{Hg}$ signatures in 2010 and 2014 higher than in 2013 and 2017 (Fig. 7). That is, in 2010 and 2014, when the chlor-alkali plant was still producing (2010) and after the sediment remediation plan (2014), MDF signatures are higher than in 2013 and 2017, where the production already had decreased (2013) or ceased completely (2017). As chlor-alkali reactions induce MDF (Wiederhold et al. 2015), and furthermore MDF in 2010 (mean $\pm$ SD: 0.59 ± 0.23) and 2014 (mean $\pm$ SD: 0.60 ± 0.10) are similar to those found in black-headed gull (mean $\pm$ SD: 0.57 ± 0.17), which feeds in rice fields, subject to a greater impact of anthropogenic contamination from the Ebro river through irrigation waters (Terrado et al. 2006; Palanques et al. 2020), this can be indicative of a greater impact of anthropogenic Hg in those years compared to 2013 and 2017. Thus, we cannot rule out an effect on Hg pollution in the Ebro Delta from the soil remediation plan in 2013-2014 at the vicinity of the chlor-alkali plant at Flix, which might have remobilized Hg within the polluted soil, confirming the results obtained in *Chapter 3*.

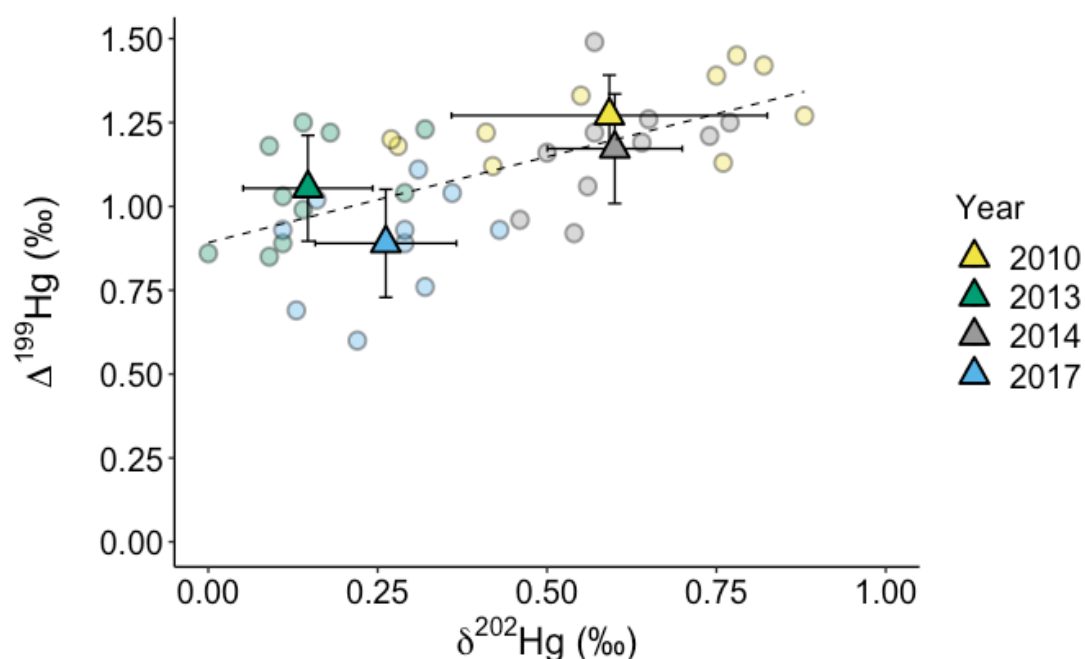


Figure 7 | Biplot of MDF $\delta^{202}\text{Hg}$ (‰) and odd-MIF $\Delta^{199}\text{Hg}$ (‰) in feathers of Audouin's gulls (IA) fledglings from the Ebro Delta breeding colony in 2010, 2013, 2014 and 2017 (N = 10 individuals for each year). Individual data are plotted as dots. Triangles and error bars represent mean $\pm$ SD. Dashed line represents linear regression between these variables for all the years studied: $y = 0.8920 + 0.5115x$; $R^2 = 0.3611$; $p < 0.0001$.

Overall, results from mercury stable isotopes are in agreement with the trophic ecologies described for the three species under study. Furthermore, they pointed out a possible influence of Hg from anthropogenic origin in Audouin's gulls in 2010, when the chlor-alkali plant was still producing chlorine and byproducts using Hg-cell technology, and 2014, when the restoration plan including the dragging and disposal of polluted sediments took place. The Hg isotopic data from these two years is similar to the signatures found for black-headed gull individuals and MDF is greater than the values obtained for 2013 and 2017 when production using Hg was reduced or completely ceased. Thus, the application of Hg stable isotopes complements the results obtained in *Chapter 3*, where the increase in Hg loads in Audouin's gulls fledglings in 2014 were found to be independent of a change in trophic position of the individuals.

4. Implications of this research

Mercury is one of the most ubiquitous and persistent pollutants in the environment (Driscoll et al. 2013). Its most toxic form is methylmercury (MeHg), which can biomagnify and bioaccumulate in organisms due to its chemical properties (Lehnherr 2014). MeHg accumulation might cause, among others, physiological, reproductive, teratogenic, and neurological health impairments to both humans and wildlife (Clarkson and Magos 2006; Díez 2009; Driscoll et al. 2013; Evers 2018). As such, it has received increasingly attention by the scientific community, from both an ecological and human health perspective (UNEP 2019).

The Ebro Delta is one of the main rice producing areas in the Iberian Peninsula, with more than 20,000 ha destined to rice farming (Mañosa et al. 2001) and a production of 120,000 tonnes/year (Day et al. 2006), constituting the main economic activity in the area. Furthermore, rice fields provide ecosystem services as seasonal habitats and foraging sites for wildlife, including birds (González-Solís et al. 1996; Ibáñez et al. 2010; Marco-Méndez et al. 2015). Thus, the evaluation of mercury dynamics within rice fields in the area is of greatest importance from a food safety and from an ecosystem health perspective. Our results show that rice field ecosystems in the Ebro Delta do constitute important sites for mercury bioavailability and biomagnification sites. Importantly, for the most compartments considered in this thesis (i.e., soil, water, plants) the total mercury levels are similar among the main agricultural management techniques employed for rice farming in the area (i.e., conventional wet, ecological wet, ecological dry). Thus, in the case of Hg pollution, it will not constitute a drawback to the application of AWD techniques to rice farming, which has been reported to have multiple benefits besides water savings (Martínez-Eixarch et al. 2021).

From a food safety point of view, total mercury levels found in whole grain (range: 1.45–7.45 µg/kg) do not exceed the 10 µg/kg set by the European Commission (Reg. EU 2018/73). Nonetheless, hydroperiod seems to affect bioaccumulation and biomagnification in fauna, with alternate wetting and drying cycles increasing THg levels in organisms. In this regard, fauna, particularly top-predators depending on rice fields resources could be exposed to higher Hg levels when feeding from AWD treated paddies.

With regard to the suitability of the Audouin's gull as an indicator organism, we found that it was sensible to Hg fluctuations in the environment due to anthropogenic activities. Particularly, there is an increase in mercury levels in the Audouin's gull colony in the Ebro Delta in the breeding season of 2014, which is not related to a shift in its trophic ecology. Additionally, mercury stable isotopes signatures obtained in this thesis point out towards the same direction, with a higher influence of anthropogenic mercury from chlor-alkali industry in 2010 and 2014, compared to the samples from 2013 and 2017. Recently, Constenla et al. (2022) reported increased histopathological alterations in *Solea solea* individuals from the Ebro Delta coastal area in 2014 and 2015, compared to 2013. Although these alterations were not directly related to heavy metal concentrations in sediments, other pollutants such as organochlorines or radionuclides present in the sludges of Flix reservoir could be involved. Thus, a leaking of legacy pollutants from dredging activities in 2013-2014 at Flix affecting downstream environments could not be dismissed.

Furthermore, we detected a slightly increase in mercury levels from 2014-2019 in Audouin's gull samples from the Ebro Delta. In this regard, mercury levels found in the majority of the Audouin's gull fledglings, were above those considered to have adverse effects on birds (5000 ng/g for feathers; Burger and Gochfeld 1997) and are much higher than the mercury levels reported in several species of seabirds breeding in sympatry in the area (e.g., Arcos et al. 2002; Pereira et al. 2019). Furthermore, it is worth noticing that the monitoring has been done by analyzing mercury body burdens in juveniles. This implies, as expected by bioaccumulation processes along the life of an individual, that mercury body loads might increase substantially over time (Souza et al. 2020). This indicates that legacy mercury pollution is still impacting the Ebro Delta areas and especially, striking top-predator species.

5. Future directions

The results of the present thesis reveal an ongoing mercury pollution within two of the main environments in the Ebro Delta (NE Iberian Peninsula), rice fields and coastal neighboring areas. On one hand, rice fields are hotspots for mercury methylation and

bioaccumulation in rice plants and fauna. The presence of mercury within the Ebro Delta area has been greatly exacerbated by the legacy pollution from a chlor-alkali plant operating until 2017 ca. 100 km upstream the river mouth. Although plant operations have already phased out, mercury pollution sequestered in the sediments around the factory site would be still released and transported by the Ebro river, thus, impacting areas far below the chlor-alkali site.

Irrigation water transports mercury into rice fields, and these, as intermittent flooded wetlands, do present propitious physicochemical conditions to enhance mercury methylation, thus being hotspots for MeHg synthesis. Moreover, some agricultural techniques employed for rice farming modify the local physicochemical environment modulating the production of bioavailable and toxic forms of mercury. Thus, further research should aim to go deeper to investigate mercury speciation within each of the compartments considered in the rice fields and get knowledge about the factors promoting MeHg formation in relation to the different rice farming practices employed, especially in *in-situ* settings. This will help to find mitigation strategies to reduce the formation of MeHg in rice fields, which would both benefit ecosystem health and food safety demands. Furthermore, other heavy metals, which are important from a toxicological point of view and are known to bioaccumulate within rice plants (e.g., arsenic, cadmium, lead), have seldom been studied within rice field ecosystems. Thus, future research investigating the presence, fate, and dynamics of these trace metals in these ecosystems should be encouraged.

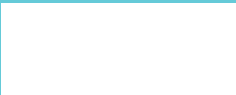
Audouin's gull has demonstrated to be a sensible species to mercury fluctuations in the environment. Furthermore, mercury body burdens in this species are among the higher within species breeding in the Ebro Delta. These results lead to different research directions to be considered in a future. On one hand, the importance of long-term biomonitoring studies should be stressed in order to detect short-, mid-, long-term impacts and trends of pollution episodes in ecosystems. This should encourage a continuous monitoring program of Hg pollution using this species as bioindicator and to relate mercury loads with population health indicators (e.g., annual demography, productivity taxes, etc.). On the other hand, might be of interest to compare the mercury levels among the different Audouin's gulls breeding colonies along the levantine coast of the Iberian Peninsula (NW Mediterranean), which would provide a picture of mercury contamination on a higher regional scale, and would allow to compare the levels of mercury in the Ebro Delta to other localities, to gain insight on the magnitude of the legacy pollution derived from the chlor-alkali plant at Flix compared to other areas. Finally, more studies are needed to understand the implications of

mercury accumulation in this species in terms of reproductive performance and physiological parameters, among other toxicity effects.

Finally, the determination of mercury stable isotopes composition in seabirds could be of help to understand different sources and processes of mercury speciation. To the best of our knowledge this is the first time that mercury stable isotopes have been analyzed in seabirds from the Mediterranean basin. Although further experimental research is still needed to be able to implement Hg stable isotopes to complex ecological problems, they look reliable to investigate the fate of Hg within environments and future research should urge its implementation in pollution monitoring programs at different spatial and temporal scales, as well as a means of investigating the transfer of mercury from different environmental compartments to biota.

07 CHAPTER 7

Conclusions



07

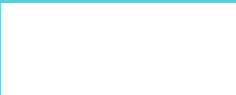
CONCLUSIONS

1. Rice field environments in the Ebro Delta, occupying a major part of the area, are hotspots for mercury sinkage and methylation, thus impacting all the environmental compartments within rice paddies, namely, soil, water, and the crop food-webs.
2. Regardless the agricultural management practices applied to rice farming (conventional wet, ecological wet or ecological dry), significant mercury concentrations occur throughout the rice growing season in soil, water, and rice plants. Furthermore, total mercury trends in these compartments throughout the rice growing season do not differ despite the agricultural practices used.
3. Rice plant parts differ in their accumulation capacities and trends: with growth, roots accumulate mercury, whereas mercury concentration in the aerial parts decreases due to a dilution effect. Mercury in roots correspond mainly to inorganic mercury, whereas methylmercury is accumulated mostly in the grain.
4. Regardless of the agricultural practices, bioaccumulation of mercury occurs in fauna inhabiting in the rice fields environment. Nevertheless, different rice farming management practices involve changes in the magnitude of Hg accumulation, with hydroperiod having a significant effect. Fields managed using alternating wet and dry cycles (i.e., ecological dry) do present higher levels of mercury bioaccumulation in fauna and slightly higher biomagnification factors than continuously flooded fields (i.e., ecological wet and conventional wet). Nonetheless, the use or disuse of pesticides and fertilizers for rice farming does not influence mercury dynamics regarding bioaccumulation in fauna and biomagnification of mercury along food webs.
5. *In-situ* mesocosm studies are an effective way to evaluate presence and to follow-up bioaccumulation processes of mercury in complex environments such as agricultural wetlands.

6. Long term monitoring of mercury levels in Audouin's gull showed a sudden increase in mercury concentrations from 2013-2014. This might be related to the dredging of the sediments at the Flix chlor-alkali factory site during those years and to the subsequent metal transport through the river, with mercury reaching the Ebro Delta and the neighboring coastal area. In this way, Audouin's gull constitutes a reliable bioindicator to trace changes of metal bioavailability in the environment.
7. Amino acid compound specific stable isotopic analyses of nitrogen (AA-CSIA) reveal that shifts observed in $\delta^{15}\text{N}$ bulk signatures in the period 2014-2019 (post-management period) compared to those in 2004-2013 (pre-management period) are not attributable to a change in the gull's trophic levels among years. Hence, increases in mercury concentrations after 2013 are not a result from changes in gull's trophic ecology.
8. The three seabird species studied to monitor mercury in different environments influenced by the Ebro river discharges revealed that mercury stable isotope signatures vary according to both, trophic ecology and foraging areas exploited by each species.
9. Odd-mass independent fractionation signatures ($\Delta^{199}\text{Hg}$) discriminate among the foraging areas used by Audouin's gull which are mainly mesopelagic, from epipelagic areas exploited by common terns. On the other hand, mercury isotopes signatures in both Audouin's and black-headed gulls points to the anthropogenic influence of mercury present in coastal areas.
10. Differences in mass-dependent mercury isotopes ($\delta^{202}\text{Hg}$) in Audouin's gulls among sampled years, point to the higher presence of anthropogenic derived mercury after the soil remediation operations at the chlor-alkali site in 2013, and thus, mercury stable isotope analysis reveals as a useful tool for tracing mercury pollution in complex environments.

R REFERENCES

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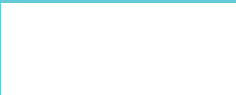
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A APPENDIX

Appendix





Effect of different rice farming practices on the bioavailability of mercury: A mesocosm experiment with common goldfish (*Carassius auratus*)

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ABSTRACT

Wetlands such as rice paddies are important ecosystems that provide habitat for a numerous range of species but are considered as a major source of mercury in the most toxic form of methylmercury. An *in situ* mesocosm experiment was conducted during the rice cultivation season of 2018 in rice paddies from the Ebro Delta (NE Spain) to investigate the bioaccumulation of mercury in fish. Common goldfish (*Carassius auratus*) were exposed to three types of rice-fields subject to different agricultural management (conventional or ecological agriculture) and different hydric practices (wet, dry) and were sampled monthly. Total mercury concentration in fish increased over time for almost all the fields under study, but the mercury increase was particularly higher for fish exposed to ecological fields. We conducted $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ measurements to follow up fish diet changes within or among fields. The results have shown there were no variations in the diet of the fish over time within fields, although differences among management practices were observed, which could explain to some extent the THg variation in fish exposed to each of the agricultural practices. Results indicate that wetting and drying rice fields might be a greater source of mercury for fish and a potential hotspot for MeHg production enhanced by physicochemical parameters. Thus, mercury bioaccumulation in fish inhabiting rice paddies depend on several factors what, in turn, could lead to biomagnification across the food webs.

1. Introduction

Mercury (Hg) is regarded as a toxic element for humans and wildlife (Clarkson and Magos, 2006; Evers, 2018). Nonetheless, several anthropogenic activities have taken advantage of this element (e.g., in chlor-alkali industry, as fungicide, pesticide, or in medicine; Clarkson et al., 2003). Hg can be found in all environmental compartments (air, soil, water and biota) and the speciation during its biogeochemical cycle is highly dependent on the local chemical conditions (Leopold et al., 2010; Driscoll et al., 2013). Methylmercury (MeHg) is the most well-known bioavailable and toxic form to humans and wildlife (Clarkson and Magos, 2006; Díez, 2009; Evers, 2018; Mergler et al., 2007). Because of its lipophilic nature, MeHg bioaccumulates in organisms and biomagnifies easily across the food webs (Gray, 2002; Driscoll et al., 2013; Lavoie et al., 2013). In the aquatic environment,

MeHg can be produced from inorganic mercury (divalent mercury Hg^{2+}) mainly by iron reducing-bacteria (IRB) and sulfate-reducing bacteria (SRB) at the surface of sediments (Compeau and Bartha, 1985; Warner et al., 2003; Podar et al., 2015) or in the water column (Eckley et al., 2005) in a process known as methylation. Model experiments have demonstrated that MeHg production by SRB can be enhanced in anoxic conditions with low pH, low salinity, and warm temperatures (Ullrich et al., 2001; Warner et al., 2003). However, complementary studies in natural conditions have shown there is no simple relationship among these physicochemical factors and bacterial methylation rates because of the presence of inorganic and organic complexing agents in water which can reduce Hg^{2+} availability for methylation or stimulate/inhibit microbial activity (Zillioux et al., 1993; Snodgrass et al., 2000; Rothenberg et al., 2014).

Wetland environments referred as continuously or periodically

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flooded fields (e.g. swamps, peatlands, fresh or saltmarshes) are often linked to high MeHg production rates (Zillioux et al., 1993; Marvin-DiPasquale et al., 2014). Progressively, with growing human population, natural wetlands are being replaced by agricultural farming sites (Verhoeven and Setter, 2010; Davidson, 2014) such as rice paddies, covering 167 million ha of land in 2017 (FAOSTAT, 2020; <http://www.fao.org/faostat/>). As flooded environments, rice paddies are subject to high MeHg production rates (Meng et al., 2011; Wang et al., 2014), a mercury form that can potentially be accumulated into organisms and be transferred to higher trophic levels in the food web (Ackerman et al., 2010). Besides anthropogenic pollution which can provide inorganic Hg available for methylation, some studies have discussed the impact of agricultural techniques used in rice farming on the bioavailability of mercury (Rothenberg and Feng, 2012; Rothenberg et al., 2011, 2014). Indeed, as in natural wetlands where the water level varies naturally, the type of management practices of agricultural soils (e.g., continuous flooding, alternating wetting and drying periods, use of pesticides) can promote microbial Hg methylation (Ullrich et al., 2001; Marvin-DiPasquale et al., 2014; Rothenberg et al., 2014; Tang et al., 2018).

During the 1990s, global climate change and increased water demand have led to the development of new rice farming techniques using less water without losing yields (Rothenberg et al., 2014). Among them, the alternating wetting and drying (AWD) technique is characterized by a drainage just after seeding followed by intermittent wetting and drying periods. However, the use of AWD techniques has been associated with higher MeHg production in agricultural wetlands, highlighting the importance of environmental factors promoted by intermittent flooding such as redox conditions, Hg^{2+} availability, or anoxic conditions enhancing overall mercury methylation (Marvin-DiPasquale et al., 2014; Rothenberg et al., 2014; Tang et al., 2018; but see Tanner et al., 2018).

Hg used as a catalyst in the chlor-alkali industry is considered as one of the main sources of Hg contamination in the aquatic environment (Carrasco et al., 2011a, 2011b). As such, the European Union committed to ban this technology from December 2017 and replace it with better available technologies that do not rely on Hg components (e.g. membrane technology; 2013/732/EU). Nowadays in Spain, chlor-alkali plants using mercury in their production processes have ceased their activity or have made the transition to membrane technology (Euro-Chlor, 2019). Nonetheless, over the past century, the Flix chlor-alkali plant (Tarragona, NE Spain), dumped around 350 000 tons of toxic waste containing more than $436 \mu\text{g g}^{-1}$ of Hg into the Ebro river (Grimalt et al., 2003; Carrasco et al., 2011a). Despite the fact that industrial activities using Hg in this chlor-alkali plant ceased in 2017, legacy mercury pollution persists, and it has already been shown that this pollutant can be transported downstream the Ebro river to its delta (Navarro et al., 2009; Carrasco et al., 2011a; Cotín et al., 2012; Sánchez-Fortún et al., 2020). The Hg dispersion caused by the river flow has resulted in a wide distribution of this metal along the riverbed, thereby affecting fish and shellfish (Carrasco et al., 2011b). Typically, fish tissues and sediment samples upstream the Flix chlor-alkali plant showed lower Hg concentrations than downstream (Navarro et al., 2009; Carrasco et al., 2011a, 2011b). In addition, water from the Ebro river is used to flood continuously or alternatively the rice fields of the Ebro Delta (Terrado et al., 2007). Consequently, the potential absorption of contaminants from the water-soil system by organisms inhabiting rice fields in the Ebro Delta can lead to higher toxicological risks for wildlife through biomagnification. This is of highest concern in the area, as the Ebro Delta is one of the most important areas in the Iberian Peninsula both as rice farming site and as a natural reserve (Mañosa et al., 2001).

The main purpose of this study is to know whether rice paddies in the Ebro Delta are suitable environments enhancing Hg bioavailability and thus promoting bioaccumulation by organisms inhabiting there. Two specific objectives were set: (i) to monitor the temporal dynamics of body mercury in fishes inhabiting the rice paddies and (ii) to understand how different agricultural management practices affect metal

bioavailability. With this aim, an *in situ* mesocosm experiment using individuals of a brownish variety of common goldfish (*Carassius auratus*) was carried out in randomly selected rice fields from the Ebro Delta area subject to different agricultural practices. To the best of our knowledge, few studies have investigated the bioavailability and bioaccumulation of Hg in agricultural wetlands using a mesocosm approach *in situ*. In this regard, such experiments will help to understand how Hg bioavailability and bioaccumulation are affected by different environmental conditions.

2. Materials and methods

2.1. Experimental design

The mesocosm study was conducted in the Ebro Delta area (NE Iberian Peninsula: $40^{\circ}33' \text{ N}$, $00^{\circ}39' \text{ E}$): comprised of ca. 30.000 ha of wetlands, 75% of the Ebro Delta is used for rice-farming purposes, 20% is protected as a natural reserve and 5% comprises urban areas (Mañosa et al., 2001). During the rice cultivation season of 2018, six rice fields from the area were randomly selected. The rice fields included in the study were cultivated by farmers using different agricultural management methods (2 rice fields per treatment), as follows: (a) conventional wet (CW) approach (flooded throughout the rice cultivation season and using pesticides and fertilizers), (b) ecological wet (EW) approach (flooded throughout the rice cultivation season and without using any pesticides or fertilizers) and (c) ecological dry (ED) approach (without using pesticides and flooded only during half the rice growing period using a mild alternating wet dry, AWD, method). Distance between rice fields managed using the same agricultural practices was ca. 7 km for CW fields and <1 km for EW and ED fields and all the fields under study layed within a 8 km^2 area.

For each rice field 4 sampling points (one in each side of the field) were selected and cages (60 cm long x 40 cm wide x 29 cm high) were installed for 60 days (within the months of June/July–August/September, corresponding with the rice growing season in the Ebro Delta). Due to the cultivation schedule of each rice-field and their availability to conduct the experiment, cages were installed in a staggered manner among rice fields in four different days in June/July, ascertaining that rice field plants were at the same stage of development (i.e., vegetative stage) in all the fields under study. Therefore, four batches of juvenile common goldfish (one for each installation date; approx. 180 individuals per batch) were obtained from an aquaculture industry. From each batch, upon the installation of the cages and before starting the experiment ($t = 0$ days), approximately 20 fishes were randomly selected to know their Hg levels prior to the experiment. Furthermore, 60 fish were assigned to each of the six rice fields, distributed in 15 fishes per cage. Cages were placed in opposite sites within the field, but deeper places were chosen preferably, especially in those rice fields subject to varying water levels (i.e., ED fields) to avoid the lack of water. Before placing them into the cages, goldfishes were weighted (g) and measured (fork-length in cm). Later on, it was intended to collect five fish per cage at 30 days ($t = 30 \text{ d}$) and five fish per cage at 60 days ($t = 60 \text{ d}$) after mesocosm installation, with the five remaining fish being placed as backup in case of the decease of some of the individuals during the experiment. Sampled fish were euthanized using MS-222 Sandoz and frozen at -20° C in zip-locked bags until analysis.

During the installation of the experimental cages and the successive samplings (i.e., 0 d, 30 d, 60 d), physicochemical parameters of the water surrounding the cage were measured (pH, oxygen concentration, salinity, conductivity, temperature).

2.2. Total mercury and stable isotopes analysis

For chemical analysis, fishes were allowed to thaw at room temperature. Then, they were rinsed with distilled water and visually examined for external defects (e.g., malformations, missing parts, scars).

Body condition Index (BCI) was recorded for each fish. Fork length (mm) and total weight (precision: 0.01 g) before evisceration were measured to determine (BCI) according to the Fulton's condition factor (Froese, 2006):

$$BCI = 100(\text{Total weight} / (\text{Fork length}^3))$$

where the total weight (with digestive tract) is expressed in g and fork length in cm. During dissection, the sex of the individuals was recorded if possible. Then, individuals were eviscerated and freeze-dried for 24–48 h until constant mass. Eviscerated fishes were entirely powdered using a cryogenic mill (Freezer Mill 6750, SpexCertiPrep Inc., Metuchen, NJ, USA) to obtain a homogenous sample for Hg analyses. 0.02 g of homogenized sample was analysed to determine the total mercury concentration ([THg] ng·g⁻¹ dry weight) by means of a Direct Mercury Analyzer 80 (DMA-80, Milestone Inc.). To assess the accuracy of measurements we analysed two certified reference materials every 10–15 samples: a dogfish liver certified reference material (DOLT-3, National Research Council of Canada; recoveries ranged from 91 to 96%) and a fish protein certified reference material (DORM-2, National Research Council of Canada; recoveries ranged from 92 to 98%).

The fish samples were also analysed for carbon and nitrogen stable isotopes in order to take into account possible differences in fish trophic ecology across time and across rice-fields. Samples were lipid extracted using 2:1 chloroform:methanol mixture according to the Folch method (Folch et al., 1957), to avoid differences in the isotopic carbon signatures related to differences in lipid content among fish. 0.30–0.35 mg sub-samples of lipid extracted fish were encapsulated in tin cups and carbon and nitrogen stable isotopes were analysed at the Scientific Technical Services of the Universitat de Barcelona (Spain) using a Thermo-Finnigan Flash 1112 elemental analyser (CE Elantech, Lakewood, NJ, USA) coupled to a Delta-C isotope ratio mass spectrometer via a CONFLO III interface (Thermo Finnigan MAT, Bremen, Germany). Isotopic signatures of N and C are expressed using the standard delta notation in parts per thousand (‰) relative to the Vienna Pee Dee Belemnite ($\delta^{13}\text{C}$) and atmospheric N₂ ($\delta^{15}\text{N}$). IAEA standards were applied every 12 samples to calibrate the system. Replicate assays of standards indicated analytical measurement errors of $\pm 0.1\%$ and $\pm 0.2\%$ for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, respectively.

2.3. Statistical analyses

Total mercury concentration values ([THg]) were log-transformed to approximate a normal distribution. Log THg concentrations in fish were modelled using linear mixed models with the *nlme* package (Pinheiro et al., 2019) including exposition time (0 d, 30 d, 60 d), agricultural rice-farming practices (CW, ED, EW) and the interaction time $\times$ agricultural management as fixed factors. The body condition index (BCI) was also included as a fixed covariate in the model. Rice field ID (CW1, CW2, ED1, ED2, EW1, EW2) was considered as a random factor.

Since for conventional rice-fields fishes were only recovered at 30 d of experiment (see “Results” section), we ran two different analyses for the model: (1) using data from all fields (CW, ED and EW fields) until 30 d of experiment, and (2) using data from ecological fields (ED and EW fields) along the entire experimental setup (0 d, 30 d and 60 d).

Concerning the stable isotopes of nitrogen ($\delta^{15}\text{N}$) and carbon ($\delta^{13}\text{C}$), a separate linear mixed model with rice field as a random effect was fitted. The model included both covariates along with BCI, exposure time and agriculture management to evaluate their effect on THg concentration in fishes. The interactions between $\delta^{15}\text{N} \times$ agricultural management and $\delta^{13}\text{C} \times$ agricultural management were also included as covariates. Data from all fields (CW, ED, EW fields) and sampling events at 30 and 60 d of exposure was used for this model.

Finally, the effect of the field type (ED or EW) and time (0, 30 and 60 d) on $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$, were evaluated fitting linear mixed models for each of the isotopic signatures as response variables, which included the

following covariates: BCI, time (0, 30 or 60 days), agricultural management type and the interaction time $\times$ agricultural management. Rice-field ID was included as a random factor. Since fishes exposed in conventional wet fields did not survive until 60 days, these fields were not included in the analyses, as we were interested in evaluating any possible change in trophic ecology throughout the experiment period when fishes were exposed to rice fields, which will be determined by examining the values of the C and N isotopic signatures between 30 and 60 days.

For all the linear mixed models fitted, significant effects were assessed by means of Wald F-test. Post-hoc comparisons were investigated using Tukey's test for multiple pairwise comparisons.

As a quality control, the four fish batches obtained from the aquaculture industry were tested for differences in [THg] using an ANOVA test. Although we assumed that differences in THg levels among fish batches provided by the aquaculture industry would be minimal, differences in log [THg] were found among them ($F(3,64) = 16.53$, $p < 0.0001$; Table 1). Hence, the four batches were considered different groups at $t = 0$ days: one group for each conventional field (Batch 1 and Batch 2), one group for ecologic wet fields (Batch 3) and one group for ecologic dry fields (Batch 4).

All analyses were performed using R v.4.0.3 (R Core Team, 2020). Statistically significant threshold was set at $\alpha = 0.05$.

3. Results

We analysed THg concentrations in whole fish ($N = 201$ samples) from six Ebro Delta rice fields subject to different agricultural management practices (2 conventional, 2 ecological dry and 2 ecological wet) at four sampling points each ($N = 24$ sampling points). Descriptive statistics of THg concentration in fish averaged by exposition days and fields are summarized in Table 1. Only for ecological rice fields (EW or ED) fish were recovered after 60 days of experiment because of their death in conventional fields before 60 days (Table 1; Table 2), although fish recovery rates were low for almost all the sites studied (Table 2), possibly due to other stressors besides mercury in the rice field environment causing the death of individuals (Lall and Tibbetts, 2009; Harper and Wolf, 2009). Most of the mercury in fish has been reported to be methylmercury (MeHg) (e.g., Marrugo-Negrete et al., 2008; Carrasco et al., 2011a, 2011b; Arcagni et al., 2018). Thus, although MeHg concentration was not determined in the present study, we will consider THg concentrations analysed to serve as a proxy for MeHg.

3.1. THg in fishes from CW, ED, EW fields from 0 to 30 days

The fitted linear mixed model considering all the data on agricultural field types until 30 d of experiment, shows a significant relationship between THg concentrations and BCI ($F(1,152) = 7.85$, $p = 0.006$). Descriptive statistics of BCI of fishes averaged by exposition days and fields are summarized in Table 2. The interaction between time and the agricultural management type was significant ($F(2,3) = 26.15$, $p = 0.012$). Pairwise comparisons of the slopes for each of the agricultural management types showed significant differences between conventional fields and ecological wet fields (CW – ED: $p = 0.011$). Nonetheless, no significant differences were detected between ecological dry and ecological wet fields (ED – EW: $p = 0.10$). Between conventional fields and ecological wet fields we detected marginally significant differences in slopes (CW – EW: $p = 0.052$). THg concentrations increased over time for ecological wet fields and ecological dry fields (EW fields: $t = 4.12$, $p = 0.026$; ED fields: $t = 7.21$, $p = 0.006$), but no significant increase over time for conventional rice-fields was observed (CW fields: $t = 0.032$, $p = 0.98$). In fact, fishes exposed to CW1 increased their THg concentrations in 30 days, but a decrease in THg levels was observed in fishes exposed to CW2 (Fig. 1).

Table 1

Sample size (N fishes) and mean $\pm$ s.e.m. THg concentrations (ng g^{-1} dw), $\delta^{15}\text{N}$ (‰) and $\delta^{13}\text{C}$ (‰) values in common goldfish (*Carassius auratus*) batches at 0 days, and caged at 30 days or at 60 days in conventional wet rice fields (CW1 and CW2), ecological wet rice fields (EW1 and EW2) and ecological dry rice fields (ED1 and ED2) from the Ebro Delta.

Rice field	0 days after setup ^a					30 days after setup ^b				60 days after setup ^b			
	Batch	N	[THg] (ng g ⁻¹)	δ ¹⁵ N (‰)	δ ¹³ C (‰)	N	[THg] (ng g ⁻¹)	δ ¹⁵ N (‰)	δ ¹³ C (‰)	N	[THg] (ng g ⁻¹)	δ ¹⁵ N (‰)	δ ¹³ C (‰)
CW1	Batch 1	10	156.47 ± 3.42	7.00 ± 0.13	-26.06 ± 0.20	12	259.82 ± 15.61	7.86 ± 0.11	-25.47 ± 0.15	na	na ^c	na	na
CW2	Batch 2	20	322.94 ± 8.61	12.02 ± 0.086	-21.15 ± 0.15	15	226.06 ± 7.55	11.62 ± 0.10	-22.69 ± 0.42	na	na	na	na
EW1	Batch 3	19	244.36 ± 21.35	11.28 ± 0.30	-22.03 ± 0.39	17	442.82 ± 35.89	9.63 ± 0.15	-26.29 ± 0.24	6	713.47 ± 52.16	9.78 ± 0.18	-26.45 ± 0.30
EW2						20	391.9 ± 22.65	9.02 ± 0.17	-25.70 ± 0.18	20	534.2 ± 30.31	9.02 ± 0.13	-25.00 ± 0.091
ED1	Batch 4	19	278.46 ± 14.82	10.93 ± 0.51	-22.41 ± 0.51	17	687.43 ± 60.21	11.67 ± 0.29	-22.95 ± 0.24	11	642.66 ± 93.54	12.13 ± 0.54	-22.55 ± 0.33
ED2						13	623.16 ± 17.81	11.35 ± 0.072	-24.38 ± 0.38	2	1143.25 ± 140.01	11.12 ± 0.027	-24.69 ± 0.29

^a Sample sizes refer to the fishes obtained from the aquaculture industries batches. 60 fishes from the same batches were introduced in each field (15 individuals $\times$ 4 cages per field).

^b Sample sizes refer to the fishes recovered from those introduced in the mesocosm cages for each field (15 individuals $\times$ 4 cages per field). See text for details on the experimental design.

^c na: non available data because of the death of all fishes caged before 60 days for conventional fields (CW1 and CW2).

3.2. THg in fishes from ED, EW fields from 0 to 60 days

The model for the ecological rice fields (EW, ED) for the entire experimental setup (0, 30, 60 days) shows that the management regime had a significant effect on THg levels in caged fish ($F(1,135) = 5.54$, $p = 0.020$). Also, the interaction between time and management type was significant ($F(1, 135) = 5.50$, $p = 0.020$). BCI showed a significant relationship with the THg levels observed in fish ($F(1,135) = 5.32$, $p = 0.023$). THg concentrations increased over time up to 60 days for ecological wet fields (EW fields: $t = 4.84$, $p < 0.0001$). However, THg concentration in fish from ecological dry fields did not show a significant increase over the whole experiment period (ED fields: $t = 0.95$, $p = 0.35$), with a β value for the slope (in log-scale) of 0.0031 (se: 0.0033) possibly as a consequence of a low statistical power, due to the low survival of individuals up to 60 days (Table 2).

After 30 days and 60 days of mesocosm exposure, THg levels in fishes caged was higher than at 0 days, independently the agriculture management employed for almost all fields (Table 1; Fig. 1). At 30-days fishes in ecological (dry or wet) rice paddies have shown higher accumulation of Hg than fishes exposed in conventional wet rice paddies. THg concentration was particularly higher in ED2 after 60 days of exposure (Fig. 1).

3.3. THg, $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ in fishes from CW, ED, EW fields

The model for THg concentration in fishes from all the agricultural type fields for 30 and 60 days including $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ as covariates and its interactions with the agricultural management results in a significant effect of $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ on $\log[\text{THg}]$ values ($\delta^{15}\text{N}$: $F(1, 118) = 17.65$, $p = 0.0001$; $\delta^{13}\text{C}$: $F(1,118) = 5.70$, $p = 0.019$). The interaction between stable isotope values and agricultural management type was significant for both $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ ($\delta^{15}\text{N} \times$ agricultural management: $F(2, 118) = 16.68$, $p < 0.0001$; $\delta^{13}\text{C} \times$ agricultural management: $F(2, 118) = 4.93$, $p = 0.009$, Fig. 2). Pairwise comparisons of the slopes for $\delta^{15}\text{N}$ against THg concentration in fishes showed significant differences between EW fields and both ED and CW fields (EW-ED: $p = 0.036$; EW-EC: $p < 0.0001$). In the EW, the relationship is significantly positive ($t = 2.51$, $p = 0.014$), whereas, in fishes from ED and CW fields we observed a negative association between THg and $\delta^{15}\text{N}$ values (ED: $t = -2.06$, $p = 0.042$; CW: $t = -2.06$, $p = 0.042$; Fig. 2). Regarding the association between $\delta^{13}\text{C}$ and THg, CW fields did not show a significant association ($t = 1.51$, $p = 0.13$), whereas EW and ED fields presented a significant negative

association (ED: $t = -2.15$, $p = 0.034$; EW: $t = -3.14$, $p = 0.002$) with no significant differences in pairwise comparisons (ED-EW: $p = 0.44$).

3.4. $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ in fishes from ED, EW fields from 0 to 60 days

Nitrogen and carbon stable isotope signatures results were compared between days of exposure (0, 30 and 60 days) only for ecological fields, as we could not recover any individual in conventional fields after 60 days of experiment. No significant effect of the agricultural type ($F(1,132) = 0.62$, $p = 0.43$), the BCI ($F(1,132) = 0.0002$, $p = 0.99$), time ($F(2, 132) = 1.20$, $p = 0.30$) was found on the $\delta^{15}\text{N}$ signatures. The interaction of time $\times$ agricultural management had a significant effect ($F(2,132) = 11.24$, $p < 0.0001$) on the $\delta^{15}\text{N}$ values measured in the fishes exposed to ecological rice-fields. Multiple comparisons for each of the agricultural management types showed any significant differences between 0, 30 or 60 days of exposure in ED fields on $\delta^{15}\text{N}$ signatures (0-30 d: $p = 0.52$; 0-60 d: $p = 0.27$; 30-60 d: $p = 0.67$), and in the case of EW fields, $\delta^{15}\text{N}$ signatures differed only between 0 and 30 d ($p = 0.0009$) and 0-60 d ($p = 0.001$), but not between 30 and 60 d ($p = 0.99$). If agricultural management types where compared for each of the exposure times, differences between ED and EW fields were observed at 30 and 60 days of exposure (30 d: $p < 0.0001$; 60 d: $p < 0.0001$).

The fitted model for the carbon stable isotope signatures showed no significant effect of the BCI of the fishes ($F(1,132) = 2.35$, $p = 0.12$) and agricultural management ($F(1,132) = 0.095$, $p = 0.76$). However, there was a significant effect of time ($F(2,132) = 4.01$, $p = 0.020$) on the $\delta^{13}\text{C}$ signatures observed. The interaction time $\times$ agricultural management was significant ($F(2,132) = 3.92$, $p = 0.022$). Multiple comparisons for each of the agricultural management types showed any significant differences between 0, 30 or 60 days of exposure in ED fields on $\delta^{13}\text{C}$ signatures (0-30 d: $p = 0.51$; 0-60 d: $p = 0.74$; 30-60 d: $p = 0.72$). In EW fields only between 0 and 30 d ($p = 0.0012$), and 0 and 60 d ($p = 0.004$), but not between 30 and 60 d ($p = 0.59$). Descriptive statistics of both $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values are summarized in Table 1.

3.5. Water physicochemical parameters in CW, ED, EW fields

Water physicochemical parameters (pH, salinity (‰), conductivity (mS), dissolved oxygen (mg l^{-1}) and temperature ($^{\circ}\text{C}$)) recorded over the rice growing season in 2018 are all summarized in Table S1. Mean pH values ranged 7.5–6 in CW fields, 7.6–5 in EW fields and 6.9–5.1 in ED fields over the experimental period, with a general trend to a

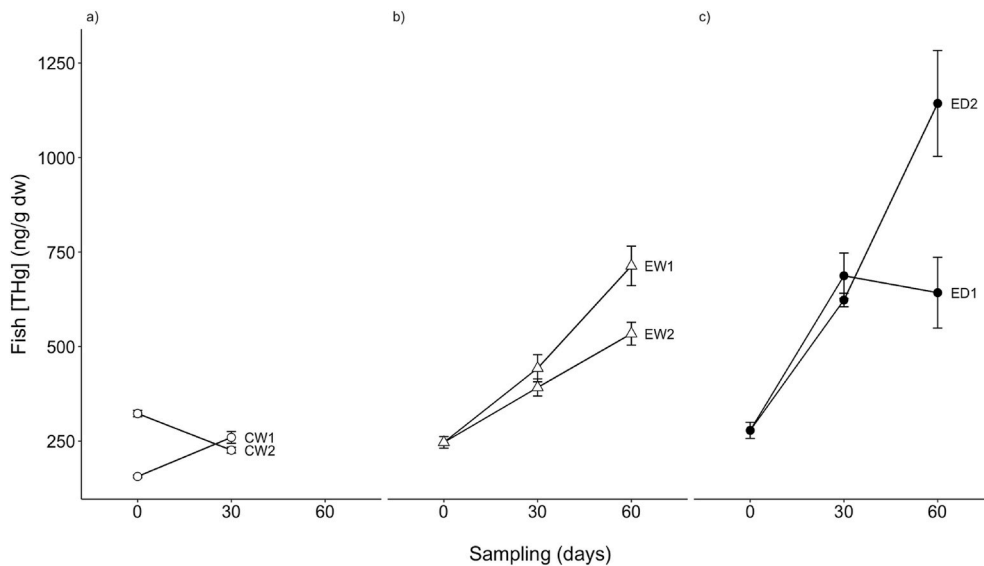


Fig. 1. Whole-body [THg] (ng/g dw) in *C. auratus* individuals under an *in situ* mesocosm experiment exposed to rice-fields under three different agricultural management practices during the rice cultivation season of 2018 in the Ebro Delta area. Two rice-fields were selected for each agricultural management practice: (a) conventional wet fields (CW1 and CW2), (b) ecological wet (EW1 and EW2), (c) ecological dry (ED1 and ED2). Data are represented as mean (points) $\pm$ s.e.m. (error bars). For details on samples sizes (N) for each agricultural management practice and for each sampling event see Table 1. See the text for details on the experimental setup.

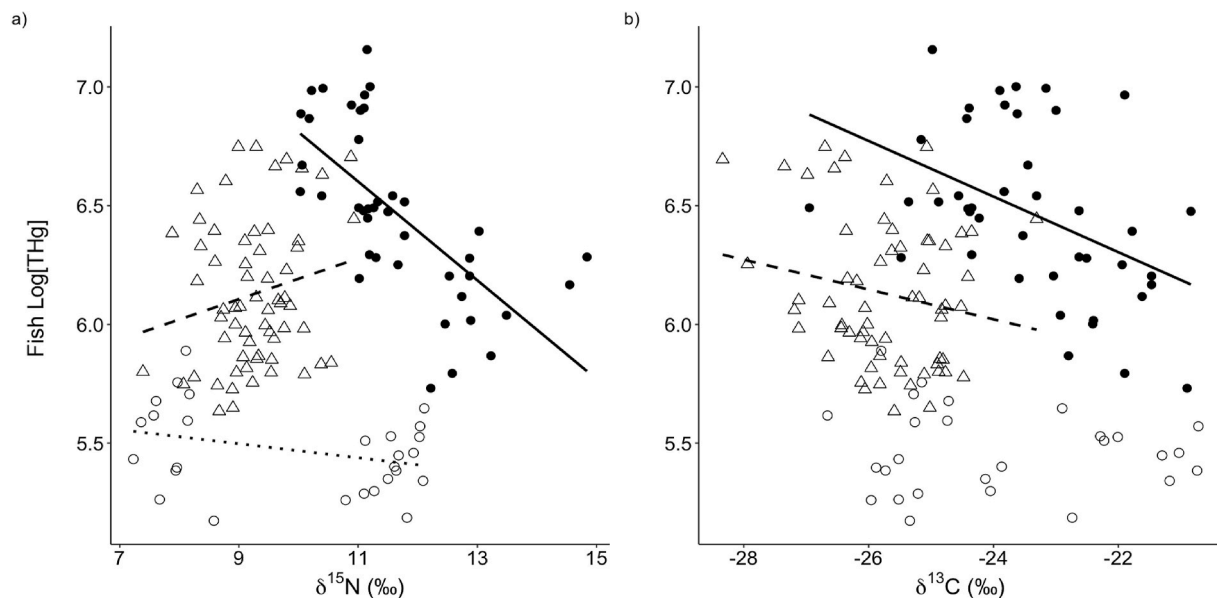


Fig. 2. Whole-body log[THg] (ng/g dw) in *C. auratus* individuals against (a) $\delta^{15}\text{N}$ and (b) $\delta^{13}\text{C}$, for conventional wet (CW1 and CW2) (open circles, dotted regression line), ecological wet (EW1 and EW2) fields (open triangles, dashed regression line) and ecological dry (ED1 and ED2) fields (filled circles, solid regression line). Only data from fishes sampled at 30 and 60 days of experiment are represented (see text for details).

bioaccumulation trends observed in the ecological rice fields, CW2 fishes exposed 30 days to the rice field environment presented a lower concentration of THg in their bodies. Amongst the causes proposed for the decrease in THg concentration over time, could be a dilution effect of THg through growth (Simoneau et al., 2005) and/or to a lower Hg intake. Our findings suggest that lower THg levels in CW2 fishes after 30-days exposure could be related to the observed poorer body condition (Table 2), probably as a result of a decrease of feeding rates due to stressors in the environment (Lall and Tibbetts, 2009). In fact, fishes in conventional wet fields did not survive up to 60 days of the experiment. Their death could be related to a lack of nutrient resources or to the presence of pesticides in water affecting their body condition (Lall and Tibbetts, 2009; Harper and Wolf, 2009; Belenguer et al., 2014; Ccancapa et al., 2016). Accordingly, we found mean BCI at 30 days for these fields were lower than BCI at $t = 0$ days (Table 2).

4.2. Ecological wet and dry fields Hg dynamics

Total mercury concentrations in fish exposed to ecological wet fields show a clear linear increasing trend in THg throughout the experiment in the two fields studied.

Conversely, although at 30 days of exposure fishes in both ecological dry fields (ED1 and ED2) show higher THg levels than fishes at 0 days, there are differences between the two fields at 30 and 60 day. This differences between dry fields could be the result of different Hg dynamics in each field. Furthermore, at 60 days of experiment, for ED fields, sample sizes are low, particularly for the field ED2 (ED1: $N = 11$ individuals, ED2: $N = 2$ individuals) as we are sampling fishes that have survived until 60 days of experimentation. Thus, the results in this case should be interpreted with caution. Furthermore, it is also worth noticing the high variation in THg concentrations of fishes exposed to ED fields throughout the experiment (Table 1), which might be due to

differences in THg levels among sampling points; in two sampling points fishes accumulated twice as Hg compared to the others, possibly due to a bioaccumulation gradient as a consequence of the transport of Hg by flooding the rice field (Ackerman and Eagles-Smith, 2010).

Several studies have demonstrated that the uptake of Hg by fish or other organisms might be enhanced by hydroperiod (Bodaly and Fudge, 1999; Snodgrass et al., 2000; Ackerman and Eagles-Smith, 2010; Ackerman et al., 2010). When comparing conventional wet, ecological wet and ecological dry fields, the THg concentrations observed in fishes from ED fields are higher than those exposed to EW and CW fields. Since diet is the main source of mercury in organisms, differences in Hg concentrations in fishes exposed to rice-fields managed differently and with different flooding regimes might be influenced by the food sources available in a particular field, with ED fields being more enriched in MeHg or fishes feeding from higher trophic levels.

4.3. THg, $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ in fishes from CW, ED, EW fields

To better understand possible differences between sites in terms of the trophic ecology of the fishes exposed to a particular environment, we analysed $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values in the whole bodies of the fishes (Bodaly and Fudge, 1999).

We found a significant relationship between THg levels and $\delta^{15}\text{N}$, that differs between EW fields, and both ED and CW fields. In the EW, the relationship is significantly positive, as expected from biomagnification processes, in which individuals feeding from higher trophic levels tend to be exposed to higher levels of Hg (Chen et al., 2009; Coehlo et al., 2013). Contrarily, in fishes from ED and CW fields we observed a negative association between THg and $\delta^{15}\text{N}$ values. However, we think that these negative associations are likely a spurious relationship due to the particular physicochemical conditions of each field and each sampling point or the presence of mercury gradients along the fields (Ackerman and Eagles-Smith, 2010).

Although only evaluated for ecological fields, $\delta^{15}\text{N}$ values differed between ED and EW field types at 30 days and 60 days of exposure. In both cases, fishes in ED fields were apparently feeding from higher trophic levels, as indicated by the higher $\delta^{15}\text{N}$ values observed (Post, 2002) than fishes exposed to EW, which might explain partly the higher Hg levels found in ED fields compared to the other types of rice fields studied.

$\delta^{15}\text{N}$ values were not significantly different after 30 days and 60 days of exposure for ED or EW fields. Thus, fishes exposed in fields managed by the same type of agriculture have not changed their overall trophic level over the period studied (Post, 2002). Consequently, this further indicates that bioavailability and bioaccumulation of mercury are the processes driving the increase in THg levels observed in fishes exposed to these fields.

Regarding $\delta^{13}\text{C}$ values, fishes exposed to EW and ED rice-fields presented a significant negative relationship between $\delta^{13}\text{C}$ and THg concentrations. Moreover, we found a similar range in $\delta^{13}\text{C}$ fish values (Table 1). These results suggest that the carbon source of fishes in the different rice paddies under study are similar (i.e., similar feeding habitat; Cherel and Hobson, 2007; Garcia et al., 2007). The negative relationship between $\delta^{13}\text{C}$ and THg indicates that to some extent there is an effect of the diet source to the Hg levels in the fishes, which probably is due to the trophic specialization of individuals within the rice paddies (Sharma et al., 2008), as reflected by the wide range in both $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, spanning more than one trophic level (DeNiro and Epstein, 1981; Post, 2002) in ecological rice-fields.

Altogether, our results support the idea that agriculture management and flooding regime play an important role in the Hg bioaccumulation by fishes, with ecological dry fields possibly enhancing Hg methylation and Hg bioavailability (Rothenberg and Feng, 2012; Marvin-DiPasquale et al., 2014, but see Tanner et al., 2018). Besides, some areas within the fields could be more suitable than others for microbial Hg methylation because of local physicochemical conditions (Ulrich et al., 2001).

4.4. Environmental conditions over the rice culture season

When comparing THg levels in fishes exposed to different agricultural management practices, after 30 days, we found that THg accumulation was significantly higher in those exposed in ecological dry fields compared to those in conventional wet fields. When comparing THg values in fishes over the whole experimental period (i.e., 60 days) in ecological rice fields, those fishes exposed to an alternating wet drying (AWD) regime tended to accumulate more THg compared to individuals exposed to ecological wet rice fields (Fig. 1). In this way, it is put forward that water regime was an important factor promoting Hg bioaccumulation in organisms. It has been shown that variations in redox conditions by intermittent flood or new flooding shallow reservoirs can promote microbial biomethylation by sulfate-reducing bacteria (SRB) at sediment anoxic surface (Snodgrass et al., 2000; Marvin-DiPasquale et al., 2014; Rothenberg et al., 2014; Hang et al., 2017). When water level decreases, anoxic sediment is exposed to oxygen and Hg is released by sediment in the aquatic system (Snodgrass et al., 2000). Therefore, when rice paddies are re-flooded and anoxic conditions recovered, Hg^{2+} dissolved in the water is made more available to be methylated (Snodgrass et al., 2000) and microbial methylation activity is enhanced (Rothenberg and Feng, 2012) increasing the MeHg concentration in the environment on paddies using AWD techniques. MeHg produced dispersed in the water column upon re-flooding can then contribute to higher accumulation in fishes.

In general, a decrease in pH was observed over the rice culture season 2018 to an acidic pH range of 6.8–5 (except for ED2 field which increases slightly the pH; Table S1). A possible explanation to this pH decrease over time could be the increase of sulphates (SO_4^{2-}) by oxidation of sulphides in the rhizosphere, which in turn promotes favourable conditions for SRB to methylate Hg over the rice growing season (Rothenberg and Feng, 2012; King et al., 2000; Ulrich et al., 2001). Therefore, besides bioaccumulation related to the time of exposure of the individuals to the rice fields, physicochemical conditions of the environment seem to enhance Hg methylation rates over the rice growing season. However, this idea has not been tested in our study and other factors such as an increase in organic matter in decomposition could be causing a decrease in the water pH over time (Hall et al., 2008). As for salinity and conductivity values measured in the fields, values remained constant along the duration of the mesocosm experiment, which suggest a weaker implication in THg accumulation by organisms in our *in situ* experiment.

Our study demonstrates that rice-fields in the Ebro Delta are hotspots for mercury bioavailability for the biota inhabiting there. Previous mesocosm experiments within agricultural wetlands using fishes or aquatics invertebrates have already shown that higher levels of Hg are accumulated by organisms in agricultural wetlands than in natural wetlands (Ackerman and Eagles-Smith, 2010; Ackerman et al., 2010). Our study highlights that agriculture type management and overall flood regime can play a role on this observation. THg accumulation by fishes caged was higher in fishes exposed to fields with a periodically flood regime. Alternance between flood and dry period could promote disturbance of physicochemical parameters and involve periodic Hg biomethylation during the rice cultivation cycle (Rothenberg and Feng, 2012). Furthermore, dietary differences as revealed by stable isotope analyses between different agricultural management types, might be also influencing the levels of THg levels observed in the organisms. Although it is difficult to estimate a degree of influence among the ultimate causes of the differences observed in Hg bioaccumulation of organisms inhabiting in complex aquatic systems such as rice-fields, it is clear that rice-paddies in the Ebro Delta area are environments suitable for Hg bioaccumulation, enhancing the mercury exposure for higher-trophic level wildlife depending on these dietary sources.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envres.2021.111486>.

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Following up mercury pollution in the Ebro Delta (NE Spain): Audouin's gull fledglings as model organisms to elucidate anthropogenic impacts on the environment^{*}

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ABSTRACT

As top-predators in marine ecosystems, seabirds are regarded as appropriate bioindicator species for a variety of contaminants. Mercury (Hg) is a global pollutant, which can biomagnify along marine and freshwater food webs. Therefore, mercury body burden in seabirds, such as gulls, will integrate information about pollution in the environment. In the Ebro Delta (NE Spain), legacy mercury pollution from a chlor-alkali industry located ca. 100 km upstream of the Ebro river mouth has been affecting the delta environment. We have analyzed a 15-year temporal series (2004–2019) of Hg in birds from a breeding colony of Audouin's gull (*Ichthyaeus audouinii*) in the Ebro Delta to understand how fluctuations in Hg levels are coupled to human activities in the industrial area in the upstream region of the river. Stable isotopic signatures of C and N ($\delta^{13}\text{C}_{\text{bulk}}$ and $\delta^{15}\text{N}_{\text{bulk}}$) are determined to characterize the trophic ecology of the species. Since only $\delta^{13}\text{C}_{\text{bulk}}$ but not $\delta^{15}\text{N}_{\text{bulk}}$ was associated with THg levels, we used compound-specific stable nitrogen isotope analysis of amino acids (AA-CSIA) to evaluate the causes of variation in $\delta^{15}\text{N}_{\text{bulk}}$ to further investigate the idea of a decoupling of $\delta^{15}\text{N}_{\text{bulk}}$ and THg over time. We found Audouin's gull to be sensitive to Hg variations in the environment due to anthropogenic changes and to be a good indicator species for this contaminant in the Ebro Delta.

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1. Introduction

Mercury is a non-essential, ubiquitous toxic pollutant, that in sufficient concentration can have deleterious effects both in humans and wildlife (Clarkson and Magos, 2006; Eagles-Smith et al., 2018; Evers, 2018). Even if mercury is naturally present in the environment, during the past two centuries the anthropogenic-derived input of this metal into the environment has increased significantly (Selin, 2009; UNEP, 2019). The major emission sources of mercury from human activities include several industrial emissions (e.g., cement, chlor-alkali, coal combustion) and artisanal gold mining activities (UNEP, 2019).

Inorganic mercury can undergo diverse transformation processes, of special importance is its methylation in aquatic environments (Gilmour and Henry, 1991; Fitzgerald et al., 2007), which transforms inorganic mercury to methylmercury (MeHg), a highly toxic form that is readily bioavailable to biota (Morel et al., 1998). Furthermore, MeHg biomagnifies in aquatic food webs and is bioaccumulated in organisms (Wiener et al., 2003; Lavoie et al., 2013; Evers, 2018). Therefore, top predators are exposed to higher mercury levels, thus being more susceptible to its effects. Seabirds have often been used as bioindicators of mercury in the environment as they often occupy the highest levels in food webs and because they are long-lived organisms, resulting in the bioaccumulation of mercury in their bodies (Monteiro et al., 1996).

The Ebro Delta (NE Iberian Peninsula; Fig. 1) has been impacted by the legacy pollution of a chlor-alkali plant located ca. 100 km upstream of the Ebro river mouth, in the locality of Flix, which has been operating since the late 19th century (ACA, 2013). During the

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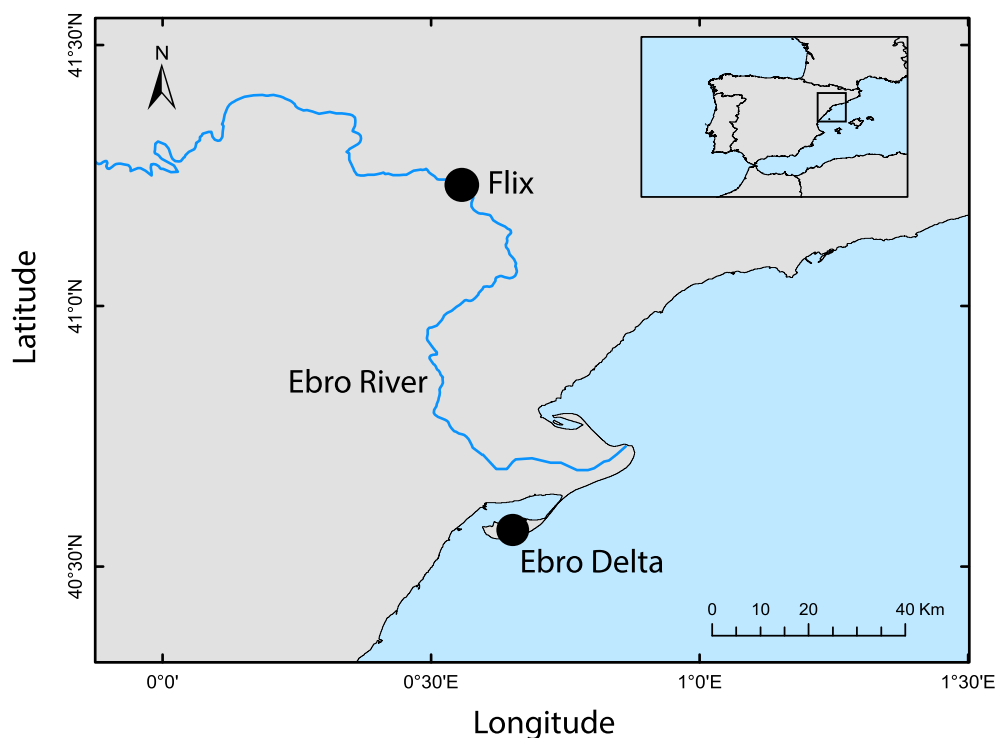


Fig. 1. Map showing the location of the Audouin's gull colony in the Ebro Delta at which samples were collected. Also shown is the location of the chlor-alkali industry upstream at Flix.

active period of the plant, 550 000–700 000 m³ of contaminated sediments, mainly with mercury and organochlorine compounds, were accumulated on the bottom of the river (Grimalt et al., 2003; ACA, 2013). Although plant operations producing chlorine using mercury ceased in 2017 (Ercros, 2019), legacy mercury pollution persists and has been impacting downstream Ebro River environments due to the transport of polluted particles along the river (Grimalt et al., 2003; Carrasco et al., 2010, 2011; Palanques et al., 2020). Moreover, during 2013–2014, a sediment remediation plan, including the dredging and disposal of polluted sediments, was implemented to remove toxicants from the riverbed in the vicinity of the factory (Ercros, 2019).

The Ebro Delta comprises 30 000 ha of wetlands, with 75% of its area used for rice-farming, 20% protected as a natural reserve, and 5% is urbanized (Mañosa et al., 2001). It is one of the most important wetlands in the western Mediterranean for the protection of birds. It is well known that wetland areas can constitute important mercury sinks and environments with high MeHg production rates (Boening, 2000). This is due to the presence of anoxic environments where sulfate- and Fe(III)-reducing bacteria biomethylate inorganic mercury into MeHg (Podar et al., 2015). Furthermore, due to its biogeochemical activity, mercury and methylmercury levels in the Mediterranean Sea are high compared to other oceanic regions (Cossa and Coquery, 2005); this is reflected in mercury concentrations in fishes (Llull et al., 2017; Junqué et al., 2018). Therefore, organisms feeding on prey associated with the Ebro Delta aquatic food webs will be exposed to MeHg. This is the case in top predators such as the Audouin's gull (*Ichthyophaga audouinii*), an emblematic species breeding in the area. Audouin's gulls exploit freshwater resources, mainly rice-field prey, marine food resources, coming from fisheries discards in the neighboring area, and to a lesser extent prey from terrestrial environments (Oro et al., 1996; Ruiz et al., 1996; Pedrocchi et al., 2002).

Bulk stable isotope signatures of carbon ($\delta^{13}\text{C}_{\text{bulk}}$) and nitrogen

($\delta^{15}\text{N}_{\text{bulk}}$) in feathers are often analyzed in order to characterize the trophic ecology of birds (Forero and Hobson, 2003; Jaeger et al., 2010). Because stable isotope baselines can be highly variable between years in highly dynamic systems such as river deltas (Bouillon et al., 2011), these isotopes only provide rough estimates of gull trophic ecology (mostly, on trophic position exploited). More recently, compound specific stable isotope analysis of amino acids (AA-CSIA) of nitrogen is being used to estimate the trophic position of individuals providing more accurate insights into the trophic ecology of seabirds (McMahon et al., 2015; Gagne et al., 2018; Hebert and Popp, 2018). Given that, in addition to measuring total mercury levels (THg), $\delta^{13}\text{C}_{\text{bulk}}$, and $\delta^{15}\text{N}_{\text{bulk}}$, we carried out AA-CSIA of nitrogen isotopes. An advantage of AA-CSIA is that it does not require estimating $\delta^{15}\text{N}_{\text{bulk}}$ in primary producers of food webs in order to generate a $\delta^{15}\text{N}$ baseline that is necessary to estimate trophic position of consumers (Hebert et al., 2016). The rationale behind AA-CSIA is the ability to estimate the trophic position of a consumer based on the main metabolic pathways that different amino acids undergo in cells (Chikaraishi et al., 2007). Those amino acids known as “trophic” amino acids, e.g., glutamic acid (Glu), undergo deamination and transamination reactions, cleaving N–C bonds, which results in nitrogen isotope fractionation. Thus, $\delta^{15}\text{N}$ values in trophic amino acids will be greater in consumers compared to their diet. Conversely, “source” amino acids, e.g., phenylalanine (Phe), only undergo deamination/transamination reactions to a very limited degree, thus, “source” AA will reflect $\delta^{15}\text{N}$ values at the base of the food web (Chikaraishi et al., 2007; Popp et al., 2007; Hebert et al., 2016).

Our work evaluates temporal trends in mercury levels in Audouin's gulls from the Ebro Delta colony from 2004 to 2019. Fledglings were used as biomonitors since they are mainly fed prey from the area around the colony (Ruiz et al., 1996). Mercury concentrations were analyzed in mantle feathers as feathers are one of the main pathways of methylmercury excretion in birds (Goede and

De Bruin, 1986; Bond and Diamond, 2009). Specifically, we (i) describe temporal trends of mercury in Audouin's gulls from the Ebro Delta breeding colony during two periods: 2004–2013 (during which the chlor-alkali plant reduced its activity) and 2014–2019 (the period following sediment remediation which could have affected the bioavailability of legacy Hg in the environment), and (ii) evaluate the contribution of both trophic position and foraging area to the observed Hg levels in gulls from the Ebro Delta colony.

2. Materials and methods

2.1. Sample collection

The present study was conducted in the Ebro Delta Natural Park (NE Iberian Peninsula: 40°33' N, 00°39' E) during the breeding seasons of 2004, and from 2008 to 2019 (from 2005 to 2007 no field work was carried out at this colony). For each of the years, we sampled 10–30 chicks at the fledging stage (approx. 3 weeks old). We sampled one chick per brood to avoid pseudoreplication. From each individual we took 10–15 mantle feathers and stored them in polyethylene bags until analysis. Upon sample collection, we ringed each individual with a metal and plastic ring to aid identification.

2.2. Total mercury determination

Feathers were washed using 0.25 M NaOH and rinsed with distilled water (following Bearhop et al., 2002). After cleaning, samples were dried to constant mass at 50 °C and then ground to a homogenous powder with an impactor mill (Freezer Mill 6850, SpexCertiPrepH Inc., Metuchen, NJ, USA) operating at liquid nitrogen temperature.

Total mercury (THg) concentration in feathers (ng/g dry weight) was determined by ICP-MS (samples from 2004, 2008–2014) or by Direct Mercury Analyzer (Milestone® DMA-80; samples from 2015

Delta-C isotope ratio mass spectrometer via a CONFLO III interface (Thermo Finnigan MAT, Bremen, Germany). Stable isotope signatures are expressed using the standard delta notation relative to Vienna Pee Dee Belemnite ($\delta^{13}\text{C}_{\text{bulk}}$) and atmospheric nitrogen ($\delta^{15}\text{N}_{\text{bulk}}$). IAEA standards were applied every 12 samples to calibrate the system. Replicate assays of standards indicated analytical measurement errors of $\pm 0.1\%$ and $\pm 0.2\%$ for $\delta^{13}\text{C}_{\text{bulk}}$ and $\delta^{15}\text{N}_{\text{bulk}}$, respectively.

2.4. Compound specific stable isotopic analysis of amino acids (AA-CSIA)

In a subset of $N = 10$ samples for each of the years 2010, 2013, 2014, 2017 we performed nitrogen AA-CSIA. Briefly, 3 mg of sample were acid-digested in 1 mL of HCl 6 M for 20 h at 110 °C. Samples were then evaporated until completely dry (approximately 2 h) under a stream of N_2 . Next, AA derivatization was performed with the addition of 50 μL of pyridine and 50 μL of N-tert-Butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA) to the samples and allowed to react at 70 °C for 1 h. Finally, derivatized AA were injected into a gas chromatography column to separate them and $\delta^{15}\text{N}$ for each of the AA was determined by means of a Trace GC ULTRA coupled to a Delta V Advantage isotope ratio mass spectrometer via a GC-Isolink interface (ThermoFisher Scientific). For each sample, we determined $\delta^{15}\text{N}$ values in 11 AA (Ala, Gly, Val, Leu, Ile, Pro, Ser, Thr, Phe, Asp, Glu) adjusted by an internal standard (norleucine, Nor) with known isotopic composition (Nor: $\delta^{15}\text{N}$ (mean $\pm$ SD): 14.60 ± 0.94) co-injected with each sample.

From the AA-CSIA determinations, the trophic position (TP) of the individuals was estimated using a multi-TDF (trophic discrimination factor) approach (Hebert et al., 2016; see the Supplementary Material, Table S1 for a comparison between TP calculation approaches), based on glutamic acid (Glu) as “trophic” amino acid, and phenylalanine (Phe) as “source” amino acid:

$$\text{TP}_{\text{AA-CSIA}} = 1 + \left[\frac{\Delta\delta^{15}\text{N}_{\text{GluPhe}} + f_{(\text{aq})} \times \beta_{(\text{aq})} + (1 - f_{(\text{aq})}) \times \beta_{(\text{terr})}}{\text{TDF}_{(\text{GluPhe})\text{avian}}} \right]$$

to 2019). For ICP-MS mercury determinations, approximately 30 mg of feather sample were acid digested in a microwave oven using HNO_3 (1–2 mL) and H_2O_2 (0.5–1 mL) in Teflon containers. For DMA-80 mercury determinations, feather samples (approx. 20–30 mg) were directly analyzed by combustion. To check the accuracy of measurements using both methodologies, we analyzed a human hair certified reference material (NIES No. 13 CRM), every 10–15 samples. Mean recoveries for ICP-MS and DMA-80, ranged from 98 to 101% and 90–94%, respectively. Therefore, no corrections were applied to the THg values of the samples prior to statistical analyses.

2.3. C and N bulk stable isotope signature determination

Bulk stable isotopic analyses for carbon and nitrogen and AA-CSIA for N, were carried out at the Scientific and Technical Centers of the Universitat de Barcelona (Spain). For the bulk C and N analyses, 0.30–0.35 mg of feather powder were placed into tin capsules and analyzed using a Thermo-Finnigan Flash 1112 elemental analyzer (CE Elantech, Lakewood, NJ, USA) coupled to a

where $\Delta\delta^{15}\text{N}_{\text{GluPhe}}$ is the difference in feather $\delta^{15}\text{N}$ values of Glu and Phe (i.e., $\delta^{15}\text{N}_{\text{Glu}} - \delta^{15}\text{N}_{\text{Phe}}$), $f_{(\text{aq})}$ is the fractional abundance of aquatic food in the diet (we set it to 0.8, according to the results by Navarro et al. (2010), although other studies have reported higher proportions of marine prey items [e.g., Pedrocchi et al., 2002; Sanpera et al., 2007; García-Tarrasón et al., 2013]). $\beta_{(\text{aq})}$ represents the difference between $\delta^{15}\text{N}$ values of Glu and Phe of primary producers associated with aquatic food webs. It has been estimated to be -3.4% for primary producers in aquatic food webs (McClelland and Montoya, 2002; Chikaraishi et al., 2010). $\beta_{(\text{terr})}$ represents the difference in $\delta^{15}\text{N}$ between Glu and Phe of primary producers associated with terrestrial food webs, including those from rice-fields ($+8.4\%$ for terrestrial C3 plants; Chikaraishi et al., 2010). Finally, $\text{TDF}_{(\text{GluPhe})\text{avian}}$ is the trophic discrimination factor between food sources and avian-consumers in Glu relative to Phe (i.e., $\Delta^{15}\text{N}_{\text{Glu}} - \Delta^{15}\text{N}_{\text{Phe}}$) for marine food webs. In the case of seabirds, $\text{TDF}_{(\text{GluPhe})\text{avian}}$ has been estimated to be 3.5% for gentoo penguin (*Pygoscelis papua*; McMahon et al., 2015), which we found appropriate as a $\text{TDF}_{(\text{GluPhe})\text{avian}}$ proxy for the species in our study.

Table 1
Descriptive statistics of annual total mercury concentrations, bulk $\delta^{13}\text{C}$, bulk $\delta^{15}\text{N}$, and amino acid-specific $\delta^{15}\text{N}$ values for Glu and Phe in feathers from fledgling Audouin's gulls.

Year	N	Feather [THg] (ng/g dw)		$\delta^{13}\text{C}_{\text{bulk}}$ (‰)		$\delta^{15}\text{N}_{\text{bulk}}$ (‰)		N	$\delta^{15}\text{N}_{\text{Glu}}$		$\delta^{15}\text{N}_{\text{AA}}$ (‰)	
		Mean	SD	Mean	SD	Mean	SD		Mean	SD	Mean	SD
2004	39	5250.9	1268.9	-17.09	0.38	12.30	0.35					
2008	15	7031.4	2782.6	-17.25	0.40	13.61	0.57					
2009	14	3166.2	1020.4	-17.69	0.71	13.03	0.32					
2010	16	4762.0	1408.6	-18.58	0.91	11.99	0.82	9	12.79	2.13	3.49	1.20
2011	12	2418.3	635.6	-18.50	0.46	12.80	0.41					
2012	15	3172.9	745.5	-17.60	0.30	12.98	0.40					
2013	15	2222.6	474.6	-20.44	2.40	12.60	0.29	9	13.33	0.86	4.40	1.07
2014	19	7884.4	2354.9	-17.14	0.87	13.71	0.41	10	13.89	1.57	4.01	1.10
2015	15	5444.2	1596.5	-17.34	0.32	13.96	0.41					
2016	19	4246.8	1286.6	-17.55	0.59	13.80	0.48					
2017	29	8448.5	1472.7	-17.88	0.78	13.85	0.39					
2018	14	5257.2	1384.4	-17.50	0.59	13.52	0.30	10	13.86	1.05	4.80	0.97
2019	15	7501.9	1392.2	-17.78	0.70	13.80	0.28					

2.5. Statistical analyses

T-tests were used to compare THg concentrations, $\delta^{15}\text{N}_{\text{bulk}}$ between the two periods studied: before the dredging of sediments (from 2004 to 2013, hereafter “before management”) and after the dredging of sediments (from 2014 to 2019, hereafter “after management”). Regarding $\delta^{13}\text{C}_{\text{bulk}}$ values, due to highly skewed data distribution and the presence of influential values, we tested the difference in the medians between the two periods of study (before and after the management) using a permutation test that was run for 1000 iterations.

We used generalized least squares (GLS) to assess the association between THg levels in feathers and the two periods studied: before management and after management. The model included the following covariates: time (years), management period (before-management and after-management) and the interaction between these variables, while accounting for variance among years. A GLS framework was used to control for residual heteroscedasticity of THg among years (Fligner-Killeen test: $\chi^2 = 26.991$, $p = 0.007$), when estimating the parameters in the model (Zuur et al., 2009). A second model was fitted by updating the previous model adding the explanatory variables $\delta^{15}\text{N}_{\text{bulk}}$, $\delta^{13}\text{C}_{\text{bulk}}$ and their respective interactions with the factor management period ($\delta^{15}\text{N}_{\text{bulk}} \times \text{Management}$, $\delta^{13}\text{C}_{\text{bulk}} \times \text{Management}$). Relationships between $\delta^{15}\text{N}_{\text{bulk}}$ with THg and $\delta^{13}\text{C}_{\text{bulk}}$ with THg are given as partial correlation coefficients (r_p) and 95% CI. The significance of the effects was tested by t-tests.

In addition, GLS models were fitted separately for $\delta^{15}\text{N}_{\text{bulk}}$ and $\delta^{13}\text{C}_{\text{bulk}}$ to evaluate the association between these response variables, and time (years), management period and the interaction between time $\times$ management period. All GLS models are fitted using the function `gls()` from the *nlme* R package (Pinheiro et al., 2019).

On the subset of samples analyzed using AA-CSIA methodology, separate multiple regression models were fitted to evaluate the effects of (i) time (years) and (ii) management period on the estimated trophic position (TP) of the individuals ($\text{TP}_{\text{AA-CSIA}}$) or $\delta^{15}\text{N}_{\text{bulk}}$. Partial correlation tests were performed to evaluate the relationship between THg or $\delta^{15}\text{N}_{\text{bulk}}$ versus $\text{TP}_{\text{AA-CSIA}}$ in the subset of samples analyzed using AA-CSIA. All statistical analyses used a significance level of $\alpha = 0.05$ unless otherwise specified. All statistical analyses were performed using R v. 3.5.1 (R Core Team, 2018).

3. Results

3.1. Total mercury temporal trends, relationship with $\delta^{15}\text{N}_{\text{bulk}}$ and $\delta^{13}\text{C}_{\text{bulk}}$

We analyzed feather samples from 237 individuals sampled in 2004 and 2008–2019. Mean total mercury (THg) concentration in fledgling feathers ranged from 2222.6 to 8448.5 ng/g (Table 1). Concentrations of THg in feathers were higher after sediment management than before ($t = 8.51$, $p < 0.0001$; Fig. 2). $\delta^{15}\text{N}_{\text{bulk}}$ values were significantly higher after management than before ($t = 15.95$, $p < 0.0001$). The permutation test for the difference of medians did not detect significant differences regarding the $\delta^{13}\text{C}_{\text{bulk}}$ before and after the management ($p = 0.54$; Fig. 2).

From the GLS model evaluating the effect of time and management period on THg concentrations in feathers we found a significant interaction between management period and time ($t = -5.11$, $p < 0.0001$). From 2004 to 2013 (before management period), feather THg concentrations decreased significantly ($t = -12.99$, $p < 0.0001$). In 2014, feather THg levels increase and from 2014 to 2019 there is an increase in feather THg levels

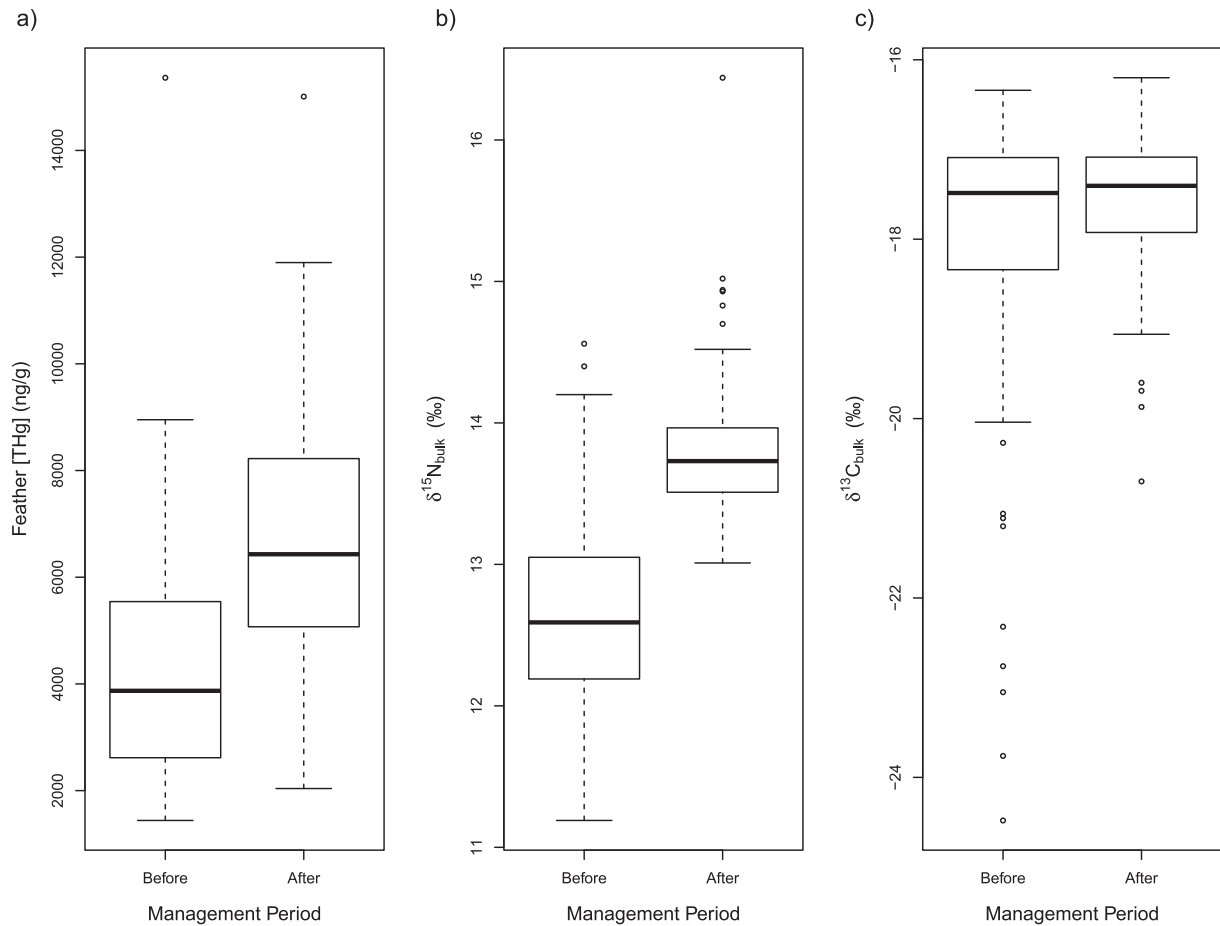


Fig. 2. Boxplots of (a) [THg] (ng/g), (b) $\delta^{15}\text{N}_{\text{bulk}}$ (‰) and (c) $\delta^{13}\text{C}_{\text{bulk}}$ (‰) measured in mantle feathers of fledgling Audouin's gulls collected from the breeding colony in the Ebro Delta. Management periods are before (2004–2013) and after (2014–2019) sediment management activities associated with remediating the chlor-alkali plant site at Flix in 2013–2014 (see text for details). Black bars represent median values, boxes represent the interquartile range, and circles represent data outliers.

through time ($t = 2.34$, $p = 0.020$; Fig. 3).

The model for THg as response variable, with the addition of $\delta^{15}\text{N}_{\text{bulk}}$ and $\delta^{13}\text{C}_{\text{bulk}}$ and their interactions with management period, showed a significant association between THg and $\delta^{13}\text{C}_{\text{bulk}}$ ($r_p = 0.36$, 95%CI: 0.24–0.47, $p < 0.0001$). However, we did not find any statistically significant association between THg levels and $\delta^{15}\text{N}_{\text{bulk}}$ ($r_p = 0.06$, 95%CI: –0.07 – 0.18, $p = 0.41$). Interactions of $\delta^{15}\text{N}_{\text{bulk}}$ and $\delta^{13}\text{C}_{\text{bulk}}$ with management period were not significant ($\delta^{15}\text{N}_{\text{bulk}} \times \text{Management}$: $t = -0.97$, $p = 0.33$; $\delta^{13}\text{C}_{\text{bulk}} \times \text{Management}$: $t = -0.01$, $p = 0.99$).

Regarding $\delta^{13}\text{C}_{\text{bulk}}$, we detected an association with time ($t = -3.43$, $p = 0.0007$) and management period ($t = -3.90$, $p = 0.0001$; Fig. 3), but no significant interaction between these two variables was observed ($t = 1.44$, $p = 0.15$). $\delta^{13}\text{C}_{\text{bulk}}$ decreased from 2004 to 2013 and from 2014 to 2019. Compared to the rest of the years, $\delta^{13}\text{C}_{\text{bulk}}$ values were anomalously low in 2013 ($t = -4.49$, $p < 0.0001$; Table 1).

We found significant differences between $\delta^{15}\text{N}_{\text{bulk}}$ and management period ($t = -5.67$, $p < 0.0001$) with higher values after management (Fig. 3). There was a significant interaction between time and management for $\delta^{15}\text{N}_{\text{bulk}}$ ($t = 2.91$, $p = 0.004$). $\delta^{15}\text{N}_{\text{bulk}}$ values increased slightly during the period before management ($t = 5.61$, $p < 0.0001$), while after management there was no significant trend ($t = -0.50$, $p = 0.62$); however, $\delta^{15}\text{N}_{\text{bulk}}$ values remained higher than before management. Higher $\delta^{15}\text{N}_{\text{bulk}}$ values could be explained by gulls feeding at a higher trophic position

(Blight et al., 2015) or a change in baseline $\delta^{15}\text{N}_{\text{bulk}}$ values (Cole et al., 2004) resulting in an overall change in food web $\delta^{15}\text{N}_{\text{bulk}}$ values. Therefore, to evaluate these two scenarios we estimated gull trophic position using CSIA-AA ($\text{TP}_{\text{CSIA-AA}}$) in a subset of samples.

All the GLS models were fitted again excluding 2013 data, however, there was no interpretation difference in the results obtained and therefore, the discussion of the results is based on the models including the data from 2013.

3.2. Compound-specific stable isotope analysis of amino acids (AA-CSIA)

We analyzed $N = 10$ samples of each of the following years: 2010, 2013, 2014 and 2017. For all, but one sample from 2010 and one sample from 2013 (both samples excluded from the analyses since we could not measure AA $\delta^{15}\text{N}$ values for Glu), we determined $\text{TP}_{\text{AA-CSIA}}$ (TP estimated using $\delta^{15}\text{N}$ values in Phe and Glu; Table 1; Table S1). In the subset of samples selected for AA-CSIA of nitrogen, there were no significant differences in $\text{TP}_{\text{AA-CSIA}}$ among years ($F_{(3,34)} = 0.59$, $p = 0.62$), nor considering before management and after management years as a factor ($F_{(1,36)} = 0.42$, $p = 0.52$; Fig. 4). We found significant differences among years ($F_{(3,34)} = 19.24$, $p < 0.0001$) and management periods ($F_{(1,36)} = 60.69$, $p < 0.0001$) on $\delta^{15}\text{N}_{\text{bulk}}$ in this subset of samples. $\text{TP}_{\text{AA-CSIA}}$ and $\delta^{15}\text{N}_{\text{bulk}}$ were not significantly correlated ($r_p = -0.20$, $p = 0.23$; Fig. 4). Furthermore, $\text{TP}_{\text{AA-CSIA}}$ was not correlated with THg in feathers ($r_p = 0.20$,

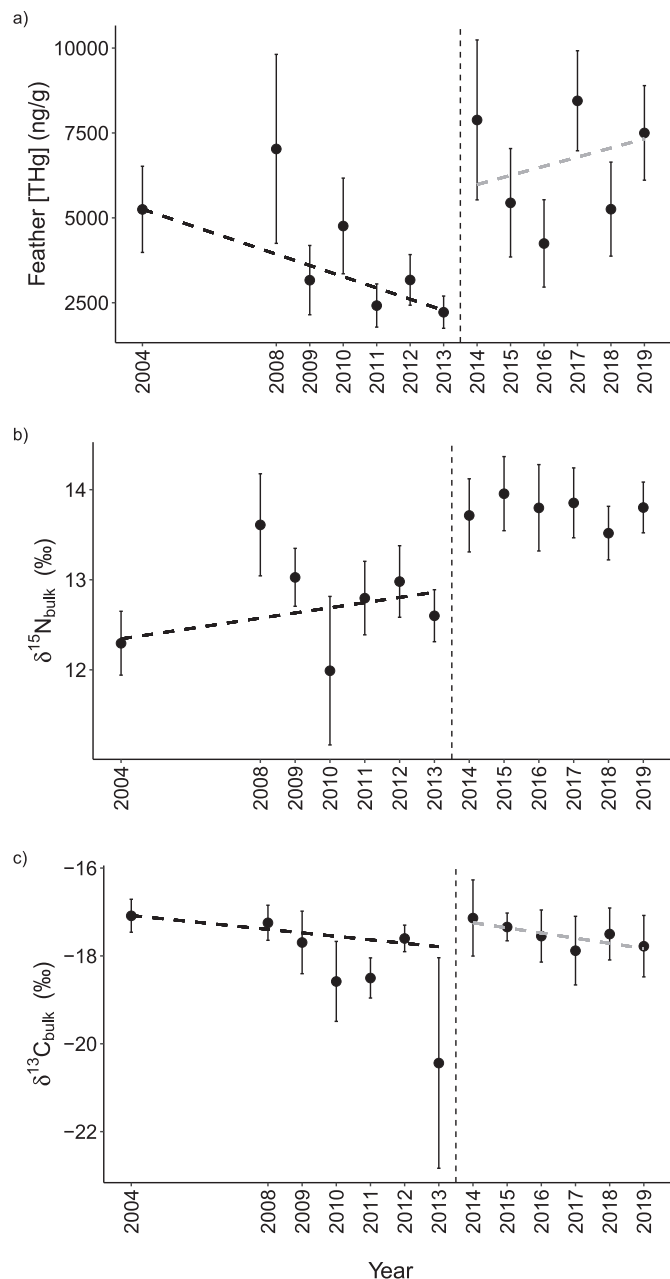


Fig. 3. Temporal trends of (a) [THg] (ng/g), (b) $\delta^{15}\text{N}_{\text{bulk}}$ (‰) and (c) $\delta^{13}\text{C}_{\text{bulk}}$ (‰) measured in mantle feathers of Audouin's gull fledglings taken from the breeding colony in the Ebro Delta. Points are means $\pm$ SD (solid lines). When statistically significant, regression trends are shown (black and grey dashed lines). Vertical dashed-line divides periods before and after sediment remediation at the chlor-alkali site (see text for details).

$p = 0.24$; Fig. 5).

4. Discussion

Effects of mercury on birds have been extensively studied elsewhere (Seewagen, 2010; Whitney and Cristol, 2017; Evers, 2018). In 54% of the fledglings studied, THg levels in feathers were above those considered to have adverse effects on birds (5000 ng/g for feathers; Burger and Gochfeld, 1997), although no experimental studies have been performed with this species to confirm these thresholds. Furthermore, we acknowledge that such

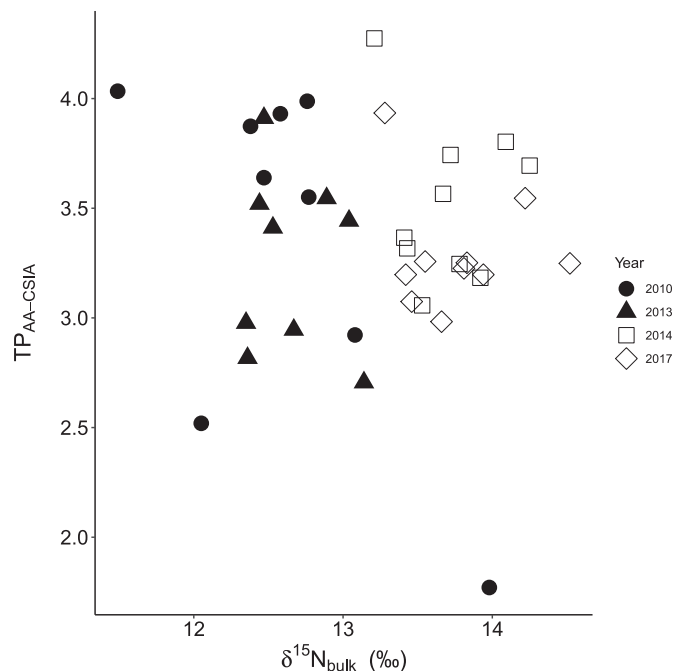


Fig. 4. $\delta^{15}\text{N}_{\text{bulk}}$ (‰) versus trophic position estimated from AA-CSIA ($\text{TP}_{\text{AA-CSIA}}$). Different symbols represent years analyzed (2010: N = 9; 2013: N = 9; 2014: N = 10; 2017: N = 10). Filled symbols represent the years before sediment remediation at the chlor-alkali site and empty symbols the years after sediment management. No significant correlation was found between $\delta^{15}\text{N}_{\text{bulk}}$ (‰) and $\text{TP}_{\text{AA-CSIA}}$ values.

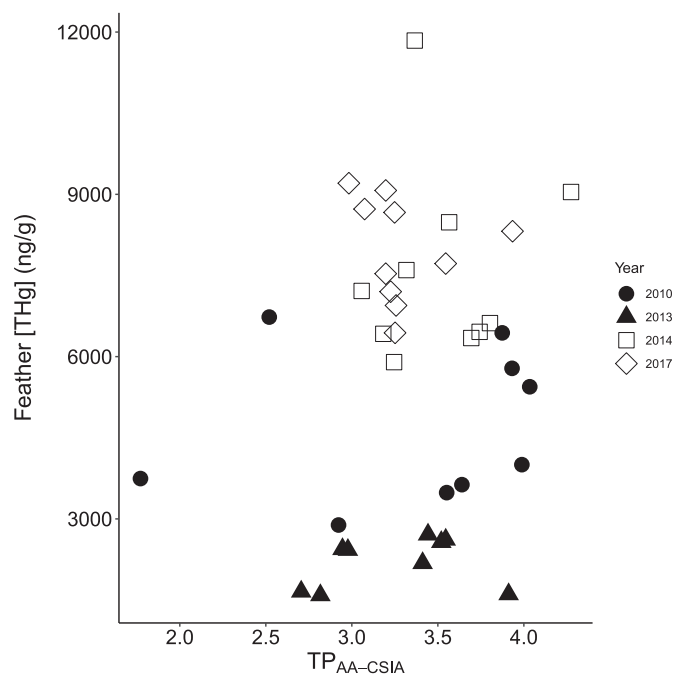


Fig. 5. Scatterplot of feather [THg] versus trophic position estimated by AA-CSIA ($\text{TP}_{\text{AA-CSIA}}$). Different symbols represent years analyzed (2010: N = 9; 2013: N = 9; 2014: N = 10; 2017: N = 10). Filled symbols represent the years before sediment remediation at the chlor-alkali site and empty symbols the years after sediment management. No significant correlation between $\text{TP}_{\text{AA-CSIA}}$ values and [THg] in feathers was found.

thresholds could vary, not only because they could be species-specific, but also in relation to particular environmental conditions, both biotic and abiotic (Goutte et al., 2014).

In the Ebro Delta, Audouin's gull is the species in which highest total mercury (THg) levels have been found, both in adults (Arcos et al., 2002) and in eggs (Morera et al., 1997). As far as we know, THg levels at the fledgling stage in Audouin's gulls from the Ebro Delta, might be among the highest levels found in seabird species inhabiting that area (Cotin et al., 2011). The reason could be the differential dietary resources Audouin's gulls exploit, feeding mostly on fisheries discards but also on prey originating from rice-fields and terrestrial sites (Oro et al., 1996; Ruiz et al., 1996; Sanpera et al., 2007; García-Tarrasón et al., 2013). Fisheries discards are mainly comprised of large proportions of mesopelagic and benthic fish species (Pedrocchi et al., 2002) which occupy higher trophic levels than epipelagic fishes (Arcos et al., 2002), and inhabit environments in the water column which enhance mercury methylation (Monteiro et al., 1996; Chouvelon et al., 2012), thus potentially accumulating and transferring more MeHg to consumers. Rice-field prey include the red crayfish (*Procambarus clarkii*; Navarro et al., 2010), which is known to bioaccumulate Hg in polluted environments (Suárez-Serrano et al., 2010) transferring it to higher trophic levels of food webs (Geiger et al., 2005). Furthermore, compared to other Audouin's gull breeding colonies along the Iberian Peninsula, available data suggest that fledglings from the Ebro Delta colony have the highest levels of THg, this has been attributed to a higher Hg pollution in the Ebro Delta area (Sanpera et al., 2007; García-Tarrasón et al., 2013).

THg in Audouin's gulls from the Ebro Delta breeding colony increased from 2004 to present. However, mercury trends look markedly different when considering the years before and after management of the chlor-alkali site. First, we found a significant decrease in feather mercury levels from 2004 to 2013, possibly reflecting the reduction of activities associated with the chlor-alkali plant in 2004 (Ercros, 2019) and a consequent decrease in Hg emissions (Pujadas, 2016) with the possibility of ecosystem recovery (Kopeck et al., 2019). From 2014 onwards, although the plant operations were decreasing (Pujadas, 2016) until the complete cessation in 2017 (Ercros, 2019), there was a slight increase in feather THg levels over time. Thus, an effect of the 2013 management actions that took place near the chlor-alkali plant at Flix on the environmental availability of Hg cannot be discounted. Dredging of sediments could have remobilized legacy mercury (Foxgrover et al., 2019) leading to an increase in THg levels in gull fledglings from 2014 onwards. Therefore, as observed in other studies with lower trophic level fauna (fishes: Carrasco et al., 2011, arthropods: George and Batzer, 2008, mussels: Carrasco et al., 2008), foodwebs in mercury-affected areas integrate information on mercury levels in the environment and fluctuations in Hg availability.

Regarding the relationship of THg levels and trophic ecology isotopic markers (C and N), we found a positive correlation between THg and $\delta^{13}\text{C}_{\text{bulk}}$, and significant variation in $\delta^{13}\text{C}_{\text{bulk}}$ values across years. In particular, $\delta^{13}\text{C}_{\text{bulk}}$ values in 2013 were lower than in the rest of the years, suggesting that fledglings were fed a higher proportion of freshwater or terrestrial prey in that year (Bearhop et al., 1999). This is consistent with the fact that $\delta^{13}\text{C}_{\text{bulk}}$ values observed in 2013 ($\delta^{13}\text{C}_{\text{bulk}}$ [mean $\pm$ SD]: -20.44 ± 2.40) were closer to those observed in other *Laridae* species breeding in the area and which forage mainly on freshwater aquatic prey (reported in Cotin et al., 2011): Whiskered terns (*Chlidonias hybridus*; $\delta^{13}\text{C}_{\text{bulk}}$ [mean $\pm$ SD]: -24.6 ± 0.7) and Gull-billed terns (*Sterna nilotica*; $\delta^{13}\text{C}_{\text{bulk}}$ [mean $\pm$ SD]: -24.8 ± 0.5). Although the lower $\delta^{13}\text{C}_{\text{bulk}}$ signatures in 2013 might be partly related to the lower levels of THg in that year, no firm conclusion can be made unless we reconstruct the diet to estimate the proportion of freshwater prey in chick diets in 2013 compared to other years. Recently developed methodologies to estimate the contribution of different sources of Hg to the

total mercury burden in organisms such as Hg stable isotope analyses (Renedo et al., 2018; Feng et al., 2019) seem a promising tool to that aim.

In the case of $\delta^{15}\text{N}_{\text{bulk}}$ values, the expected correlation with THg (Coelho et al., 2013), as $\delta^{15}\text{N}_{\text{bulk}}$ is usually regarded as a proxy for trophic position and THg biomagnifies along food webs, was not encountered in our study. However, $\delta^{15}\text{N}_{\text{bulk}}$ values were significantly higher in the years after sediment management than before. Remarkably, $\delta^{15}\text{N}_{\text{bulk}}$ increased significantly between 2013 and 2014, and afterwards, $\delta^{15}\text{N}_{\text{bulk}}$ values remained higher than during the 2004–2013 period. Even if both $\delta^{15}\text{N}_{\text{bulk}}$ values and THg concentrations increased in the years after sediment management the models fitted did not find any relationship between $\delta^{15}\text{N}_{\text{bulk}}$ and THg in our study system, indicating that both processes were uncoupled and that THg levels were not related to changes in trophic position across years but rather to concurrent higher levels of both ^{15}N and Hg in the environment (Rumbold et al., 2018). To further explore the possible causes of $\delta^{15}\text{N}_{\text{bulk}}$ variation we conducted AA-CSIA analyses (Hebert et al., 2016).

In this study, AA-CSIA was used to estimate the trophic position of individual birds during periods before and after sediment remediation. Gulls, as tertiary consumers, are expected to occupy a trophic position above 3.0 (Chikaraishi et al., 2009). In our study this is the case of most individuals when using a multi-TDF approach to estimate the trophic position. Otherwise, using a single-TDF approach (Chikaraishi et al., 2009) on the same dataset did provide underestimated trophic position values (TP < 3.0) for most of the gulls (Table S1). Thus, in the case of top-predators feeding from both terrestrial and marine resources, it is advisable to use a multi-TDF approach (Hebert et al., 2016). Even if using a multi-TDF approach, the trophic position of some individuals in our study is underestimated (TP < 3.0). This could be due to the TDF_{GluPhe} value applied to compute TP_{AA-CSIA}. A recent meta-analysis on TDF_{GluPhe} among different wildlife groups has determined that there is high variability on this value among different animal taxa (Nielsen et al., 2015) and ideally, a specific TDF_{GluPhe} value for the model species is needed to improve TP_{AA-CSIA}. Estimating specific TDF_{GluPhe} needs to be done in experimental conditions, which are often difficult to attain for a wild species. Nevertheless, even of this underestimation of TP, since we are dealing with the same species and applying the same formula to calculate the TP for all the individuals, this must not be an issue regarding the results obtained. Interestingly, we did not find any significant difference between the pre-management and the post-management period regarding the TP_{CSIA-AA} of the gulls, and therefore, the increase in $\delta^{15}\text{N}_{\text{bulk}}$ is likely due to causes other than a trophic shift of the species. For instance, due to the eutrophication of the environment (Cole et al., 2004; Cotin et al., 2011). Both past and ongoing eutrophication could have led to an increase on the $\delta^{15}\text{N}_{\text{bulk}}$ at the base of the food web translating to an overall $\delta^{15}\text{N}_{\text{bulk}}$ increase through the food web. Wastewaters from industries or fertilizer applications could have been one of the multiple causes leading to the eutrophication observed (McClelland and Valiela, 1998; Hebert and Wassenaar, 2001; Cole et al., 2004; Anderson and Cabana, 2006). These results point out that after 2013 the bioavailability of THg in the environment increased along with an eutrophication episode, which, in parallel, lead to an increase in $\delta^{15}\text{N}_{\text{bulk}}$ in the food webs of the Ebro Delta area. Although limited in sample size, we demonstrate how AA-CSIA can be used to assess possible changes in TP over time and explore relationships with mercury bioavailability or mercury sources.

Correlations between $\delta^{15}\text{N}_{\text{bulk}}$ and biomagnifying pollutants such as mercury in biota inhabiting systems where changes in baseline $\delta^{15}\text{N}_{\text{bulk}}$ values may be taking place should be interpreted with caution. Possible shifts in baseline $\delta^{15}\text{N}_{\text{bulk}}$ values need to be

considered when assessing possible TP changes (Post, 2002; Dolgova et al., 2018; Hebert and Popp, 2018). The assumed relationship between $\delta^{15}\text{N}_{\text{bulk}}$ and biomagnifying pollutants in systems where possible eutrophication could have taken place in conjunction with pollution episodes needs to be explored more in depth in order to avoid confounding interpretations of $\delta^{15}\text{N}_{\text{bulk}}$ increase. To that aim, compound specific stable nitrogen isotope analysis of amino acids can be a reliable tool to assess potential shifts in the trophic position of individual organisms.

In summary, we found that Audouin's gull fledglings were good bioindicators of Hg contamination in the Ebro Delta. Long-term monitoring studies focused on this high trophic level integrator species could aid in the evaluation of ecosystem Hg loads as well as other pollutants.

Credit author statement

MSF: Conceptualization, Investigation, Formal analysis, Writing original draft, Review and edition of the manuscript, Project administration; JOC: Investigation, Review and edition of the manuscript; CJ: Investigation, Review and edition of the manuscript; MGT: Investigation, Review and edition of the manuscript; JLC: Conceptualization, Formal analysis, Review and edition of the manuscript, Supervision; CS: Conceptualization, Investigation, Review and edition of the manuscript, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envpol.2020.115232>.

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