Geographic, seasonal and ontogenetic variation in cadmium and mercury concentrations in squid (Cephalopoda: Teuthoidea) from UK waters

G. J. Pierce[1], G. Stowasser[1], L.C. Hastie[1], P. Bustamante[2*]

1. School of Biological Sciences [Zoology], University of Aberdeen, Tillydrone Avenue, Aberdeen AB24 2TZ, UK.

2. Centre de Recherche sur les Ecosystèmes Littoraux Anthropisés, UMR 6217 CNRS-IFREMER-Université de La Rochelle 22 Avenue Michel Crépeau, Université de La Rochelle, 17042 La Rochelle, France.

* Corresponding author. *E-mail address:* pbustama@univ-lr.fr

Abstract: Cadmium (Cd) and mercury (Hg) levels were measured in the tissue samples of two loliginid (*Alloteuthis* sp. and *Loligo forbesi*) and two ommastrephid (*Todarodes sagittatus* and *Todaropsis eblanae*) squid species collected from research cruise and fishery (market) samples in UK waters during 2004–05. Concentrations of Cd were generally higher in the ommastrephids, in all tissues except muscle. Hg concentrations were higher in *T. sagittatus* than in the loliginids. In *L. forbesi*, metal concentrations differed between tissues and also varied in relation to body size, geographic origin and season. Cd levels decreased with increasing body size. This may be related to a shift in the diet with growth, since small *L. forbesi* feed on benthic invertebrates that have relatively high Cd concentrations, whereas larger individuals prey mainly on fish that have low Cd concentrations. Hg levels increased with body size, indicating its retention, and they were highest at the end of the spawning season and in squid from the English Channel and the Scottish West Coast. It is likely that the ambient concentration of Hg in seawater plays an important part in its accumulation in squid tissues. As it is a short-lived species, *L. forbesi* may therefore function as a bioindicator species for Hg contamination of the marine environment. Our results indicate that there is no significant danger to humans from consuming squid from UK waters.

Keywords: cephalopods; heavy metals; trace elements; bioaccumulation; risk for human consumption

Introduction

The commercial significance of cephalopods to world fisheries is of relatively recent, but growing importance (Boyle and Pierce, 1994; Boyle and Rodhouse, 2006). In the 1990s alone there was a 40% increase in squid catches worldwide (FAO data, 2003). In UK waters, the main commercial cephalopod species are the demersal long-fin squid *Loligo forbesi* and *L. vulgaris*, and the cuttlefish *Sepia officinalis* (Pierce et al., 1994a,b, 1998; Dunn, 1999; ICES, 2006). Annual landings of long-fin squid into the UK from adjacent waters varied from 1400 to 3000 tonnes during 1997-2004. These figures include landings of *L. forbesi*, *L. vulgaris* and the smaller *Alloteuthis* spp. Much of the squid landed in the UK is exported to southern Europe (Shaw, 1994). Landings of cuttlefish into the UK ranged from 1600 to 4900 tonnes during 1997-2004 (ICES, 2006). All these species occur in coastal, continental shelf waters. While *S. officinalis* and *L. vulgaris* are common only in the English Channel, the distribution of *L. forbesi* extends throughout UK waters (Roper et al., 1984). Three species of the mainly pelagic and oceanic short-fin squid family Ommastrephidae also occur in UK waters, namely *Illex coindetii*, *Todarodes sagittatus* and *Todaropsis eblanae*. All are of lesser commercial importance in UK waters, although some landings are reported from England and Wales (ICES, 2006). The lesser flying squid *T. eblanae* is most frequently recorded ommastrephid in coastal areas and occasionally occurs in very large numbers (Hastie et al., 1994). All squid are carnivorous, feeding mainly on crustaceans, small fish and other cephalopods, including conspecifics (Collins et al., 1994; Pierce et al., 1994c; Collins and Pierce, 1996; Lordan et al., 1998; Quetglas et al., 1999). Squid are themselves important prey items for large fish, seabirds and marine mammals (Croxall and Prince, 1996; Smale, 1996 ; Pierce and Santos, 1996; Santos et al., 2001).

The UK continental shelf is of high economic interest for present and future hydrocarbon exploration. Oil production activities could lead to increased releases of heavy metals resulting in increased bioaccumulation in marine biota, particularly in cephalopods, since they are very efficient accumulators of various trace elements (e.g. Martin and Flegal, 1975; Miramand and Bentley, 1992; Bustamante et al., 2002a). Toxic metals such as cadmium (Cd) and mercury (Hg) are very efficiently bioaccumulated and retained in squid (Bustamante et al., 1998a, 2006a) and consequently passed on to predators, thus potentially increasing the contaminant load in higher trophic levels, including humans (Bustamante et al., 1998a; Lahaye et al., 2005; Storelli et al., 2005, 2006).

Metals are also toxic for marine biota above a certain threshold. The early life stages (embryos and juveniles) of invertebrates are particularly sensitive to contaminants (Calabrese et al., 1973; Martin et al., 1981; Warnau et al., 1996). This is especially obvious when the spawning and the subsequent embryonic development occur directly in seawater, allowing direct contact of waterborne contaminants with the embryos and larvae, as in the case of squids (Villanueva and Bustamante, 2006). In addition to the potential direct effects of metals on squid embryos and juveniles, an increase of contamination levels in the environment is also likely to affect the reproductive tissues, potentially impacting on fertility and egg production (e.g. Gerpe et al., 2000; Craig and Overnell, 2003; Seixas et al., 2005; Miramand et al., 2006; Villanueva and Bustamante, 2006).

Overall, there is a lack of published data concerning the natural variations in metal concentrations in squids from UK waters. Such baseline information is needed to quantify the impact of anthropogenic contaminant sources (e.g. oil production activities) on these species. The main aim of this study was to provide baseline data on Cd and Hg concentrations in the tissues of different squid species from different seasons and regions around the UK and to identify and quantify sources of variation (e.g. seasonal, regional and ontogenetic). Biological data were also collected to provide a contemporaneous picture of the distribution of size, maturity and sex ratio. Although this investigation focused on *L. forbesi*, due to its wide distribution in coastal waters and high commercial importance, tissues from other common squid species in UK waters (*Alloteuthis* sp., *T. eblanae* and *T. sagittatus*) were also analysed for comparison.

Material and Methods

Sampling and biological data collection

A total of 3309 squid was collected and measured over 13 months (March 2004 to March 2005). The majority of data collected for *L. forbesi* (n = 1902) arose from five demersal trawling surveys carried out by the FRS Marine Laboratory between March 2004 and March 2005 in British waters (Table 1). During these surveys, samples of *Alloteuthis* sp. (n = 1216), *T. eblanae* (n = 86) and *T. sagittatus* (n = 17) were also collected. Additional samples of *L. forbesi* were obtained from a research cruise in the Irish Sea in October 2004 (n = 50) and

from a market sample of *L. forbesi* fished in the English Channel in July 2004 (n = 50). For all these squid, dorsal mantle length (DML), wet weight (g), sex, and maturity stage were recorded. DML was measured to the nearest 10 mm. A standard 5-point scale of sexual maturity for *L. forbesi* was used (Pierce et al., 1994b, adapted from Lipinski, 1979), in which stage 1 is 'immature', stages 2–3 are 'maturing' and stages 4–5 are 'mature'. Specimens with no visible gonad development were classified as 'juveniles' and allocated to maturity stage 1.

Sub-samples of whole squid from each sample were stored frozen (-20 ºC) for subsequent tissue sampling. A total of 282 squid from four species, i.e. 171 *L. forbesi* (mean DML: 129 ± 78 mm), 74 *Alloteuthis* sp. (mean DML: 67 ± 15 mm), 25 *T. eblanae* (mean DML: 100 ± 41 mm) and 12 *T. sagittatus* (mean DML: 343 ± 100 mm) was analysed for concentrations of Cd and Hg. The total number of squid analysed was limited by available funding. During each cruise, squid were selected by stratified random sampling, with the aim of obtaining a representative size range for both sexes from as many area/season combinations as possible.

Metal analysis

Metal concentrations were measured in weighed samples (around 5g) from digestive gland, mantle, gonads, gills and the homogenised combined remaining tissues, including head, arms and viscera. Normally, each tissue of each animal was processed separately but if the total weight of the tissue was very small (e.g. digestive glands of *Alloteuthis* sp.), samples were pooled for animals of the same sex and similar maturity stages or size for each haul. Gonads of juvenile and stage 1 squids were too small to sample, as were gonads, gills and combined remaining tissues of *Alloteuthis* spp.

Tissue samples were weighed, homogenised and freeze-dried. Dry weight of the samples was then recorded (and original moisture content estimated) prior to preparation for trace element analysis by atomic absorption spectrophotometry. For Cd analysis, the samples were digested with 4:1 v:v 65% ultrapure HNO_3 and ultrapure 70 % $HClO_4$ at 80°C for 2 days. The acids were then evaporated and the residues were dissolved in 0.3N ultrapure nitric acid (Bustamante et al. 1998a). Cd analyses were carried out using an atomic absorption spectrophotometer Hitachi Z-5000 with Zeeman background correction. For Hg analysis, two aliquots ranging from 10–50 mg of dried material were directly analysed in an Advanced Mercury Analyser spectrophotometer (Altec AMA 254) as previously reported (Bustamante

et al., 2006a). Reference tissues - dogfish liver (DOLT-3, NRCC) and lobster hepatopancreas (TORT-2, NRCC) - were treated and analysed in the same way as the samples. Results were in good agreement with the certified values and recoveries of the elements ranged from 97–103 % (n = 5). Detection limits were 0.004 and 0.005 $\mu g.g^{-1}$ dwt for Cd and Hg, respectively. Concentrations of both metals were expressed relative to both the dry weight of tissue ($\mu g.g^{-1}$ dwt) and the wet weight of tissue ($\mu g.g^{-1}$ wwt). The former values are used for statistical analysis, to eliminate any effects of varying moisture content while the latter are used for calculating risks associated with consumption of squid tissue.

Data analysis

Differences in metal concentrations ($\mu g.g^{-1}$ dwt) between species were tested on log-transformed data using ANOVA with Tukey tests. For *L. forbesi*, Gaussian GAMs were fitted to the log-transformed data on metal concentrations, using BRODGAR 2.5.1 statistical software, to identify spatial, temporal, sex- and size-related trends. Season and location were treated as categorical explanatory variables. Haul locations were grouped into the following area codes: IS = Irish Sea (52^o 35' N – 54^o 19' N and 3^o 54' W – 5^o 46' W), IW = Irish West Coast (52^o 42' N – 55^o 57 N and 8^o 51 W – 11^o 30 W), SWC = Scottish West Coast (56^o 09 N – 59^o 20' N and 5^o 09' W – 9^o 30' W), MF = Moray Firth (57^o 40' N – 58^o 13' N and 2^o 34' W – 3^o 25' W), NSUK = North Sea Coast of the UK (55^o 54' N – 57^o 45' N and 1^o 49' W – 2^o 14' W), EC= English Channel (data not available) and NS = North Sea (54^o 43' N – 57^o 40' N and 2^o 37' E – 0^o 59' W). Months were grouped into three "seasons", January to April, May to August and September to December (Table 1).

Since between-species differences in metal concentrations were not consistent across all organs, when possible, we also estimated average concentrations across all organs, as a weighted average of the individual organ concentrations. Complete data were available for 71 *L. forbesi*, 4 *T. eblanae* and 5 *T. sagittatus.* Although the sample sizes for the ommastrephids are very small we used ANOVA on log-transformed data to test whether differences between species were significant.

The health effects of Cd and Hg on humans from dietary sources have been repeatedly assessed by the Joint FAO/WHO Expert Committee on Food Additives (JECFA). The Committee allocated a Provisional Tolerable Weekly Intake for Cd, total Hg and methyl Hg

of 7, 5 and 1.6 $\mu g.kg^{-1}.wk^{-1}$ for humans, respectively (WHO 2003). The weight of cephalopod tissue that would need to be eaten to reach these limits for Cd and total Hg was calculated for each species (in Kg) for an average person of 60 Kg and is called "maximum safe weekly consumption" (MSWC). Estimations of the amount of squid flesh to reach the PTWI for methyl Hg were based on reported data concerning organic Hg proportion in cephalopods from the North East Atlantic waters (Bustamante et al. 2006).

Results

Biological data

Loligo forbesi of both sexes were smallest in spring 2004 and reached maximum average size in winter. The proportions of mature animals were highest in winter and spring and lowest from April to June (Table 1). Squid caught in spring 2005 were significantly larger and more mature than squid caught in spring 2004 (Table 1). Mature *L. forbesi* were recorded in the English Channel in July. Apart from the Scottish sample collected in July, the proportion of male squid amongst the mature squid caught was always higher than that of female squid. Small immature specimens were present in most months, although the proportion of these "recruits" increased during the summer.

Between-species differences in Cd concentration

Concentrations of Cd were highest in the digestive gland for all species (Table 2), ranging from 1.83 to 98.0 $\mu g.g^{-1}$ dwt for *T. eblanae* and from 9.61 to 215 $\mu g.g^{-1}$ dwt for *T. sagittatus* showing the highest concentrations. These concentrations were up to 30 times higher than those in *L. forbesi* (1.51-55.0 $\mu g.g^{-1}$ dwt) and *Alloteuthis* sp. (5.65-1.05 $\mu g.g^{-1}$ dwt). In *L. forbesi*, the combined remaining tissues showed the second highest Cd concentration (after digestive gland), while both *T. eblanae* and *T. sagittatus* had higher metal concentrations in their gills (Table 2). The tissue with the lowest Cd concentrations was muscle for *L. forbesi* and *T. sagittatus* and gonadal tissue for *T. eblanae*. Cd concentrations in muscle tissue were highest for *Alloteuthis* sp. and *T. eblanae*.

ANOVA confirmed that Cd concentrations ($\mu g.g^{-1}$ dwt, log-transformed) in digestive gland varied significantly between species ($F_{3,139} = 10.35$, $P<0.001$), being significantly higher in *T.*

sagittatus than in all the other species and significantly higher in *T. eblanae* than in *L. forbesi* (Tukey tests).

Although there was significant variation between species in muscle Cd concentrations ($\mu g.g^{-1}$ dwt, log-transformed) ($F_{3,152}$ = 14.08, P<0.001), trends were different to those in digestive gland, with Cd concentrations being significantly higher in *Alloteuthis* sp. and *T. eblanae* than in *T. sagittatus* and *L. forbesi*, and significantly higher in *T. sagittatus* than in *L. forbesi*.

For Cd concentrations in gills, gonads, combined other tissues, and the overall average for the body, statistical comparisons were possible only for the three larger species. There was significant between species variation in Cd concentration ($\mu g.g^{-1}$ dwt, log-transformed) in gills ($F_{2,88}$ = 44.37, P<0.001). Concentrations were significantly higher in both ommastrephids than in *L. forbesi.* There was also significant between species variation in Cd concentration in gonads ($F_{2,54}$ = 6.19, P=0.004), with significantly higher values in *T. sagittatus* than in *L. forbesi*. Between-species variation in Cd concentration of combined other tissues was marginally significant ($F_{2,87}$ = 3.23, P=0.044). Although the lowest values were seen in *L. forbesi* none of the paired comparisons indicated a significant difference. Overall average Cd concentration in the body varied significantly between species ($F_{2,77}$ = 29.32, P<0.001), with significantly higher concentrations in both ommastrephids than in *L. forbesi.*

Between-species differences in Hg concentration

Hg concentrations tended to be slightly higher in the digestive gland than in other tissues of all species except for *Alloteuthis* sp., in which Hg levels were similar to those found in the muscle tissue (Table 2).

There was significant between-species variation in Hg concentrations ($\mu g.g^{-1}$ dwt, log-transformed) in digestive gland ($F_{2,139}$ = 7.80, P<0.001). Concentrations were significantly higher in *T. sagittatus* than in *T. eblanae* and *A. subulata*, while *L. forbesi* also had higher concentrations than *A. subulata.* For muscle tissue, again overall variation was significant ($F_{2,149}$ = 12.08, P<0.001); concentrations were higher in *T. sagittatus* than the other three species and higher in *L. forbesi* than in *A. subulata*.

Analysis for other tissues was restricted to the three larger species. Although *T. sagittatus* tended to have the highest concentrations, between species variation in Hg concentration in gills was not significant ($F_{2,87}$ = 2.73, P=0.071). There was significant variation for gonad ($F_{2,54}$ = 4.49, P=0.016), other tissues ($F_{2,87}$ = 4.64, P=0.012) and the all tissues average ($F_{2,77}$ = 4.52, P=0.014), with higher concentrations in *T. sagittatus* than in *L. forbesi* in all cases.

Trends in Cd concentrations in Loligo forbesi

A summary of Cd concentrations in the different tissues of *L. forbesi* from the different sampling areas and at different seasons is presented in Table 3. The final GAM for Cd concentrations ($\mu g.g^{-1}$ dwt, log-transformed) in the digestive gland tissue of *L. forbesi* explained 80% of deviance (N = 105, AIC = -62.4). There was no difference between the sexes but the effect of body size (DML) was highly significant (P<0.0001). Cd concentration decreased (df = 2.73) with DML, although with a steeper decline in small animals (Fig 1a). There were also significant effects of the season, with values in March being higher than in January and values in the last four months of the years being lower than those in the first four months (P<0.0001). There was also significant geographic variation with concentrations in squid from the English Channel (selected as the reference area) being lower than those from other areas (P<0.0001) (Fig 2).

The final model for Cd in muscle explained 54% of deviance (N=101) and was similar in that Cd concentration decreased with DML (df=2.58, P<0.001). However, seasonal differences were non-significant and the only area effect was for higher concentrations to be seen in the English Channel than in the Irish Sea (P=0.002).

The final model for Cd in gills explained 58% of deviance (N=78) and included effects of DML (d.f. = 3.73, P<0.001; a negative trend up to around 200 mm DML after which no further crease is seen) and area: all areas except the west coasts of Ireland and Scotland showed lower Cd concentrations than in the English Channel.

In the case of gonad tissue, the final model included DML (df=1, P=0.008), in this case a positive linear effect, season (lower values in the last four months of the year than in the first four months, P=0.005) and area (lower concentrations in the North Sea and Moray Firth than

in the English Channel, P=0.023 and P=0.037 respectively). This model explained 42% of deviance (N=47).

The final model for Cd concentrations in other tissues included DML (df=2.61, P<0.001) – an initially negative relationship reaching an asymptote around 250 mm – and season, with higher values in the first four months of the year than in the second and third periods (P<0.001).

Trends in Hg concentrations in L. forbesi

A summary of Hg concentrations in the different tissues of *L. forbesi* from the different sampling areas and at different seasons is presented in Table 3. The final GAM for Hg concentrations ($\mu g.g^{-1}$ dwt, log-transformed) in the digestive gland tissue of *L. forbesi* explained 84% of deviance (N = 105, AIC = -126.6). There was marginally significant trend for higher values in males (P=0.038) but this was dropped from the final model to increase sample sizes (around 20 animals could not be sexed). No difference between the sexes but the effect of body size (DML) was also highly significant (P<0.0001). Hg concentration increased linearly with increasing DML (Fig 1b). There were also significant effects of season, with values in January to April being higher than in the rest of the year (P<0.001). Hg concentrations in the English Channel were higher than in all other areas except the North Sea (P<0.001).

In the case of muscle, the best model of Hg concentration explained 85% of deviance (N=101), including a positive linear effect of DML (P<0.001), higher concentrations in the English Channel than in the North Sea (P=0.011), Moray Firth (P<0.001) and North Sea coastal waters (P<0.001), and higher concentration in the first four months of the year than in thre last 4 months (P<0.001).

For gills, the best model explained 90% of deviance (N=78), including a positive effect of DML (df=2.05, P<0.001), higher concentrations in the first four months of the year than in the second and third periods (P<0.001) and higher concentrations in the English Channel than in the North Sea, Moray Firth and North Sea coasts (P<0.001 in all cases).

The best model for Hg in gonads explained 83% of deviance (N=47) and included a weak positive effect of DML (df=1.71, P=0.031), higher values in the first four months of the year than the last four (P<0.001) and higher values in the English Channel than in the North Sea (P=0.014) and Moray Firth (P<0.001).

Finally, the best model for Hg in other tissues explained 86% of deviance (N=77) and included a positive linear effect of DML (P<0.001), higher values in the first four months of the year than the last four (P<0.001) and higher values in the English Channel than in the North Sea (P=0.026), Moray Firth (P<0.001) and North Sea coasts (P<0.001) and higher values in the Irish Sea than in the English Channel (P=0.034).

As was apparent from the modelling results, data from different tissues of *L. forbesi* give somewhat different pictures of the geographical distribution of metal concentrations (Fig 2). These plots do however show that Hg levels were consistently highest in all tissues of squid from the English Channel. Interestingly, these squids also displayed the lowest Cd concentrations in their tissues, with the exception of the gonads.

Implications for public health

To exceed the PWTI for Cd (i.e. 7 $\mu g.kg^{-1}.wk^{-1}$), it would be necessary to ingest from 27 to 333 g of squid muscle per kg of body weight for an average human body weight of 60 kg. This represents consumption of 1.6 to 20 kg of squid muscle in a week (Table 2). However, the PTWI would be reached with only 15 $g.wk^{-1}$ for the digestive gland of the four squid species and for the gills of *T. sagittatus* (Table 2). This may be an issue in countries (e.g. Japan), where squid digestive glands are consumed. In southern Europe, small squids are often eaten whole but the viscera of larger squid would not normally be eaten.

In relation to Hg intake, the amount of squid tissue to reach the PTWI for total Hg (5 $\mu g.kg^{-1}.wk^{-1}$) would be around 9 kg of *Loligo* or *Todaropsis* flesh per week or around 4 kg of *Todarodes* flesh. Even for the tissue with the highest Hg concentration, i.e. the digestive gland of *Todarodes*, it would be necessary to eat more than 2.7 $kg.wk^{-1}$ to exceed safe limits (Table 2). Knowing that from 70 to 90% of Hg in squid tissues would be under organic form (Bustamante et al. 2006), the amount of squid flesh to reach the methyl Hg PTWI would be

3.5 to 4.5 times lower compare to the values for total Hg, i.e. between 2 to 2.6 kg of *Loligo* or *Todaropsis* flesh per week and between 0.9 to 1.1 kg of *Todarodes* flesh.

Discussion

The biological data collected are largely consistent with previous findings for *L. forbesi* in Scottish waters (e.g. Boyle and Pierce, 1994; Collins et al., 1997). The *L. forbesi* population sampled in spring 2004 was already past the peak of its spawning season. Mature and large squid were absent in August in the Northern part of the North Sea, suggesting that they had either died or emigrated from fished areas. Following recruitment of the next generation, maturity subsequently increased in autumn and winter. However, mature squid were recorded from the English Channel in July 2004, which could represent the summer spawning group that has previously been identified in the English Channel (Holme, 1974). The presence of small immature specimens in most months suggests that some recruitment occurs throughout the year, as previously recorded by Lum-Kong et al. (1992). A higher proportion of mature animals was found in March 2005 than March 2004. Since both surveys covered the same areas, this suggests that the spawning season started earlier in 2005. Interannual variation in the timing of life-cycle events (e.g. migrations, maturation) has been previously documented in this species and is likely to be linked to environmental variation (Sims et al., 2001; Pierce et al., 2005).

Higher Cd concentrations were found in the digestive gland of all species compared to the other tissues, reflecting the digestive gland's role in the storage and detoxification of this metal (e.g. Miramand and Guary, 1980; Tanaka et al., 1983; Finger and Smith, 1987; Miramand and Bentley, 1992; Bustamante et al., 2002a,b). For Hg, the concentrations recorded in all tissues were generally in the same order of magnitude compared to the digestive gland. Such different patterns of accumulation for Cd and Hg suggest that cephalopods have different mechanisms/rates of uptake and/or sequestration of these two metals. Sources of metals to cephalopods are 1) seawater, as it passes through the skin and through the gills and 2) diet, which probably represents the main pathway for many elements - as previously shown for Am, Cd, Co or Zn (Koyama et al., 2000; Bustamante et al., 2002a, 2004, 2006b; Miramand et al., 2006). To the best of our knowledge, Hg uptake and retention has not been fully investigated in cephalopods. Mechanisms of uptake and depuration of Hg can be influenced by the speciation of the metal in both seawater and prey. However,

compared to Cd, the role of the digestive gland in the storage of Hg appears to be relatively limited in loliginid and ommastrephid species in UK waters (see Table 3). This may be due to: 1) an excretion function of Hg by the digestive gland, and 2) a preferential redistribution of Hg to muscular tissues where it bound to the sulphydryl groups of proteins (Bloom, 1992; Bustamante et al., 2006a). Further research focusing on Hg detoxification, storage and excretion in cephalopods would be worthwhile.

In contrast to Hg, Cd concentrations in the digestive gland exhibit high variability in the four species tested, i.e. from 9.48 to 65.3 $\mu g.g^{-1}$ dwt, with the lowest concentrations found in loliginids and the highest in ommastrephids (Table 2). This result is in accordance with previous observations in the north east Atlantic waters (e.g. Bustamante et al., 1998a) and from the Mediterranean Sea (e.g. Bernardi et al., 2004). Since dissolved Cd concentrations are higher in coastal waters than the open ocean, loliginids might be expected to have higher Cd concentrations in their tissues than ommastrephids, which tend to have a less coastal distribution. Since the majority of the Cd in cephalopod tissues is probably derived from the diet (Koyama et al., 2000; Bustamante et al., 2002a), differences in feeding could contribute to differences in Cd contamination. Both *L. forbesi* and *T. sagittatus* are largely piscivorous but the latter may take more pelagic and fewer demersal and benthic prey (Collins et al., 1994; Pierce et al., 1994c; Quetglass et al., 1999). Differences in physiological characteristics of loliginids and ommastrephids may also explain the differences observed. With regard to tissue ultrastructure, the digestive gland cells of loliginids do not contain the "boule" structures characteristic of most cephalopod species (Boucher-Rodoni and Boucaud-Camou, 1987). These are generally considered to be heterolysosomes and heterophagosomes involved in intracellular digestion (Boucaud-Camou, 1976; Boucaud-Camou and Yim, 1980). A lack of "boules", therefore could result in a reduction of particle capture, limiting intracellular digestion (Boucher-Rodoni and Boucaud-Camou, 1987). Overall, the lysosomal system of loliginids is less developed than in other cephalopod species and these squids could be physiologically limited to storing and detoxifying Cd via binding to insoluble compounds. Alternatively, these squids may also have developed mechanisms favouring the excretion of Cd (Bustamante et al., 2002b).

The more extensive sampling of *L. forbesi* allowed investigation of the variation of Cd and Hg concentrations with biological and environmental factors, such as age (size), sex, lifestyle and geographic origin. In this species, Cd concentrations decreased with increasing body size

and tissues showed significantly different Cd loads according to the geographic origin of the animals sampled. The decrease of Cd concentrations could reflect a shift in their diet with increasing body size. Juvenile *L. forbesi* have a crustacean-dominated diet whereas the adults consume more fish (Collins and Pierce, 1996). Fish generally exhibit lower Cd concentrations than invertebrates (Cossa and Lassus, 1988). Therefore the diet of smaller squid feeding on invertebrates would be richer in Cd than the diet of bigger squid feeding predominantly on fish. Furthermore, the decrease of Cd concentrations may be strengthened by the dilution of the metal in the tissues due to the very fast growth of this squid. A similar ontogenetic decrease of Cd concentrations has been reported for the mantle muscle of other cephalopod species, e.g. *Octopus salutii* and *Eledone cirrhosa* from the Mediterranean Sea (Barghigiani et al., 1993, Storelli and Marcotrigiano, 1999). For *O. salutii*, this trend was related to its reproductive cycle, since adult females approach the coast to lay their eggs. Therefore, young octopods, having very high trophic activity, are proportionally more exposed to pollution in coastal sites (Storelli and Marcotrigiano, 1999). However, an opposite trend was found for the cuttlefish *S. officinalis* from the English Channel, especially when considering Cd concentration in the digestive gland (Miramand et al., 2006). In cuttlefish, Cd appears to be strongly retained in this tissue, with a biological half-life exceeding eight months (Bustamante et al., 2002b). These contradictory results among cephalopod orders show that investigations on Cd metabolism should be carried on a wider range of cephalopod species, including octopus and squid species.

Unlike the other tissues, the Cd concentrations in gonads increased with size, the highest values being found in winter when squids reach sexual maturity. Higher Cd levels were also reported in the gonads of mature squid *Illex argentinus* from the South Atlantic Ocean compare to immature ones (Gerpe et al., 2000). Interestingly, Cd and Hg varied positively, suggesting a common elimination through the gonads. Although difficult to assess, Cd and Hg accumulation in gonadal tissues may occur as a result of the accumulation of essential elements to supply the embryo's needs (Villanueva and Bustamante, 2006). Essential elements such as Zn are stored in metal-containing enzymes and metalloproteins within the ovary. Cd and Hg are well-known for its affinity to Zn binding sites, particularly in SH-containing proteins such as metallothioneins (Niboer and Richardson, 1980, Cosson et al., 1991, George and Olsson, 1994) and could therefore accumulate bound to such proteins. To the best of our knowledge, there are no studies reporting the occurrence of metallothionein in the gonads of cephalopods. As there were significant differences of Cd and Hg levels between

areas, the effect of this metal accumulation on the squid fertility and the egg production should be evaluated in the future.

Environmental factors, i.e. geographic origin and season also influenced Cd concentrations in *L. forbesi* tissues (Table 3, Fig 2). However, the pattern of variation was different according to the tissue considered and to the season. With the exception of the gonads and gills, Cd concentrations were generally lower in squids from the English Channel, suggesting that they were less exposed to the metal from the dietary pathway. This is, at first sight, surprising, in that anthropogenic activities would be expected to increase the Cd contamination in the food webs from this area. However, Cd occurs naturally at very high concentrations in cephalopods from remote and pristine areas such as the Austral Ocean and Northern Atlantic waters (Bustamante et al. 1998ab). As a general rule, Cd concentrations in various phyla increased "naturally" from the temperate zone to sub-polar and polar waters (e.g. Rainbow 1989, Zauke & Petri 1993, Bustamante et al. 1998a, 2003). Even though our sampling represents a restricted latitudinal range, it is possible that the low Cd concentrations in squids from the English Channel result from a natural latitudinal trend.

In contrast to Cd, Hg levels increased with size of *L. forbesi* and were highest at the end of the spawning season. In aquatic animals, Hg concentrations may vary according to their trophic level and age. As it is mainly stored in the methylated form in biota, Hg consistently biomagnifies through the food chain, with predators showing higher tissue concentrations than are found in their prey. In cephalopods, it is generally agreed that Hg tissue concentrations are positively correlated with body size (e.g. Rossi et al., 1993; Storelli and Marcotrigiano, 1999) and a similar relationship has also been reported for loliginid squid from the Azores, the Bay of Biscay, the Celtic Sea and the Faroe Bank (Monteiro et al., 1992; Bustamante et al., 2006a). Monteiro et al. (1992) reported a higher accumulation rate of Hg in *L. forbesi* females, suggesting physiological and/or ecological differences between the sexes. However, no sex-related differences were evident in the present study.

In addition, Hg levels were highest in squid from the English Channel and the Scottish West Coast and there was also seasonal variation. Higher Hg concentrations found in the tissues of *L. forbesi* from the English Channel (Fig. 2) could be related to human activities in the area. However, considering data for all species, no clear habitat (open water *vs* coastal) or latitude (north *vs* south) differences could be found in Hg loads. Since squid have annual life-cycles

and undertake regular seasonal migrations, it is difficult to differentiate ontogenetic, seasonal, and regional variation in metal concentrations. Although the relative contributions of dietary and waterborne pathways have not been assessed in cephalopods, it is expected that food would be the main source for Hg accumulation in squid tissues (Bustamante et al., 2006a). As cephalopods are short-lived species, they might therefore function as good indicator species of the variation of Hg concentrations in seawater and for marine pollution (Seixas et al. 2005).

Cephalopods may constitute a significant seafood source of human tissue burdens of Cd and Hg (Storelli and Marcotrigiano, 1999; Storelli et al., 2005). In most European countries, viscera are removed before consumption. In Italy and Spain, however, small *Loligo* spp. and *Alloteuthis* spp. are eaten whole, and people from these countries consume much of the *L. forbesi* landed in the UK and Northern France (Shaw, 1994). Moreover, in Japan, the digestive gland is consumed and is considered to be a delicacy. It was therefore important to evaluate the implication of metal levels in squid tissue for public health.

The maximum permitted levels of Cd and Hg for human consumption are 1.0 and 0.5 $mg.kg^{-1}$ fresh weight respectively (based on the consumption of seafood). The Joint FAO/WHO Expert Committee on Food Additives (JECFA) has established a Provisional Tolerable Weekly Intake (PTWI) of 7 and 5 $\mu g.kg^{-1}.wk^{-1}$ for Cd and Hg, respectively (WHO, 1989, 1992, 2003). The quantities of Cd and Hg in the muscle of *L. forbesi*, (i.e. the tissue predominantly used for human consumption) were such that thresholds are very unlikely to be exceeded, with average levels of Cd measured at 0.02 $\mu g.g^{-1}$ and average levels of Hg measured at 0.04 $\mu g.g^{-1}$. For an adult human of 60 kg body weight, it would be necessary to eat at least 1.6 kg wk^{-1} of *T. eblanae* muscle to reach the PTWI. For the other squid species studied, larger amounts would have to consumed, up to 20 $kg.wk^{-1}$ in the case of *L. forbesi.* In southern Europe, the complete consumption of bodies of small squid (loliginids) may also contribute significantly to Cd intake, since their digestive glands account for ~5% of total body mass. Based on a weighted average of Cd concentrations in muscle and digestive gland, the critical amounts of whole squid consumed to reach PTWI would be around 1.6 $kg.wk^{-1}$ for *Alloteuthis* spp. and around 2.3 $kg.wk^{-1}$ for *L. forbesi*. For total Hg, the critical amounts of squid flesh ingested to reach the PTWI were always > 3.8 $kg.wk^{-1}$ and even for digestive gland was consistently >2.7 $kg.wk^{-1}$ (Table 2). For methyl Hg, the PTWI is only 1.6 $\mu g.kg^{-1}.wk^{-1}$ (WHO 2003), and assuming that from 70 to 90% of the total Hg is under organic form

in squid tissues (Bustamante et al. 2006), the critical amounts of squid muscle and digestive gland ingested were always > to 1.1 and > 0.6 $kg.wk^{-1}$, respectively. The results of this study indicate that squid fished in the areas investigated were, in terms of Cd and Hg concentration in selected tissues, safe for human consumption.

Conclusions

As heavy metal contamination of squid has potential implications for human health and conservation of marine mammals, it should be monitored on a regular basis. The observed differences between Cd levels in loliginids and ommastrephids need to be investigated further, in order to determine the possible influence of physiological differences and/or dietary changes during development. The digestive gland plays a major role in Cd bioaccumulation and detoxification in squid, but it seems to have a limited role in storage of Hg. Observed seasonal variations indicate that squid can be used to monitor ambient Hg levels in the environment.

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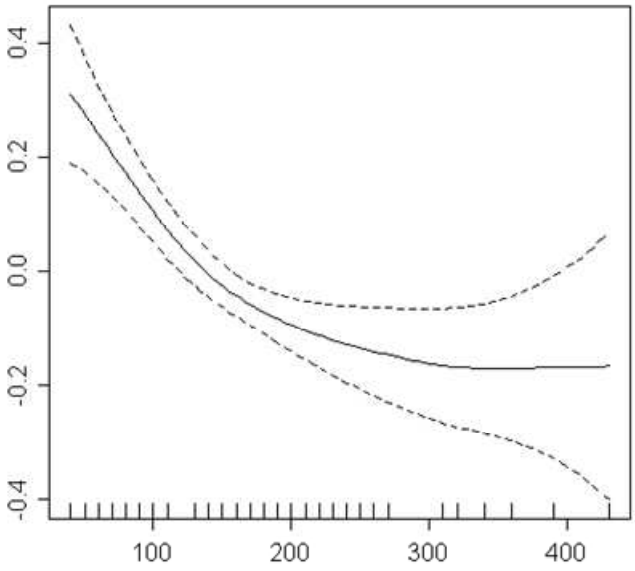
Caption to figures

Figure 1. Smoothers for effects of dorsal mantle length (DML) on (a) cadmium (Cd) and (b) mercury (Hg) concentrations ($\mu g.g^{-1}$ dwt) in the digestive gland of *Loligo forbesi*. The y-axis shows the contribution of the smoother to the predictor function (in arbitrary units). Smoothers illustrate the partial effect of DML, i.e. the effect of DML once the effects of all other explanatory variables in the model have been taken into account. Dashed lines represent 95% confidence bands for the smoothers.

Figure 2. Cadmium (Cd) and mercury (Hg) concentrations ($\mu g.g^{-1}$ dwt) in tissues of *L. forbesi* for areas sampled. CNS = Central North Sea, EC = English Channel, IS = Irish Sea, IW = Irish West Coast, MF = Moray Firth, NSUK = North Sea Coast UK, SWC = Scottish West Coast.

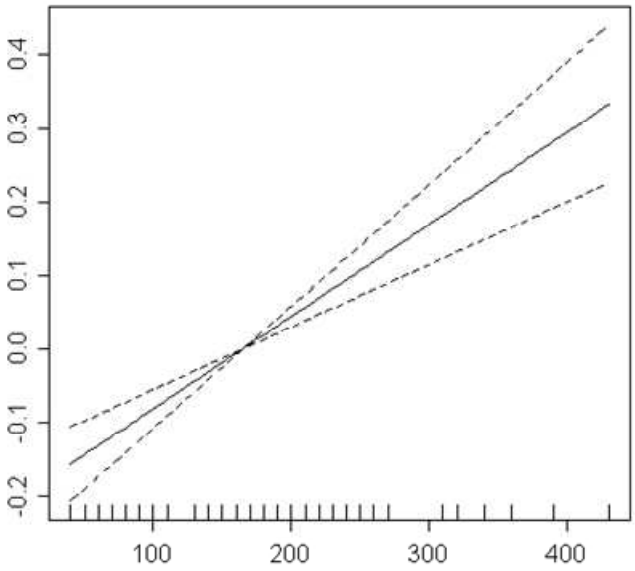
Figure 1.

(a)



Dorsal mantle length (mm)

(b)



Dorsal mantle length (mm)

Figure 2

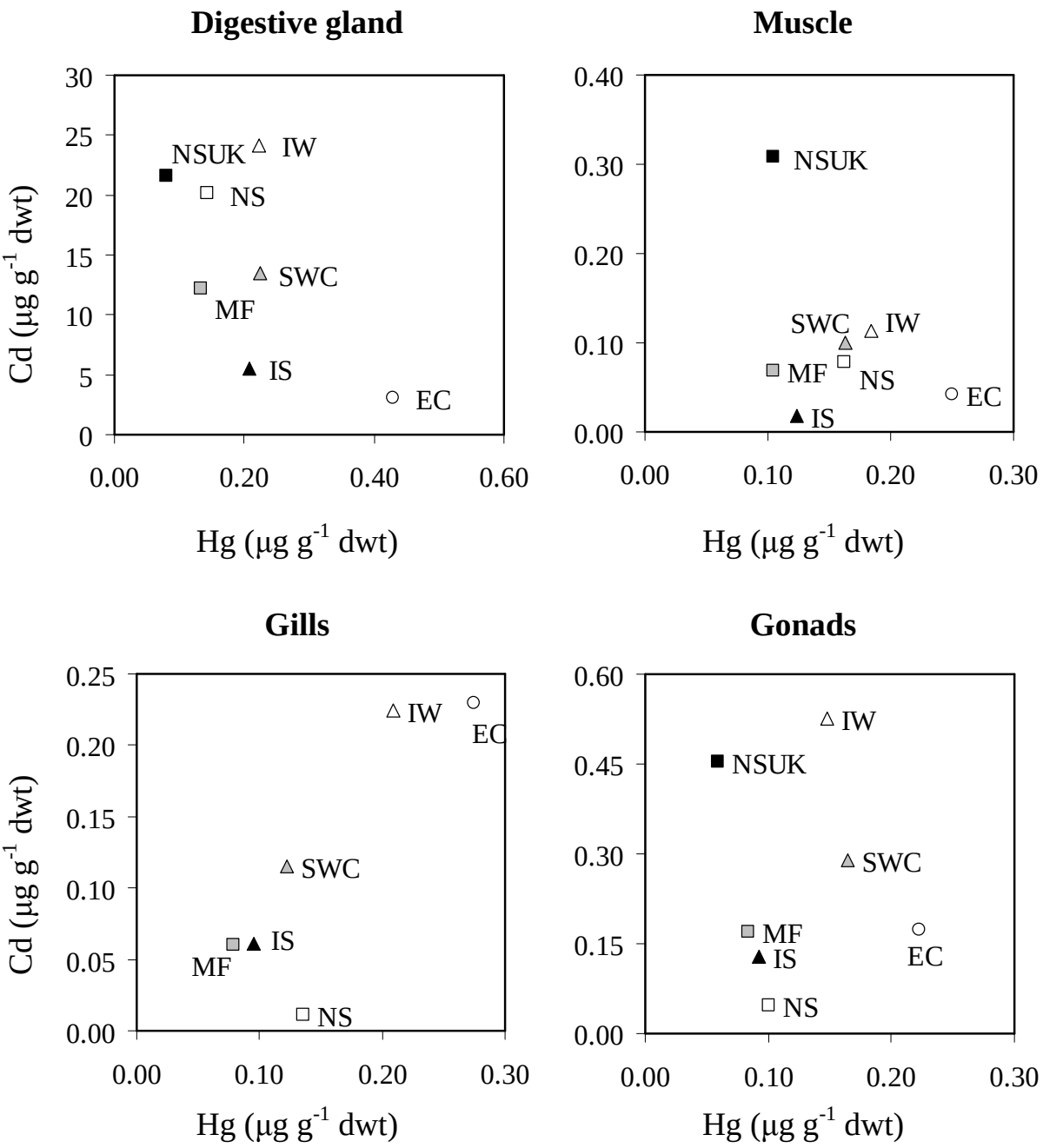

Digestive gland
Cd (µg g-1 dwt)
Hg (µg g-1 dwt)
NSUK
IW
NS
SWC
MF
IS
EC
Muscle
Hg (µg g-1 dwt)
NSUK
SWC
IW
MF
NS
EC
IS
Gills
Cd (µg g-1 dwt)
Hg (µg g-1 dwt)
IW
EC
SWC
IS
MF
NS
Gonads
Hg (µg g-1 dwt)
IW
NSUK
SWC
MF
IS
EC
NS

Table 1. Size and maturity in sampled *L. forbesi*, grouped according to sex, for each quarter of the year. For dorsal mantle length (DML), the table shows the mean ± 1 standard deviation. For maturity, the range and median values for the maturity stage index are given. The proportion of mature squids refers to stages 4 and 5.

	Males				Females			
Season/year	n	DML (mm)	Maturity	Proportion of mature squids (%)	n	DML (mm)	Maturity	Proportion of mature squids (%)
Spring 04	136	105 ± 87	1-5 (1)	18	119	106 ± 66	1-5 (2)	24
Summer 04	171	128 ± 57	1-3 (2)	0	131	110 ± 42	1-4 (1)	5
Autumn 04	256	161 ± 93	1-5 (3)	32	232	156 ± 54	1-4 (3)	8
Winter 05	20	241 ± 72	2-5 (4)	65	18	203 ± 43	1-5 (4)	67
Spring 05	77	150 ± 69	1-5 (4)	51	51	169 ± 65	1-5 (4)	67

Table 2. Concentrations of Cd and Hg (mean ± 1 standard deviation) in the tissues analyzed for all species. Also shown are the mean mantle length (mm, ± 1 standard deviation) and the maximum safe weekly consumption (MSWC) (kg) of each tissue of each species. The MSWC is the weight of cephalopod tissue that must be consumed, assuming 100% assimilation, to reach the PTWI (7 µg $Cd.kg^{-1}.wk^{-1}$ and 5 µg $Hg.kg^{-1}.wk^{-1}$) for an average person of 60 kg body weight.

Species and tissues	N	Cd concentration		MSWC	Hg concentration		MSWC
		$\mu g.g^{-1}$dwt	$\mu g.g^{-1}$ wwt	(kg)	$\mu g.g^{-1}$ dwt	$\mu g.g^{-1}$ wwt	(kg)
Loliginidae							
Loligo forbesi							
Digestive gland	105	12.2 ± 9.92	3.31 ± 2.31	0.127	0.216 ± 0.176	0.065 ± 0.052	4.6
Muscle	101	0.093 ± 0.146	0.021 ± 0.033	20.0	0.153 ± 0.081	0.035 ± 0.019	8.6
Gonads	47	0.116 ± 0.160	0.028 ± 0.036	15.0	0.140 ± 0.090	0.034 ± 0.022	8.8
Gills	78	0.245 ± 0.318	0.055 ± 0.066	7.6	0.128 ± 0.083	0.029 ± 0.019	10.3
Remaining tissues	77	0.533 ± 0.639	0.110 ± 0.121	3.8	0.129 ± 0.067	0.028 ± 0.015	10.7
Alloteuthis sp.							
Digestive gland	5	9.48 ± 2.30	2.27 ± 0.62	0.185	0.072 ± 0.011	0.017 ± 0.002	17.6
Muscle	20	0.797 ± 0.204	0.159 ± 0.033	2.6	0.098 ± 0.011	0.017 ± 0.005	17.6
Ommastrephidae							
Todaropsis eblanae							
Digestive gland	23	25.1 ± 25.9	8.41 ± 8.64	0.050	0.128 ± 0.099	0.042 ± 0.031	7.1
Muscle	23	1.55 ± 2.15	0.256 ± 0.311	1.6	0.206 ± 0.201	0.035 ± 0.035	8.6
Gonads	4	0.600 ± 0.871	0.143 ± 0.211	2.9	0.162 ± 0.109	0.039 ± 0.026	7.7
Gills	6	5.69 ± 5.09	1.23 ± 1.10	0.341	0.187 ± 0.133	0.041 ± 0.030	7.3
Remaining tissues	6	1.20 ± 1.30	0.238 ± 0.218	1.8	0.169 ± 0.143	0.029 ± 0.022	10.3
Todarodes sagittatus							
Digestive gland	11	65.3 ± 61.1	30.7 ± 27.5	0.015	0.280 ± 0.105	0.110 ± 0.066	2.7
Muscle	12	0.304 ± 0.292	0.068 ± 0.065	6.2	0.425 ± 0.194	0.080 ± 0.054	3.8
Gonads	6	1.00 ± 1.37	0.211 ± 0.289	2.0	0.286 ± 0.106	0.061 ± 0.026	4.9
Gills	7	8.67 ± 8.98	1.68 ± 1.63	0.250	0.245 ± 0.136	0.047 ± 0.036	6.4
Remaining tissues	7	1.41 ± 1.56	0.305 ± 0.356	1.4	0.289 ± 0.146	0.064 ± 0.034	4.7

Table 3. Concentrations of Cd and Hg (mean ± 1 SD μg.g^{-1} dwt), by area and month, in tissues of *L. forbesi*. No gonad tissue was analysed for samples from the Irish West Coast.

Area / tissue sampled	Cd		Hg	
Moray Firth	January	August	January	August
Digestive gland	9.14 ± 3.57	14.9 ± 3.7	0.192 ± 0.063	0.081 ± 0.012
Muscle	0.048 ± 0.029	0.085 ± 0.058	0.120 ± 0.019	0.091 ± 0.009
Gills	0.099 ± 0.086	0.204 ± 0.195	0.138 ± 0.043	0.057 ± 0.014
Gonad	0.077 ± 0.090	0.026 ± 0.009	0.087 ± 0.029	0.064 ± 0.010
Scottish West Coast	March	November	March	November
Digestive gland	28.8 ± 19.0	11.9 ± 10.3	0.314 ± 0.054	0.126 ± 0.080
Muscle	0.144 ± 0.117	0.099 ± 0.147	0.281 ± 0.081	0.107 ± 0.035
Gills	0.543 ± 0.448	0.337 ± 0.460	0.225 ± 0.054	0.094 ± 0.048
Gonad	0.290 ± 0.160	0.083 ± 0.119	0.202 ± 0.032	0.095 ± 0.034
Irish West Coast	April	November	April	November
Digestive gland	18.1 ± 2.94	30.2 ± 8.18	0.363 ± 0.265	0.081 ± 0.014
Muscle	0.065 ± 0.038	0.162 ± 0.167	0.270 ± 0.038	0.098 ± 0.053
Gills	0.121 ± 0.060	0.930 ± 0.563	0.221 ± 0.052	0.074 ± 0.039