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SIMULTANEOUS EXPERIMENTAL STUDY OF DIRECT AND DIRECT PLUS TROPHIC CONTAMINATION OF THE CRAYFISH *ASTACUS ASTACUS* BY INORGANIC MERCURY AND METHYLMERCURY

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Abstract—An experimental study was carried out to investigate direct and direct plus trophic contamination routes of the crayfish *Astacus astacus* by inorganic mercury (Hg(II)) or methylmercury (MeHg). Direct exposure was based on low contamination conditions, 300 and 30 ng/L in the dissolved phase, respectively, during 30 d at 20°C. Trophic exposure was based on daily consumption of the Asiatic clam *Corbicula fluminea*, previously contaminated during 40 d with similar exposure conditions. The Hg concentrations in the bivalves were very similar: 1,451 ± 287 ng/g for Hg(II) and 1,346 ± 143 ng/g for MeHg. In the crustaceans, Hg bioaccumulation was analyzed at the whole-organism level and in eight organs (gills, stomach, intestine, hepatopancreas, tail muscle, green gland, carapace, and hemolymph), after 15 and 30 d of exposure. Analysis of the results showed marked differences between Hg(II) and MeHg accumulation in favor of MeHg: for the direct route, the ratio between metal concentrations was close to 8; for the trophic route, no significant increase in Hg accumulation was observed for Hg(II) even when the ratio between Hg concentration in the direct plus trophic contamination route and Hg concentration in the direct contamination route was 1.6 for MeHg, with an estimated trophic transfer rate close to 20%. Mercury organotropism was also specifically connected to the exposure conditions, especially at the biological barrier level according to the route of exposure: gills and carapace for the direct route and digestive tract including hepatopancreas for the trophic route.

Keywords—Mercury Bioaccumulation Exposure routes Crayfish

INTRODUCTION

The contamination of aquatic organisms by trace metals in natural conditions is the result of a series of complex mechanisms, which lead in turn to a wide and varied range of responses in the processes of bioaccumulation and in the toxic effects produced. Among these mechanisms, the exposure modalities of living creatures can play a prominent part, with direct (D) exposure routes, from the surrounding aquatic environment, and trophic (T) routes, through ingested prey. According to the metals studied, the contamination conditions, and the type of organism considered, the predominance of one or the other of these two routes can vary considerably.

In the case of mercury (Hg), a metal with a very high capacity for accumulation and bioamplification along food webs, contamination of predatory species via the T route is often described as being predominant [1–3]. However, such transfers are very much dependent on the chemical form of the metal supplied by the prey, in particular the percentages of methylmercury (MeHg) compared with those of the inorganic form (Hg(II)). In the natural environment, quantifying the respective proportions of the D and T routes is extremely difficult, if not impossible, because bioaccumulation measured in organisms or in organs sampled is the result of a combination of these two routes, with simultaneous depuration processes.

We set up large indoor experimental systems (ESs) to study simultaneously the D and direct plus trophic (D + T) contamination routes of the two chemical forms of mercury (Hg(II) or MeHg) in two freshwater predator species: first the crayfish *Astacus astacus* with the Asiatic clam *Corbicula fluminea* as prey precontaminated with the two Hg compounds; and second a herbivorous fish, the Chinese carp (*Ctenopharyngodon idella*) with trophic intake of the metal via the consumption of contaminated rooted macrophytes (*Elodea densa*).

In this paper we present results obtained with the crayfish for fairly low contamination conditions via the D route (300 ng/L or 1.5 nM for Hg(II) and 30 ng/L or 0.15 nM for MeHg, based on Hg concentration), with the clams having been contaminated during the first phase of the experiment with similar exposure conditions to those for the predatory organisms. Over a period of several years, *C. fluminea* has shown a very strongly invasive dynamic in freshwater systems in France, comparable with that observed in the United States at the end of the 1930s [4,5]. This species has a strong capacity to bioaccumulate metals, which is closely related to its ventilatory activity, for respiratory and nutritional purposes [6–8]. A very limited number of predators have been described in their natural environment, one of which is the crayfish [9]. The bioaccumulation of the two chemical forms of Hg was analyzed at the prey level to obtain similar metal burdens in the soft bodies, and thus simplify a comparative analysis of trophic transfers. In the predatory organisms, Hg was analyzed at the whole-organism level and in eight organs, after 15 and 30 d of exposure via the two contamination routes, D and D + T.

MATERIALS AND METHODS

Structure of the experimental systems

The indoor ESs consisted of resin polyester tanks (110 × 80 × 95 cm) lined with plastic bags (Plastiluz, alimentary standard, Bordeaux, France) and containing 470 L of aerated tap water and 100 kg (wet weight) of a natural sediment mix-

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ture: 50% pure sand (98% silica; granulometry: 0.8–5 mm, Silaq, France) and 50% natural sediment, collected from the banks of the Garonne River upstream from Bordeaux (France). This sediment was made up of fine particles (80% <16 μm in diameter) and consisted of 51% clays, 47% nonclay minerals, and only 2% organic matter. This mixture was homogenized by mechanical mixing then a layer 10 cm deep was spread over the bottom of the ES. An area 20 × 80 cm, at one end of the tanks, was also covered with a layer of sand, 2 to 3 cm deep, for the bivalves. The ESs were located in a thermoregulated room (20 ± 0.5°C) with light conditions monitored separately for each ES: photoperiod 12 h of light/d; light intensity at the water–air interface 45 $\mu E/m^2/s$. The water column was constantly aerated by bubbling air through at a depth of about 5 cm beneath the surface of the water column (aerator 301, Rena, France). Frequent measurements were taken of the pH, oxygen concentrations and saturation levels, and conductivity (Multiline F\SET-3, WTW, Weilheim, Germany) at different points in the ES water column.

After a three-week period for the stabilization of the biotopes, 72 cuttings of the rooted macrophyte *E. densa* (length: 25 ± 0.5 cm, Ets Quentin, France) and 230 benthic bivalves (*C. fluminea*; shell length: 2.1 ± 0.2 cm; soft body weight: 0.36 ± 0.07 g [fresh weight]), collected from a reference site (Cazaux-Sanguinet Lake, southwestern France), were introduced into each ES. The bivalves were placed in the area where the sediment was covered with sand so that sampling would be easier and also to avoid bioturbation effects. They fed during the experiment from bacteria and algae naturally occurring in the ES, notably at the sediment–water interface. These two species were later used as the trophic support for contaminating the predators during the second phase of the experiment.

After two weeks, contamination of the ES by the two chemical forms of Hg was begun, with three ESs for Hg(II) and three ESs for MeHg. Two hundred bivalves were collected from each ES after 40 d of exposure and stored at −20°C; 12 samples (four from each of three ESs) were used to determine the average Hg concentrations in the soft bodies for the two exposure conditions. The bioaccumulation kinetics of Hg in the clams were analyzed over an 80-d period, with four individuals taken from each ES every 20 d. After 87 d of contamination, corresponding to the end of the first phase, the macrophytes were removed by cutting the stems at the sediment surface; identical batches of biomasses (60 g [fresh weight]) were formed. These were then reintroduced into separate tanks to study trophic transfers into the herbivorous fish (*C. idella*; one fish per tank; length: 7 ± 1.2 cm; weight: 4.5 ± 0.9 g [fresh weight]), over 15 and 30 d. During this second phase (30 d), contamination of the water column in the ES was maintained to study simultaneously the D + T and the D contamination routes. At the same time, 12 crayfish (*A. astacus*) were introduced into each ES. Eight were introduced into individual chambers (cylinders made from plastic-coated netting; 0.7-cm mesh, 20-cm diameter) to study the D + T contamination route, with each crayfish receiving daily one clam from the stock of bivalves contaminated during the first phase of the experiment; the other four crayfish were fed on noncontaminated bivalves. The crayfish were obtained from a breeding company in northeastern France (Franckhauser, Sarreguemines); they were 18-month-old males, with an average weight of 14.1 ± 3.3 g (fresh weight). They were acclimated in the laboratory for a period of four weeks, mainly to become accustomed to eating the soft bodies of the bivalves. Note that if the soft bodies are not in their shells the crayfish do not eat them; they must be inside the shells, which have already been opened. Daily intake of one soft body per crayfish corresponds on average to a food ration equivalent to 2.5% of the weight of the predators; under semi-intensive breeding conditions, food intake is 4% on average. Each of the contaminated bivalves introduced on a daily basis into the containers was weighed (soft body + shell) after the shell was opened and the water in the palleal cavity was eliminated. At the end of the two exposure periods (15 and 30 d), the shells were recovered and weighed; in this way we closely monitored the biomasses consumed by each crayfish. During the acclimation phase of the crayfish and during the experiment, several observations revealed that consumption of the soft bodies was a very rapid process, with the prey invariably being ingested after 2 h.

For both exposure periods the crayfish were removed 24 h after their last meal, which gave sufficient time, according to the available data in the literature [10,11], for all the various stages of the digestion process to be completed. Each crayfish was weighed (total fresh weight) after eliminating surface water with absorbent paper sheets. Organs or tissue samples were taken from each individual to determine Hg organotropism: hemolymph (0.5–0.7 ml on average) taken by aspiration using a 1-ml syringe, with the needle pushed into the articular membrane of the fifth leg; tail muscle (whole); hepatopancreas or digestive gland (whole); gills (all five pairs); stomach (stomacal pouch and mesenteron, which is very small); intestine (whole); green gland (whole); and carapace (sample collected from the dorsal zone of the cephalothorax without the internal ectoderm coating). Each sample was weighed (fresh weight) and stored in the freezer until the dosage stage. After dissection, the rest of the body was mechanically ground, then three samples of this mixture were collected for Hg determination. By combining results from the other compartments sampled and the rest of the body we were able to determine metal concentrations and burdens at the whole-body level.

Method for Hg contamination of the water column

Contamination of the water column in the ESs was based on daily additions of aqueous solutions of methylmercury (MeHg: CH_3HgCl, 5 mg/L, Merck, Darmstadt, Germany) or inorganic mercury (Hg(II): $HgCl_2$, 20 mg/L, Merck) to obtain nominal dissolved concentrations of 30 ng/L and 300 ng/L, respectively (0.15 and 1.5 nM). Two main reasons existed for selecting these concentrations: preliminary experiments indicated that these two contamination pressures give similar bioaccumulation levels in the soft body of *C. fluminea* after 40 or 50 d of exposure (O. Simon, unpublished data), thus facilitating the comparative study of trophic transfers of the two chemical forms of the metal; and several recent studies have shown that MeHg concentrations in freshwater systems are between 5 and 20% of the concentration of total Hg in the dissolved fraction [12–14]. The reference to the dissolved fraction is also linked with preliminary studies that showed that, in experimental conditions similar to ours, the particles in suspension in the water column of the systems, consisting essentially of low-density planktonic algae (diatoms), brought about a high degree of Hg complexation. The bioavailability of metals for organisms such as the crayfish is mainly centered in the dissolved fraction of the metals in aquatic environments, through adsorption and absorption processes at the interfaces (gill barrier and carapace) [15,16].

The amounts of Hg added daily to each system were adapted to compensate for the decrease in metal concentrations over the 24-h cycles, which were measured on a regular basis by analyzing unfiltered and filtered water samples. Our experience acquired in the application of this procedure to many experimental models shows that addition is necessary on average of twice the difference between the nominal concentration desired and the concentration measured at the end of the daily cycle. This procedure, although extremely constraining, is nevertheless essential to ensure satisfactory monitoring of the contamination conditions of the organisms, given the many factors that contribute to the decrease in Hg(II) and MeHg concentrations in the ES water columns.

Mercury analysis

Two methods of total Hg analysis were used, according to the nature of the samples. Concentrations in the water samples were determined by flameless atomic absorption spectrometry (Varian M 6000, Walnut Creek, CA, USA). A bromine salt treatment was systematically applied to water samples before the addition of stannous chloride [17]. Concentrations of dissolved Hg were determined after filtration at 0.2 μm (Minisart filters NSM 17597K, Sartorius, France). Tests were carried out on solutions with a low concentration of the two Hg chemical forms to check the absence of metal fixation on the filters (data not shown). Two calibration ranges were used, depending on the nominal concentrations selected: 0, 25, 50, 100, and 150 ng/L for samples collected in the MeHg ESs (detection limit: 2 ng/L); and 0, 100, 250, 500, and 1,000 ng/L (detection limit: 25 ng/L) for the Hg(II) ESs.

Total Hg concentrations in the biological samples were determined by flameless atomic absorption spectrometry using an apparatus specifically designed for the dosage of this metal, without the need for a preliminary digestion phase, which is carried out automatically after drying, by thermal decomposition at 750°C, under an oxygen flow (AMA 254, Leco, Livry Gargan, France; detection limit: 1 ng/g, [dry wt]). The validity of the analytical method was checked during each series of dosages against two standard biological reference materials (TORT-2, lobster hepatopancreas and DOLT-2, dogfish liver from National Research Council of Canada [Conseil National de Recherches Canada], Ottawa, ON, Canada). The Hg values were consistently within the certified ranges (data not shown).

The MeHg concentrations in the soft body of the bivalves were determined in organisms collected after 40 d of exposure, to control exposure conditions for the crayfish via the T route. The technique used was one devised by Bloom [18] and later modified by Saouter and Blattmann [19] for semiautomation of the on-line analytical procedure. After an extraction step by potassium hydroxide in methanol at 75°C for 3 h, the samples underwent an aqueous phase ethylation, followed by the separation of the Hg volatile forms by gas chromatography at 90°C, electrothermic atomization at 800°C, and Hg° determination by cold vapor atomic fluorescence spectrometry (Brooks Rand, Seattle, WA, USA). The two reference materials used for total Hg determinations were also applied for quality controls; MeHg values were consistently within the certified ranges (data not shown).

Data treatment

The data were produced in the context of a complete experimental factorial design, with a minimum of three replicates from the three ESs set up for each condition studied. The effects of the two factors considered, contamination routes D and D + T and exposure duration, on the bioaccumulation of the two chemical forms of Hg were studied by a general two-way analysis of variance (ANOVA) followed by a least-square deviation test (Statistica, Version 5, 1997, StatSoft, Tulsa, OK, USA). Means and standard errors of the mean (SEM, $p < 0.05$) were calculated for all parameters.

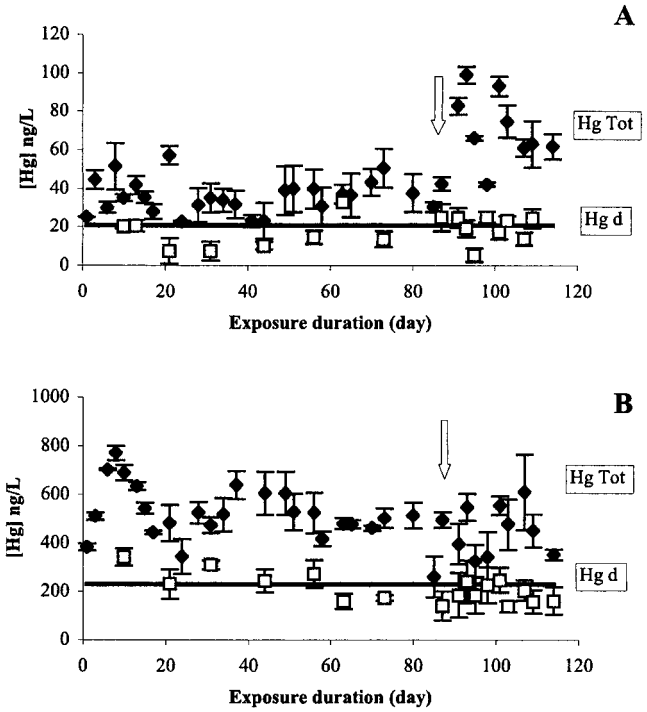

Fig. 1. Average minimal mercury concentrations in the water column (unfiltered samples = $[Hg]_{Tot}$; filtered samples = $[Hg]_d$) measured at the end of the 24-h cycles, over a 117-d exposure period with the two chemical forms of mercury: methylmercury (**A**) and inorganic Hg (**B**). Vertical bars = standard error of mean (SEM; three replicates for each exposure duration). Horizontal lines = average minimal Hg concentrations during the 117 d. Vertical arrows = introduction of the predators (crayfish, fish) in the experimental systems at 87 d.

RESULTS AND DISCUSSION

*Exposure conditions of the prey (*C. fluminea*) and predator (*A. astacus*) species*

Direct route: Evolution of Hg concentrations in the water column. The evolution of the concentrations of the two chemical forms of the metal, measured on unfiltered ($[Hg]_{Tot}$) and filtered ($[Hg]_d$) water samples collected at the end of the 24-h cycles, is shown in Figure 1. Five hundred sixty dosages were carried out and, based on these, daily additions of Hg into the systems were adapted, according to the nominal concentrations initially defined for the dissolved fraction: 300 ng/L for Hg(II) and 30 ng/L for MeHg.

During the first contamination phase (0–86 d), distribution of the two Hg compounds in the particulate and dissolved phases (0.2 μm) was similar and relatively constant, with average values for the ratio $[Hg]_d/[Hg]_{Tot}$ of 0.49 for Hg(II) and 0.51 for MeHg. Average Hg concentrations in the dissolved phase determined from all the dosages carried out at the end of 24-h cycles during the first phase of the experiment were 263 ± 13 ng/L for Hg(II) and 18 ± 1.1 ng/L for MeHg. To maintain the contamination pressure, additions of Hg and MeHg were required on an almost daily basis, which corre-

sponded on average and in each ES to burdens of 33 μg/d (70 ng/L) for Hg(II) and 7 μg/d (15 ng/L) for MeHg. Thus, the average contamination pressure via the dissolved phase can be estimated at 298 and 25 ng/L, respectively, during the first 87 d of exposure.

The second phase of the experiment (87–117 d) immediately followed the introduction of the predators into the systems (crayfish, herbivorous fish). Remember that the majority of the bivalves were collected after 40 d of contamination then stored in a freezer to provide a daily food supply for the crayfish exposed via the double D + T route. The rooted macrophytes were removed at the end of the first phase, by cutting them off at the base of the main stem; the samples were collected to produce homogeneous biomasses and then introduced into the tanks containing the fish to be contaminated via the double D + T route. Despite all the precautions taken, these interventions in the ESs stirred up some sediment particles into suspension, and this resulted in a low degree of turbidity in the water column for 3 to 4 d, at the beginning of the second phase. This turbidity then gradually decreased and the swimming activity of the fish contributed to maintaining a residual degree of turbidity until the end of the 30-d exposure period. The impact of this particulate phase on the evolution of the Hg(II) concentrations in the water column (Fig. 1B) was negligible: the 10 dosages carried out during this period indicate a good stability in average concentrations in the dissolved fraction at the end of the cycles, with no particular modifications to the level of concentrations measured in the unfiltered samples ($C[Hg]_{Tot}$). In contrast, for the MeHg (Fig. 1A), dosages carried out on unfiltered samples presented a large increase in concentrations (two times), whereas readings taken in the dissolved fraction remained similar to those obtained during the first phase of the experiment. The return to suspension of contaminated sediment particles during the first phase of the experiment, via Hg transfers between the water column and the upper sediment layers, may be the reason for these phenomena. Thus, for the inorganic Hg, the contamination pressure during the 30 d of exposure of the crayfish varied between a minimal concentration of dissolved Hg (average of readings at the end of the cycles) of 193 ng/L and a maximal concentration after additions of 320 ng/L, that is, an average value of 256 ng/L. For the MeHg, the minimal and maximal concentrations were 18 and 39 ng/L, or an average value of 28.5 ng/L. Also important is that little variation occurred in concentrations measured in the three replicates set up for each Hg chemical form; ratios between average Hg concentrations and the corresponding SEM were close to 5%.

Concentrations measured in the water column of the ESs corresponded to the dosages of total Hg, but these data do not take into account any transformations of the two Hg compounds: reduction or methylation in the case of the Hg(II), demethylation for the MeHg [1,13]. Note that the daily additions method, based on controlled solutions of the two chemical forms of the metal, minimizes the importance of these processes. In this experiment we were not able to use the method of analysis for the MeHg with sodium tetraethylborate and detection by atomic fluorescence spectrometry, which for the samples of natural water requires a preliminary extraction phase. However, we did obtain this information indirectly by using the properties of bromine salts as breakdown agents for the MeHg [17]. In the water samples from systems contaminated by MeHg, the suppression of the bromination reaction led systematically to the nondetection of the metal (detection limit: 1 ng/L). On the other hand, for the systems contaminated by Hg(II), this operation did not lead to any significant modifications in determinations, thus reflecting the nondetection of organic Hg in the samples.

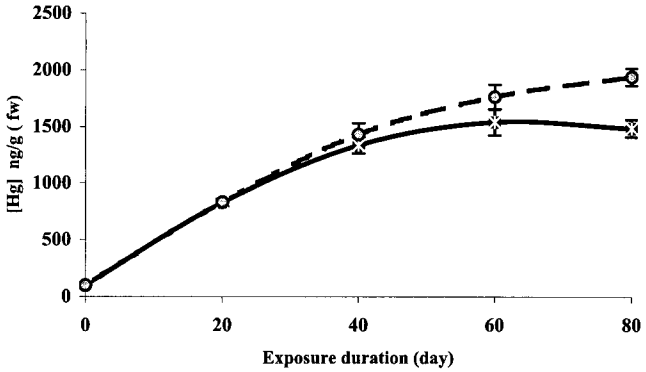

Fig. 2. Average mercury concentrations in the soft body of *Corbicula fluminea* over 80 d of exposure. Broken curve = inorganic Hg. Bold curve = methylmercury. Vertical bars = standard error of mean (SEM; three replicates for each condition).

Frequent measurements of the physicochemical parameters of the water column revealed only minor variations over the two stages of the experiment, with a good level of homogeneity over all the ESs and in the different layers of the water column: temperature: 20.1 ± 0.5°C; pH: 8.6 ± 0.3; dissolved oxygen: 9.3 ± 0.7 mg/L; conductivity: 456 ± 18 μS/cm.

Trophic route: Mercury concentrations in the soft bodies of C. fluminea. The clams used for trophic contamination of the crayfish were exposed to the two Hg compounds during the first 40 d of the experiment. Total Hg determinations made on 12 samples from the three ESs for each chemical form of the metal showed similar bioaccumulation at the whole soft body level: 1,451 ± 287 ng/g (fresh weight) for Hg(II) and 1,346 ± 143 ng/g (fresh weight) for MeHg. The average ratio between the fresh and dry weights of the soft bodies was 5.5. Remember that the average contamination pressure, on the basis of concentrations in the dissolved fraction of the water column, was about 12 times higher for the Hg(II) during this exposure phase. These results reflect the very high accumulation capacity for MeHg in bivalves via the D exposure route [20]. Analysis of the relationship between the biomasses of the soft bodies and the Hg concentrations revealed no significant correlation between these two criteria ($p < 0.05$).

The accumulation kinetics of Hg in the soft bodies of the bivalves during the 80 d of exposure are shown in Figure 2. For MeHg, a plateau was reached after 40 d: the differences between the three average concentrations measured at 40, 60, and 80 d are not statistically significant (one-way ANOVA and least-square deviation test). For Hg(II), the plateau was less distinctive: only the averages measured after 60 and 80 d of exposure were not statistically different; however, they were significantly higher than those at 40 d. These results are in agreement with earlier studies carried out in comparable conditions, with Hg concentrations that were nevertheless considerably higher in the water column [21]. These two accumulation kinetics enable us to determine bioconcentration factors ($[Hg]_{soft\ body\ (fresh\ weight)}/[Hg]_d$ or $[Hg]_{Tot\ (water)}$) at the plateau phase (60 or 80 d). Bioconcentration factors based on Hg concentrations in the dissolved phase are close to 6,000 for Hg(II) and 55,000 for MeHg. When Hg concentrations in the

unfiltered samples are taken into account, the corresponding bioconcentration factors are 3,000 and 33,000, respectively.

These results are in agreement with the initial objectives of this experiment, in which we hoped to obtain similar bioaccumulation levels in the soft bodies of the prey for the two Hg compounds. Note that the concentrations observed after 40 d of exposure were very close to maximal accumulation capacities, corresponding to the plateau tendency for the exposure conditions studied (Fig. 2).

As was the case with the water column, bioaccumulation data for the bivalves was based on analysis of total Hg. With a view to carrying out comparative study of the rates of trophic transfer of the two Hg compounds between *C. fluminea* and *A. astacus*, MeHg concentrations were measured in the soft body of the bivalves sampled at 40 d. The metal bioaccumulated in the organisms from the systems contaminated by MeHg was only in the methylated form (100 ± 4%, $n = 3$). For the bivalves exposed to Hg(II), 96 ± 2% of the Hg accumulated in the soft bodies was in the inorganic form, with 4% being in the monomethylated form. This 4% corresponds to a concentration of 58 ng/g (fresh weight), or about one half of the background level measured in bivalves collected at time zero. This value is identical to the average MeHg concentration measured in the control samples (64 ± 8 ng/g [fresh weight]; $n = 4$).

Bioaccumulation of Hg in crayfish after exposure via the D or D + T routes

No mortality was observed after 15 or 30 d of exposure in any of the 72 crayfish introduced into the six ESs. Note that the crayfish were collected after 24 h without food; according to the available data in the literature, this was enough time for the complete elimination of all food residue in the digestive tract [10,11].

Analysis at the whole-organism level. The concentrations measured in the predator organisms after 15 and 30 d of exposure are shown in the two graphs in Figure 3. For Hg(II) (Fig. 3A), the exposure duration factor had a significant effect ($p < 0.05$) on Hg bioaccumulation, whereas the exposure route and the interaction term are not significant (two-way ANOVA). For the D route, where the organisms were fed with noncontaminated bivalves, a linear increase in concentrations was found. After 30 d, the average concentration was 307 ± 82 ng/g (fresh weight), or 6.1 times the background value; a least-square deviation test showed that differences between Hg concentrations after 15 and 30 d were not statistically significant. The scattering of the data, evident from the SEM values on the graph, is important and increased with the length of exposure. These results indicate a very low level, or even a complete absence, of trophic transfer of Hg(II) in our experimental conditions, if we consider a strict addition between bioaccumulation via the D and T exposure routes.

For MeHg (Fig. 3B), the increase in concentrations of the metal according to the length of exposure is linear for the two contamination modalities studied. For the D route, the average value after 30 d was 256 ± 19 ng/g (fresh weight), or 5.1 times the background value. The daily consumption of prey precontaminated with MeHg led to a bioaccumulation level of 416 ± 52 ng/g (fresh weight), which was significantly greater than the corresponding level via the D route only: the ratio of the average concentrations $[Hg]_{D+T}/[Hg]_D$ was 1.6 for the two exposure periods studied. The variability of the data relating to MeHg was markedly less than that observed for Hg(II);

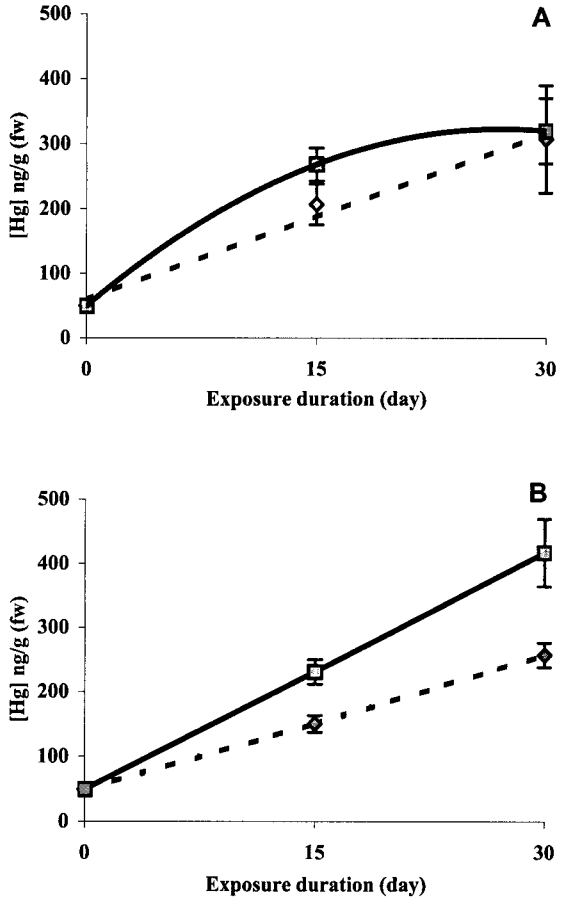

Fig. 3. (**A**) Average mercury concentrations in the crayfish *Astacus astacus* at the whole-organism level according to the direct (broken curve) and direct plus trophic (bold curve) routes of exposure to the inorganic form of mercury (Hg(II)). (**B**) Average mercury concentrations in the crayfish *A. astacus* at the whole-organism level according to the direct (broken curve) and direct plus trophic (bold curve) routes of exposure to the methylated form of mercury (MeHg). Symbols correspond to the mean measured values (three replicates for each exposure duration). Vertical bars = standard error of mean (SEM).

several experimental studies, carried out with different aquatic species (rooted macrophytes, crustaceans, insect nymphs, and fish), have made similar observations [22,23].

A comparison of the two chemical forms of Hg, for the D exposure route, confirms the predominance of MeHg bioaccumulation. Indeed, the ratio between the average concentrations measured at the end of the experimental period was 1.2 in favor of Hg(II), whereas the contamination pressure exerted by this chemical form was about nine times greater, on the basis of average concentrations in the dissolved fraction of the water column during the second phase of the experiment. Determination of the bioconcentration factors, despite the absence of a plateau tendency, indicates average values of 1,000 for Hg(II) and 7,200 for MeHg, with background levels having been deducted. A comparative analysis of contamination via the T route will be undertaken when transfer rates between the quantities of Hg brought in by the prey and the metal burdens measured in the predatory organisms will be estimated (see the section *Inorganic and methylmercury transfer rates*).

Analysis at the organ level. The distribution of Hg in the crayfish was analyzed in eight organs or tissue samples. These were representative of the digestive tract (stomach, intestine, and hepatopancreas), the barriers in direct contact with the

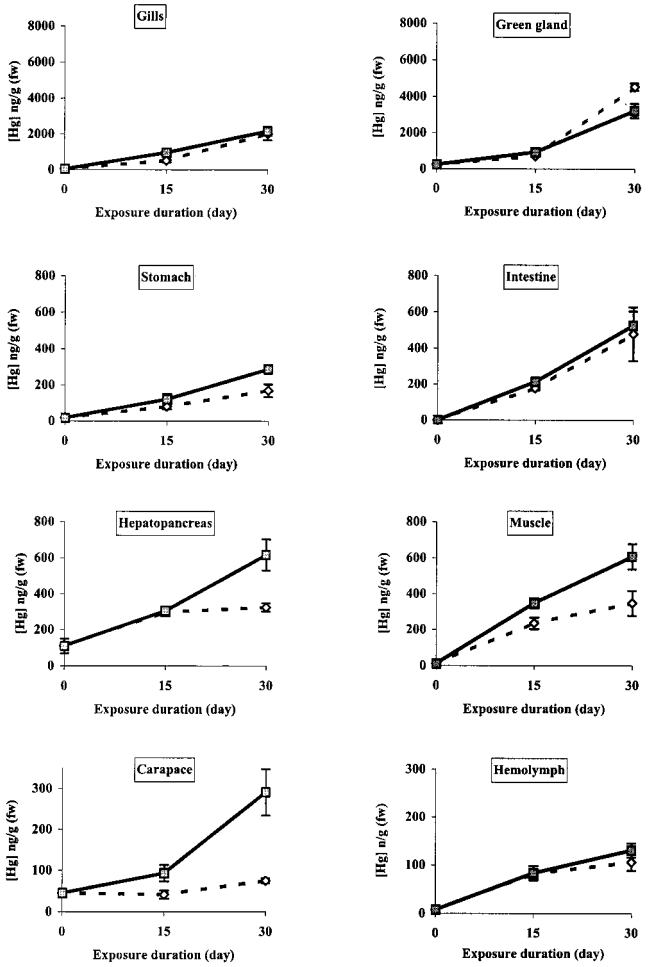

Fig. 4. Average mercury concentrations in the eight organs of the crayfish *Astacus astacus* according to direct (broken curves) and direct plus trophic (bold curves) routes of exposure to the inorganic form of mercury (Hg(II)). Symbols correspond to the mean measured values per exposure (three replicates for each exposure duration). Vertical bars = standard error of mean (SEM).

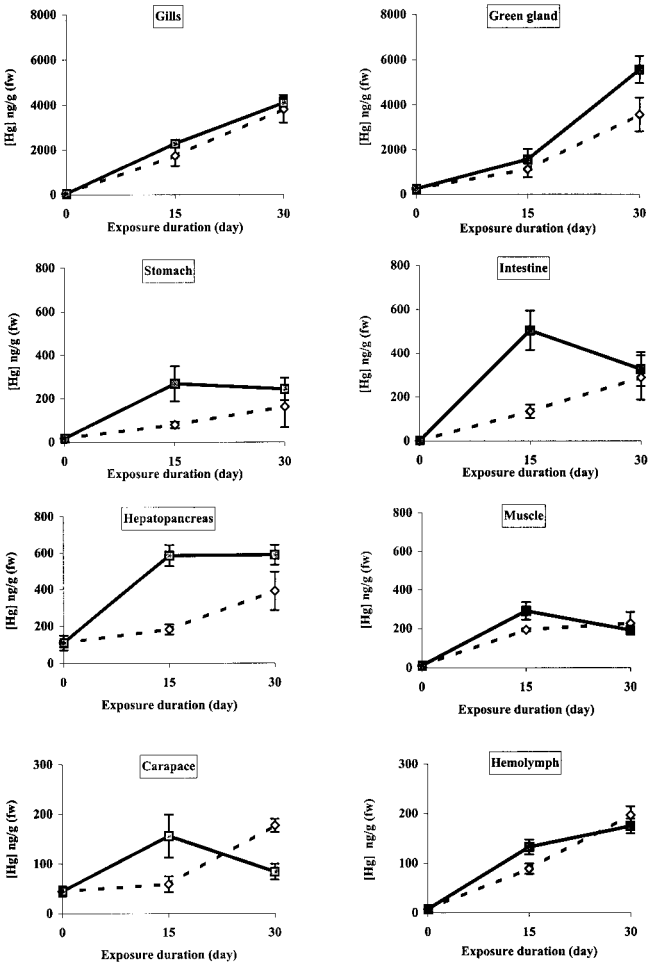

Fig. 5. Average mercury concentrations in the eight organs of the crayfish *Astacus astacus* according to direct (broken curves) and direct plus trophic (bold curves) routes of exposure to the methylated form of mercury (MeHg). Symbols correspond to the mean measured values per exposure (three replicates for each exposure duration). Vertical bars = standard error of mean (SEM).

surrounding aquatic environment (gills and carapace), the excretory system (green gland), the muscle tissue (tail muscle), and the internal medium (hemolymph). The average Hg concentrations measured in the different organs of *A. astacus*, after 15 and 30 d of exposure via the D and D + T routes, are shown in Figures 4 and 5.

Inorganic Hg concentrations (Fig. 4). For the majority of the organs, the D contamination route gives a linear, or close to linear increase in metal concentrations, according to exposure duration. This is a similar trend to that observed at the whole-organism level. Only the carapace, more precisely the dorsal and posterior part of the cephalothorax, collected without the internal ectodermic lining, had a very low level of accumulation after two weeks of exposure ($[Hg]_{15d}/[Hg]_0$ = 1.3), followed by a very marked increase in concentrations after the second phase ($[Hg]_{30d}/[Hg]_{15d}$ = 3.9). Two groups of organs can be distinguished according to their Hg concentrations measured at the end of the experiment: first, the gills and the green gland, with very high concentrations (4,000 and 5,560 ng/g [fresh weight], respectively), corresponding to 93 and 23 times the background levels measured at time zero; and second, the other organs (stomach, intestine, hepatopancreas, tail muscle, carapace, and hemolymph), where average concentrations were lower (between 150 and 300 ng/g [fresh weight]). However, note that for some organs, such as the tail muscle or the hemolymph, wide variations from the background levels are found, greater than a factor of 20. These results are in agreement with the data available in the literature for other species of crustaceans. Thus, the gills represent the route via which the Hg(II) entered the organism and the gills have a very high accumulation capacity, given that they have a vast surface area for exchanges with the external environment and the almost permanent renewal of water in the gill cavity [21,24,25]. Autometallography studies on the gills of the common shore crab (*Carcinus maenas*) after direct exposure of the organisms or contamination of isolated and perfused gills, have shown that Hg(II) was accumulated in the superficial layers of the cuticule and within vacuoles in the nephrocytes, with this cell type playing an important phagocytic role [26].

In crayfish, the green gland is described as an excretory organ that is well irrigated [27]; in many aquatic and terrestrial species, the kidneys represent key targets for the accumulation of inorganic Hg [28]. This accumulation requires absorption of Hg(II) and transport by the hemolymph; the quantities of metal accumulated in this organ are nevertheless compara-

tively low, representing less than 5% compared with the total burden at the whole-organism level.

For the organs of the digestive tract (stomach, hepatopancreas, intestine), levels of accumulation of Hg(II) after 30 d of contamination via the D route only are very high, compared with the very low background levels measured at time zero. If we consider that crayfish do not drink water [29], the metal additions must necessarily derive from internal transfers, via the hemolymph. Further studies are clearly necessary on this point, in particular to verify that no water entered the stomach while food was being ingested.

The trophic supplies of Hg, via the daily ingestion of one soft body of *C. fluminea* per crayfish, together with contamination via the D route, gave rise to different levels and accumulation trends, depending on the organ. For the components of the digestive tract, a wide difference was observed after 15 days of exposure in favor of the D + T route, with the maximal difference being observed in the hepatopancreas ($[Hg]_{D+T}/[Hg]_D = 3.2$), followed by a maintaining of concentrations, or even a decrease by the end of the experiment; the differences between the two contamination modalities were not globally significant except in the stomach and hepatopancreas. The animals went 24 h without food before being collected from the ESs, which gave time for the digestive tract to be emptied. The Hg measured in these organs was therefore adsorbed or absorbed on the biological barriers and reflects accumulation capacities after 15 and 30 d of exposure. For the gills, the levels of accumulation measured after 15 and 30 d of exposure via the D route were not modified significantly by the intake of Hg(II) via ingested prey; in these conditions, the internal transfers of Hg via the hemolymph would seem to be negligible. Analysis carried out on the hemolymph samples revealed minor differences between the two contamination conditions, although these differences were not significant. Among the organs analyzed, only the green gland presented concentrations that were higher at the end of the experiment for the crayfish that had consumed contaminated clams, but once again, variance analysis revealed that the duration of exposure had a significant effect and the contamination route factor did not, without a significant effect of the interaction term. Lastly, note the surprising results for the carapace, presenting very marked differences between the two contamination conditions, which were completely reversed after the two exposure durations.

These results are in agreement with the very low absorption capacities of Hg(II) through the digestive barrier (see the estimates for transfer rates below), whereas the compartments of the digestive tract can accumulate considerable quantities of metal. In the fish, for example, high concentrations of Hg(II) were measured in the intestine after contamination by the T route alone, with very low levels of accumulation in the internal organs, apart from the kidneys. However, when ingestion of contaminated prey ceased, a very large proportion of Hg in the intestine left this organ during the first days of the depuration phase and did not pass into the blood; the Hg was eliminated with the feces, indicating a reversible storage on the external face of the enterocytes, especially at cell coat level [30].

Methylmercury concentrations (Fig. 5). As before, considerable differences emerged between organs, according to the contamination modalities and the exposure duration. For the D contamination route, the two organs that were most contaminated after 30 d of exposure were the green gland (4,527 ± 203 ng/g [fresh weight]; 19 × background level) and the gills (2,013 ± 247 ng/g [fresh weight]; 49 × background level), with metal bioaccumulation showing a clear exponential trend. For the other organs (stomach, intestine, hepatopancreas, tail muscle, and hemolymph), average accumulation levels measured at the end of the experiment were between 100 and 600 ng/g (fresh weight), with concentration evolution tendency being more or less linear, other than for the hepatopancreas, for which a plateau trend was observed between 15 and 30 d of exposure. For the tail muscle after 30 d of exposure via the D + T contamination routes, the average Hg concentration was close to 600 ng/g (fresh weight). The evolution trend observed would suggest high levels of accumulation for longer exposure periods, with the risk of contamination for predatory organisms, especially humans, who consume for the most part this abdominal muscle compartment. Note that standard permitted levels for the consumption of crustaceans range from 500 ng/g (fresh weight) (USA, Canada) to 1,000 ng/g (fresh weight) (Europe). The dorsal area of the carapace, at the level of the cephalothorax, accumulated very little Hg after exposure via the D route, with the ratio $[Hg]_{30d}/[Hg]_0$ at 1.7. At the opposite of Hg(II) and in accordance with results observed at the whole-organism level, the majority of the organs analyzed presented significant differences between the two exposure conditions after 30 d, in favor of the double D + T route; ratios for average concentrations $[Hg]_{D+T}/[Hg]_D$ varied from 3.9 for the carapace to 1.2 for the hemolymph. The very small increase in MeHg concentrations in the hemolymph between the two exposure routes has already been observed in crayfish during contamination via the T route only [31]. This does not indicate an absence of absorption in the digestive tract but rather a high level of exchange capacities between the hemolymph and the other internal tissue compartments, especially the muscles. Indeed, in contrast to Hg(II), the organic compound is able to cross the digestive barriers without presenting any high capacity for accumulation in these structures. The hemolymph contains the respiratory pigment hemocyanin. This large soluble protein offers binding sites for trace metals [32]. However, by analogy with the hemoglobin of fish or mammals, the links that have been established are probably very labile, allowing rapid transfers between the different tissues, depending on irrigation rates, accessibility, and chemical affinity for the different sites at the membrane and cytoplasm levels [22]. Bioaccumulation is once again atypical in the carapace, with Hg concentrations having very noticeably increased after 30 d of exposure by the D + T route. No evidence was found of significant molting of the crayfish during the 30 d of the experiment. In the case of metal uptake from the external water compartment, decapod crustaceans are expected to have a low rate of uptake, because most of the body is covered by an impermeable cuticule, with permeability being restricted to the gills. However, decapod crustaceans are able to transfer accumulated trace metals, including zinc and cadmium, into the cuticule, which can act as both a storage site and an excretion route [33,34]. The MeHg, given the quantities transferred via the T route into the hemolymph, may behave similarly, especially in the fragment of carapace taken from the dorsal zone of the cephalothorax, which is in direct contact with the internal cavity and the hemolymphatic compartment. In the gills, the contribution of the intake of MeHg by the T route is not significant compared with the concentrations measured after exposure by the D route; for the green gland, an inversion was observed after 30 d of exposure, with bioac-

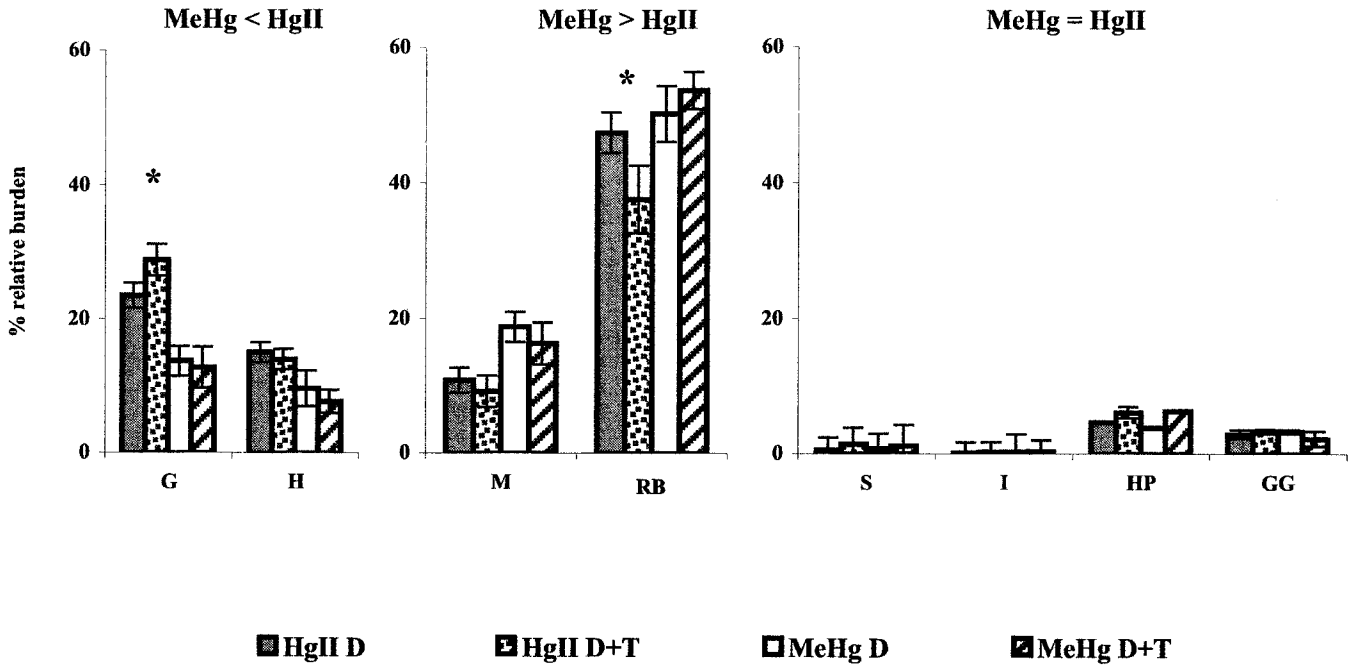

Fig. 6. Average relative burdens of the two chemical forms of mercury (inorganic Hg, Hg(II); methylmercury, MeHg) in eight organs of the crayfish *Astacus astacus* after 30 d of exposure via the direct (D) and direct plus trophic (D + T) routes. G = gills; M = tail muscle; S = stomach; HP = hepatopancreas; I = intestine; GG = green gland; H = hemolymph; RB = rest of the body. Vertical bars = standard error of mean (SEM). An asterisk (*) indicates a significant difference between $Hg(II)_D$ and $Hg(II)_{D+T}$ ($p < 0.05$).

cumulation of the metal in this organ being significantly greater after exposure via the D route alone.

Mercury relative burdens. Given that major differences exist between the mass of the organs and the tissue compartments in the crayfish, by considering relative burdens in parallel with the concentration criteria, we were able to obtain complementary data for analysis of the bioaccumulation processes for the two chemical forms of Hg. The weight measurements carried out directly during dissection indicate that relative weights ($W_{org}/W_{total} \times 100$; fresh weight basis) were homogeneous for the batch of crayfish used: gills, 2.1 ± 0.1%; tail muscle, 15.4 ± 0.5%; stomach, 1.2 ± 0.1%; hepatopancreas, 5 ± 0.2%; intestine, 0.2 ± 0.01%; and green gland, 0.3 ± 0.01%. For the hemolymph, the data available in the literature as well as our own estimations relative to *A. astacus* [31] indicate a relative mass of 28%. The Hg relative burdens were calculated for each crayfish in these seven organs and in the rest of the body, with the quantities of metal in this latter compartment being obtained by adding together the burdens measured in the carapace sample and in the mixture of tissue residue obtained after dissection (carapace, appendages, nervous system, and so on). The Hg relative burdens calculated after 30 d of exposure are given in Figure 6, with the two chemical forms of the metal and the two contamination modalities for the crayfish. We should stress that this criterion does not take into account the differences in bioaccumulation between the different conditions studied, because the bioaccumulation was standardized on the basis of a total burden equal to 100 in all the organisms. Two comments are important to make after analysis of this graph. The first is that relative Hg burdens in the various tissue compartments of the crayfish after contamination by the two chemical forms of the metal were globally similar for the two exposure routes studied (D, D + T). The only significant differences observed ($p < 0.05$) were in the gills for Hg(II) (D + T > D) and in the rest of the body for Hg(II) (D > D + T). The second is that comparison between the two Hg compounds shows marked differences in several organs. In the gills and hemolymph, the average relative burdens are higher after exposure to Hg(II) (2 times and 1.7 times, respectively). On the other hand, in the rest of the body and especially in the tail muscle, contamination by MeHg gave rise to higher relative burdens (1.4 times that in the rest of body after exposure via the D + T route; 1.8 times that in the tail muscle).

Inorganic and methylmercury transfer rates. With the experimental conditions used in this study we were not able to carry out a rigorous analysis of trophic transfer rates, demonstrating the relationship between Hg burdens measured in the predatory organisms at the end of the experiment and the quantities of metal brought in by the ingested prey. Indeed, the crayfish were not contaminated by the T route alone. Nevertheless, from data obtained relating to the soft bodies of the bivalves (details of the daily biomass consumed and determination of the average contamination levels of the batches sampled at the end of the exposure phase) and by considering that bioaccumulation via the T route can be deduced from the difference between the D + T and D routes, estimating transfer levels for MeHg is possible, although the differences between the two exposure routes are not significant for inorganic Hg. Calculations at the individual level give an average amount of MeHg brought in by the 30 soft bodies ingested of 11.7 μg, whereas the metal burden accumulated in the crayfish via the T route seems to be on average 2.5 μg; in these conditions, the estimated average transfer rate would be 21% after 30 d of exposure. This transfer rate is similar to that determined during a comparative study between trophic transfers of MeHg and cadmium between these same biological models, for a duration of 15 d and for higher contamination levels in the prey [31]. This transfer rate, compared with the available literature data, is low, indeed very low, if we compare it with

some values obtained in carnivorous fish, which reach values as high as 90%, or even higher [3,35]. Assimilation efficiency of MeHg in edible muscle of the shrimp *Pandalus borealis* contaminated by mussel consumption was estimated at about 42% [36]. The particular features of the species studied, in particular the structural and functional features of the biological barriers of the digestive tract, may be the reason for these low absorption capacities in crayfish. At the present time, our knowledge of the structure of the digestive tract in decapod crustaceans and the mechanisms involved in digestion show that during the first stage, food is ground in the gastric mill, mixed with enzymes produced by the hepatopancreas, and sorted into two fractions. The coarse fraction, containing all particles greater than 100 nm in diameter, is passed directly to the midgut; and the fine particles and liquids enter the hepatopancreas where peritrophic membranes prevent particulate material from coming into contact with the epithelium. The hepatopancreas is a complex tubular organ derived from the midgut, the functions of which include absorption and storage of nutrients, synthesis of digestive enzymes, and detoxification of trace metals and xenobiotics [32,37]. During the second stage, numerous mature B cells from the hepatopancreas are extruded into the lumen, isolated from the epithelium by the production of a new peritrophic membrane, transferred into the midgut, and then voided. During the last stage, all the material derived from the hepatopancreas is passed to the hindgut and then voided [10,11]. The crayfish's method of predation and ingestion of food may also contribute to a considerable underestimation of transfer rates. Most decapod crustaceans are macrophagous, feeding on large pieces of animal matter cut off with claws and macerated by the array of mouthparts and maxillipeds [32]. This behavior may lead to a considerable loss of Hg before the ingestion of food in the stomach, via transfers to the water column (diffusion of metal and nonabsorption of small fragments resulting from dilaceration). Losses by diffusion may also be increased by the storage of the samples at −20°C, because this could lead to cellular membrane rupture in different soft body tissue compartments. Studies are currently underway to analyze these phenomena, using a force-feeding technique by which a mixture of ground contaminated soft body is injected directly into the stomach of the crayfish and the buccal cavity is then very quickly sealed. This procedure avoids any rejection of food by regurgitation. Under these conditions and by taking into account the different stages in the digestion process in this species, measuring the amounts of metal accumulated in the predatory organism should enable us to determine the trophic transfer rates for the two chemical forms of Hg from metal burdens that have really been ingested.

For the Hg(II), the data available in the literature give low trophic transfer rates, generally less than 20% [3,38]; in crabs contaminated by cockles previously exposed to Hg(II), the transfer rate was close to 4% [39]. The absence of any significant transfer being revealed in our experimental conditions can be compared with the arguments put forward earlier in relation to MeHg. Indicating the greater variability of the results compared with the MeHg (e.g., clearly visible in Fig. 3) also is necessary because this variability limits the possibility of showing any significant differences between the two routes, D and D + T.

For the D contamination route, we do not have any readings for the quantities of Hg(II) and MeHg that were in contact with the biological barriers at the crayfish–aquatic medium interface, especially at the branchial lamellae level. Ecophysiologic studies on the ventilatory activity of the crayfish (*Astacus leptodactylus*) have estimated, via the simultaneous determination of the partial pressure of oxygen in the inspired and expired water, the water flow rate in the gill cavity. These results show marked differences between morning and evening periods, related to the activity of the organisms. At 23°C, the average flow rates were 98 and 254 ml/kg/min, respectively [40]. On an average basis of 180 ml/kg/min and an average weight of 15 g (fresh weight), the amounts of Hg brought by the water flow over the 30-d exposure period would then be 29.8 μg for Hg(II) and 3.3 μg for MeHg. The corresponding metal burdens measured in the crayfish after deducting the background level are 3.8 and 3.0 μg, respectively. For Hg(II), the transfer rate would be 13% and for the MeHg, the transfer rate would be 90%. Clearly, these figures must be considered with certain reservations, given that difficulties can be linked with extrapolating data, such as use of different crayfish species, wide differences in the masses of the organisms (15 g on average in our experiment; 30 g for the ecophysiologic studies), and specific features of the experimental conditions (which may have a strong influence on the ventilatory activity of the decapod crustaceans and include stress, feeding, motor activity, and so on).

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