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Uptake and release of Hg and Cd by some marine organisms
Role of metallothioneins

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When trying to model aquatic food webs one might be tempted to associate a flux of pollutants - say heavy metals - to the fluxes of carbon or nitrogen linking the different parts of the system. Restricting to major questions, one needs know the level of contamination of prey and predator, the rate of entry from food or directly from water, the rate of loss, the possible effects of abiotic factors (chemical speciation, partition of the metal between water column and suspended particulate matter, temperature, salinity, oxygen content, etc). Toxicity must be evaluated at all levels including man and terrestrial animals. The task seems enormous and laboratory experiments only give a partial answer. However they should be planned with the aim to understand the general scheme and to probe real systems for assessment, asking pertinent questions. The data briefly presented here have been collected with this general framework in mind.

1.- Relative uptake of heavy metals from water and food by aquatic organisms : a simple and useful laboratory set-up to study Hg distribution in a two levels food chain *Tubifex tubifex* and *Lebistes reticulata*

The reason to use the chain *Tubifex tubifex* and *Lebistes reticulata* (guppy) is that these freshwater animals are easy to keep alive and readily available at low cost. The experiments (Bouquegneau and Mercenier,

to be published) have been carried out in a 100 ℓ tank separated in three compartments by two plastic gauze screens (2 mm mesh size); water circulation and aeration is maintained by a conventional aquarium pump (250 ℓ/hour) with no filter. Two lots of 35 guppies are kept in the two first compartments; a cluster of about 20 g of Tubifex worms is kept in an open container in the third compartment. The fishes and the worms were intoxicated at the same time for 12 days by adding 10 or 50 ppb of mercury ($HgCl_2$ or CH_3HgCl). One group of fishes was fed until refusal with the intoxicated worms, the other group with non-intoxicated worms kept in a spare aquarium. The water Hg concentration was kept constant by addition of $HgCl_2$ or CH_3HgCl each 24 h and Hg content in the three groups of animals was followed in samples taken each day using the Coleman Mercury Analyser System MAS 50 and the technique described by Hatch and Ott (1968). This experimental set-up allows to simultaneously follow the Hg uptake by direct contamination (from water) and by direct plus indirect contamination (from water + food) under identical experimental conditions, taking into account for instance the possible direct intoxication from the pollutant released from faeces and the role of organic matter, dissolved or particulate, since the water was non filtered. The results are shown in figure 1.

Important Hg concentration in both fish lots and in the prey is observed but no significant difference in the uptake kinetics of both groups of guppies. The conclusion after careful analysis of the data is that the main route of uptake is direct contamination from water and that the percentage of Hg assimilated from food must be small — but higher for CH_3HgCl than for $HgCl_2$. This can be shown by fitting the experimental data to a curve of the type proposed by Pentreath (1975) :

$$C_t = C_{ss} \ (1 - e^{-kt}) \qquad (1)$$

where C_t is the Hg concentration at time t , C_{ss} the concentration at the steady state,

$$k = \frac{0.693}{t_{b\frac{1}{2}}}$$

with $t_{b\frac{1}{2}}$ being the "biological half-time". The $t_{b\frac{1}{2}}$ (days) and C_{ss} (ppm) values are given in table 1.

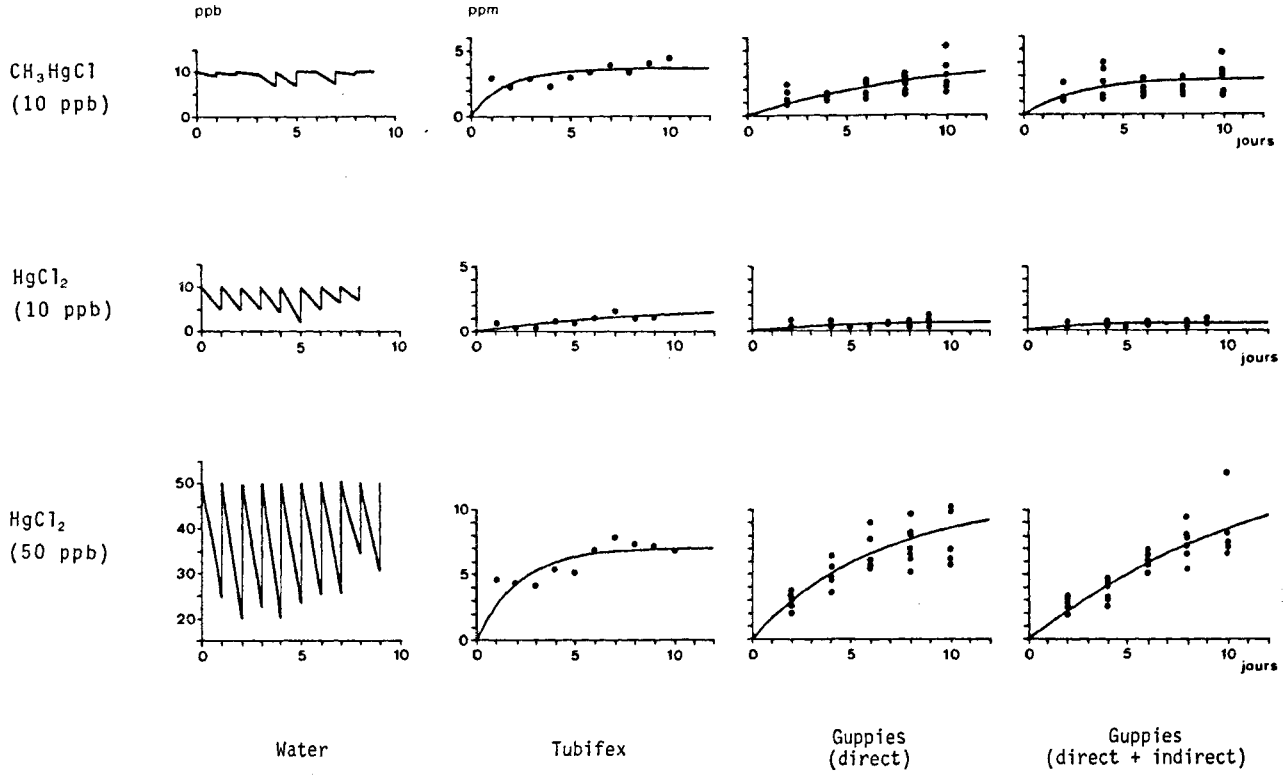

fig. 1.

Evolution of mercury content of water and organisms during intoxications by 10 or 50 ppb of mercury added daily to the aquarium water.

Table 1

Biological half-times $t_{b\frac{1}{2}}$ and concentrations at steady state C_{ss} calculated from curves of figure 1

	Tubifex		Guppies (dir.+ind. intoxication)		Guppies (dir. intoxication)	
	$t_{b\frac{1}{2}}$	C_{ss}	$t_{b\frac{1}{2}}$	C_{ss}	$t_{b\frac{1}{2}}$	C_{ss}
10 ppb CH_3HgCl	1.2	3.7	5.9	4.4	1.9	2.8
10 ppb $HgCl_2$	6.1	2.0	2.8	0.7	1.7	0.55
50 ppb $HgCl_2$	1.4	6.8	4.3	10.9	8.6	15.5

The significance of the calculated $t_{b\frac{1}{2}}$ values will be discussed later in this paper. The faster uptake of CH_3HgCl compared with $HgCl_2$ can be explained by the high solubility of CH_3HgCl in lipids and because it is mainly eliminated by the liver (bile) attached to low molecular weight molecules able to be reabsorbed in the intestine (Norseth, 1971). $HgCl_2$ is mainly excreted by the kidneys (Bouquegneau, 1975).

The fact that contamination from water is the main entry route for fish in this experiment fits with the observations of Bouquegneau (1973-1975) showing that Hg easily penetrates through the gills, in non-fed animals (*Anguilla anguilla* adapted to sea-water and *Myoxocephalus scorpius*) and accumulates in the body tissues. $HgCl_2$ induces the formation of metallothioneins and under acute intoxication, death is observed by rupture of the NaCl balance (Bouquegneau, 1975, 1977). Metallothioneins have also been detected in Tubifex intoxicated as described above. That the effect observed because of ingestion of contaminated food is small is in general agreement with observations made by other experimenters on experimental food chains, taking into account that is most cases direct and indirect contamination were combined, because of faeces and excretion products, in an uncontrolled way.

A review of the matter is presented in a paper by Bouquegneau and Noël-Lambot (1977) who point out that the lowest trophic levels *in situ* or in the laboratory (see f.ex. Aubert et al., 1972, 1974, 1975) are generally the most exposed to contamination by heavy metals.

If heavy metals assimilated from food contribute only partially to the contamination of aquatic predators it remains to evaluate the order of magnitude of this route of entry. We will show how we propose to deal with this problem in the next chapter.

2.- Determination of percentages of ingested heavy metals assimilated from food by aquatic animals in three two levels food chains : *Dunaliella bioculata — Artemia salina; Tubifex tubifex — Lebistes reticulata* and *Patella vulgata — Serranus cabrilla*

The experiments on fish (Bouquegneau et al., to be published) made in a two compartment aquarium as described under § 1 but the pump is fitted with a large charcoal-glass fiber filter to retain suspended matter and to remove the greatest part of the metals released by excretion or bacterial activity on faeces. In one compartment predators were fed with non-intoxicated food and in the other they received contaminated food. Both batches were sampled and their content in heavy metals substracted.

Blank experiments with no pollutants were also carried out. The *Patella vulgata* prey was collected in the industrially polluted area of the Bristol Channel and contained about 50 ppm Cd (w.w). The data on the planktonic chain were obtained in the following way : 100 artemias in a 25 cm plastic tube ϕ 6 cm, obturated at one end with a 1 mm mesh gauze were fed through the gauze on a batch of intoxicated algae during 1 hour per day during 8 days. The number of ingested cells was measured with a Coulter-counter. The water containing the algae was then filtered and 100 artemias in another plastic tube were exposed each day to that water for 1 h again and then fed on non-intoxicated algae for 1 h. The tubes containing the artemias either intoxicated directly only on intoxicated directly and by ingestion of contaminated food where then placed together in a stirred and aerated sea-water tank to complete the direct intoxication resulting from the presence of faeces. The heavy metal content of both algae and artemias per gram wet weight was measured by atomic absorption spectrophotometry.

The percentages of assimilated ingested heavy metal are obtained by substracting the metal concentrations of the animals intoxicated indirectly

only from that of the predators intoxicated both directly and indirectly and dividing by the amount of food metal ingested.

Table 2

Percentage of ingested heavy metals assimilated by *Artemia salina*, *Lebistes reticulata* and *Serranus cabrilla*

Dunaliella bioculata - *Artemia salina* food chain	
$CuCl_2$	non detectable
$ZnCl_2$	non detectable
$CdCl_2$	3.5 %
$HgCl_2$	5.7 %
CH_3HgCl	28.8 %
Tubifex tubifex - *Lebistes reticulata* food chain	
$ZnCl_2$	10.6 %
$CdCl_2$	0.1 %
$HgCl_2$	0.1 % - 1.4 %
CH_3HgCl	37.1 % - 53.2 %
Patella vulgata - *Serranus cabrilla* food chain	
Cd (see text)	0.8 %

The results are summarized in table 2. The data show the very low percentages of ingested metals assimilated by artemias and fishes except in the case of methylmercury where 30 to 50 percent revealed to be assimilated probably retained in lipids. The low values obtained for the other pollutants are quite interesting since it is known that a large fraction of the heavy metals present in the tissues is bound to proteins and that the percentage of ingested proteins assimilated by fishes can be taken to be about 80 %. This might indicate that heavy metals inhibit either the hydrolysis of their protein carrier or the transport through the digestive tract of peptides and amino acids to which they remain attached.

3.- Study of the direct accumulation and elimination of mercury in some marine and freshwater organisms

3.1.- THE UPTAKE OF $HgCl_2$ FROM SEA WATER BY *Serranus cabrilla* (Med. Sea)

The direct accumulation of mercury from water by fish through the gills generally flattens out in a plateau with time, as if mercury intake was compensated by loss (excretion, etc.). If this is true the curve can be represented by equation (1) (see § 1) and biological half-times can be calculated. On the other hand release of Hg by intoxicated fish can be measured by exposing the animals to non-contaminated sea-water. Obviously the calculated biological half-times from the uptake curves and those obtained from experimental evidence must coincide. If they do not, then the significance of equation (1) has to be questioned.

Table 3 shows results which confirm the validity of equation (1) and of the assumptions of Pentreath (1975) who proposed this simple treatment of the results.

Table 3

Biological half-times of mercury in some sea water fishes

Species	Pollutant	$t_{b\frac{1}{2}}$	References
Anguilla anguilla	CH_3Hg^+	1000 d	Jarvenpaa et al., 1970
Anguilla anguilla	$HgCl_2$	80 d	Bouquegneau, 1975
Pleuronectes platessa	$HgCl_2$	162 d	Pentreath, 1976a
Pleuronectes platessa	CH_3HgCl	300 d	Pentreath, 1976b
Raja clavata	$HgCl_2$	277 d	Pentreath, 1976c
Raja clavata	CH_3HgCl	no loss detectable	Pentreath, 1976c
Serranus scriba	CH_3HgNO_3	267 d	Miettinen et al., 1972

Some recent experiments by Radoux and Bouquegneau (1979) on the uptake of Hg ($HgCl_2$) by *Serranus cabrilla* show contradiction between the biological half-times $t_{b\frac{1}{2}}$ calculated and measured.

The fishes (19 to 48 g) caught off Calvi's Bay (Corsica at the Oceanographic Station of the University of Liège) were intoxicated in 80 ℓ aquaria containing aerated non filtered natural (S = 30 ‰) sea water (21 °C)

to which 100 ppb Hg ($HgCl_2$) was added. Water was changed and polluted every day. The fishes were not fed. Four were taken as samples before the experiment and after 1, 2, 4 and 10 days intoxication. The remaining fishes were divided in two batches : one continued to be intoxicated, the other was placed in aquaria containing unpolluted sea-water, filtered on charcoal glass fibers filters. The Hg concentration was measured as indicated page 86. The kinetics of uptake and experimental release is shown in figure 2 for the whole body.

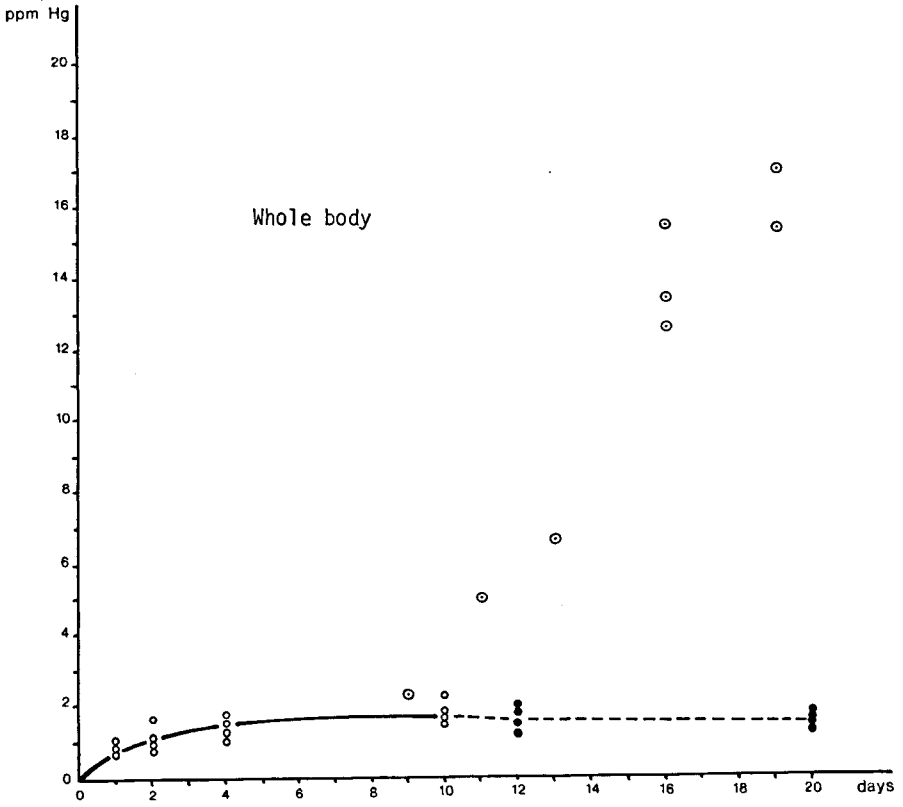

fig. 2.

Kinetics of uptake (———) and release (- - - -) of mercury in the whole body of *Serranus cabrilla*.

- o Intoxicated fishes still alive
- ⊙ Dead fishes
- • Intoxicated fishes put back in clean water

After Radoux and Bouquegneau (1979).

The slow elimination (broken line) suggests a $t_{b\frac{1}{2}}$ of about 100 days of the order of magnitude found for other marine species as indicated in table 3.

However if one uses the curve representing the uptake kinetics of fig. 2 it can be shown that it fits equation

$$C_t = 1.7\ (1 - e^{-0.5\ t})$$

suggesting a $t_{b\frac{1}{2}}$ of only 1.36 day (0.693/0.5). After eleven days of intoxication the concentration of Hg in fishes surviving in polluted water seems to be uncontrolled, the data (all corresponding to dead fishes) fit a curve seemingly parallel to the initial rate of mercury uptake.

Radoux and Bouquegneau (1979) have followed the kinetics of accumulation and experimental release in the organs of the fishes and find the same sort of discrepancy between calculated $t_{b\frac{1}{2}}$ and observed ones.

To conclude it looks as if the accumulation kinetics does not reflect a rapid elimination of the pollutant but rather a decrease of the rate of entry at least during 10 days of exposure. The limiting process has not been identified but might be linked to the production of a protective mucus layer which in some cases was observed, but unfortunately not quantified, especially on the gills. If the protective mechanism fails, the uptake kinetics would rapidly become linear in the experimental conditions and lead to death, for example by breakdown of osmoregulatory homeostasis as shown by Bouquegneau (1977) in sea-water adapted eels.

3.2.- THE UPTAKE OF $HgCl_2$ FROM SEA WATER BY *Crenilabrus ocellatus* (Med. Sea)

The same experimental protocol has been used as for *Serranus cabrilla* by Velissarides and Bouquegneau (to be published). Accumulation in gills, digestive tract plus attached organs and muscles is shown in figure 3 at the 50 ppb $HgCl_2$ level and the accumulation in the whole body is given in figure 4 together with the experimental release curve. The experimental biological half-time is long for the whole body and of the order of magnitude quoted in table 3. Gills eliminate Hg faster than the digestive tract, but muscles show an extremely slow rate of elimination if not a continuously slow accumulation.

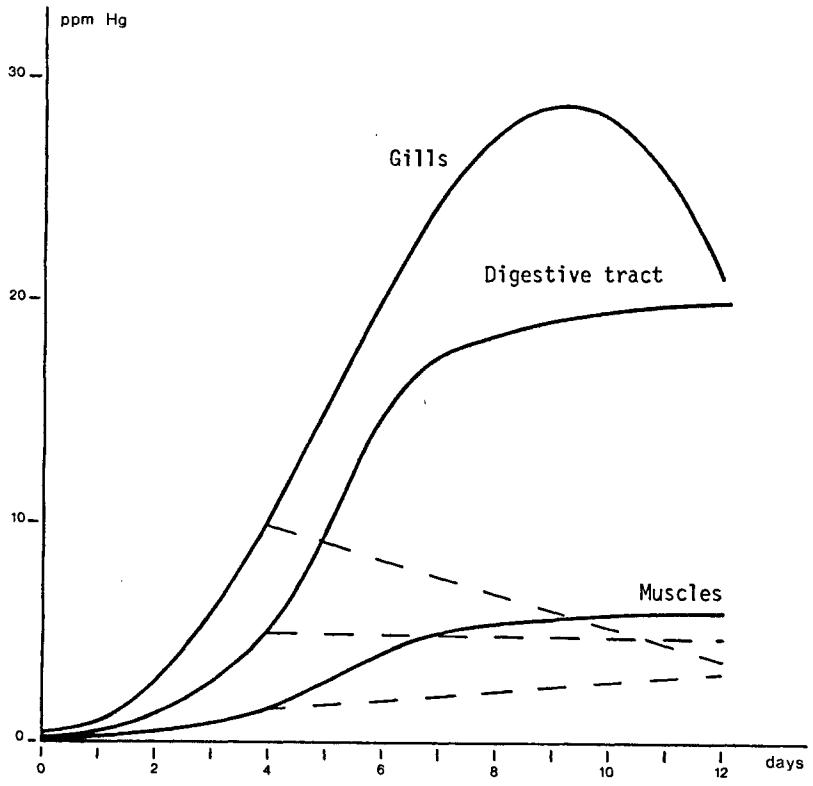

fig. 3.

Kinetics of uptake and release of mercury by gills, digestive tract and muscles of *Crenilabrus ocellatus* intoxicated in sea water containing 50 ppb Hg ($HgCl_2$) [broken line = release]

The accumulation curves are not at all classical, being sigmoïd and do not fit equation (1).

During 4 or 5 days accumulation proceeds slowly and might correspond to diffusion of Hg in the gill tissues where it attaches to -SH groups. The increase that follows is possibly related to the production in the animal of storage sites, probably metallothioneins. Extracts of the digestive tract plus attached organs shows indeed the presence of proteins with molecular weight 10 000 during this period of intoxication. To

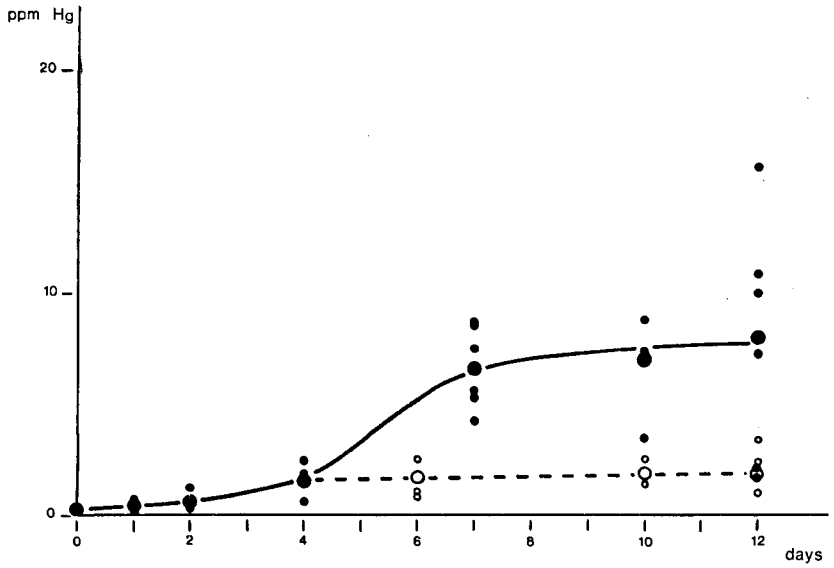

fig. 4.

Kinetics of uptake (———) and release (– – – –) of mercury by *Crenilabrus ocellatus* exposed to 50 ppb Hg ($HgCl_2$).

explain the plateau of the sigmoïd curve one is left to admit either that the gills become less permeable to Hg or capable of excreting the pollutant at a greater rate. Further studies are obviously required to answer these questions but it remains interesting to point out that an accumulation curve of the Michaelis-Menten type is by no ways universal.

3.3.- THE UPTAKE OF $HgCl_2$ AND CH_3HgCl FROM FRESH WATER BY *Lebistes reticulata*; EFFECT OF ORGANIC PARTICULATE (AND DISSOLVED) MATTER

To investigate the kinetics of direct uptake and release of $HgCl_2$ and CH_3HgCl by *Lebistes reticulata*, Mercenier and Bouquegneau (to be published) have intoxicated unfed fishes in fresh water renewed each day, containing 50 ppb $HgCl_2$ or 10 ppb CH_3HgCl . Release was measured after three and ten days in unpolluted water filtered on charcoal and glass fiber. The calculated $t_{b\frac{1}{2}}$ were respectively 3 and 13 days, the observed $t_{b\frac{1}{2}}$ was 10 to 12 and 40 to 60 days. The same sort of argument as for *Serranus*

cabrilla might be proposed : possible protective action by mucus or unknown limiting mechanism of uptake of Hg .

However if one compares the values of $t_{b\frac{1}{2}}$ with those obtained in the experiments described in fig.1, when the mercury level was adjusted each day, the fishes being kept in the same water still other values are found. Further if one now compares the uptake curves when the water is renewed each day with those corresponding to the uptake when the guppies are fed on tubifex (fig.5) it is clear that the level of intoxication is much lower in this last case. The difference can be explained in this

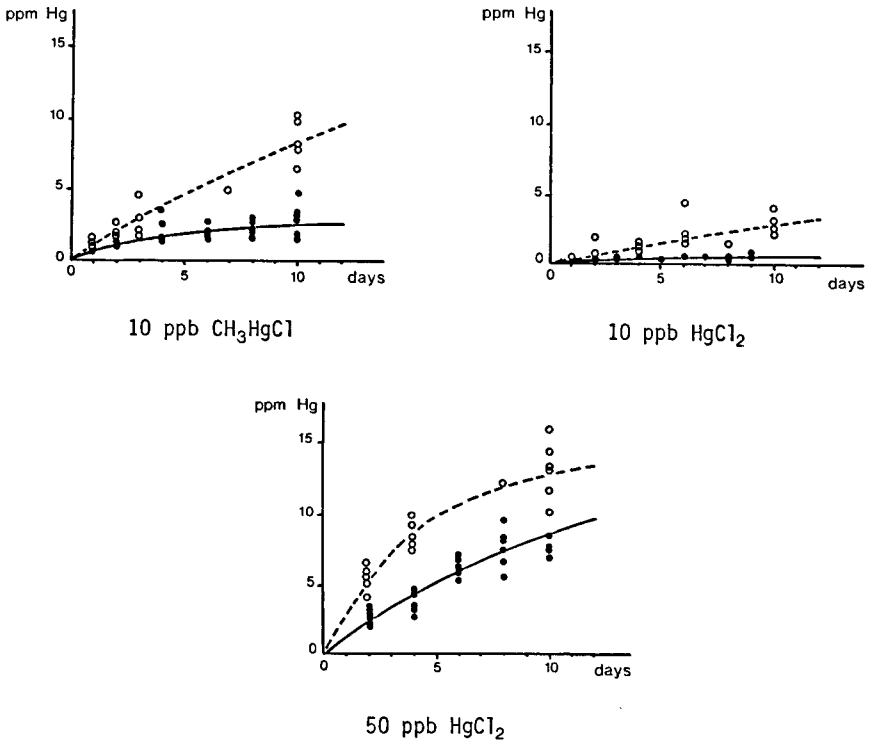

fig. 5.

Comparison between uptake kinetics of mercury by guppies whether the water is renewed (- - - -) or the mercury level is adjusted each day (———) ; the fishes are fed on Tubifex.

instance by the presence of suspended and dissolved organic matter to which Hg combines in a way or another. The metal available to the fishes is in lower concentration the higher the content in organic matter.

Mercenier and Bouquegneau have filtered the aquarium water on Millipore filter, type GS 0.22 µm , to retain the so-called particulate matter and the filtrate was passed on an Amicon UM2 membrane to separate molecules at the 2000 molecular weight level.

Typical results are given in table 4.

Table 4

Aquarium water renewed each day (fishes unfed) : 50 ppb HgCl					
Sampling date	Hg concentration	% Hg on suspended matter	% Hg in solution		
			Total	MW > 2000	MW < 2000
Day 9 , $t_{0\,h}$	50 ppb	2	98	71	29
$t_{24\,h}$	21 ppb	9	91	79	21
Aquarium water not renewed (fishes fed)					
A.- 50 ppb $HgCl_2$ adjusted each day					
Day 1 , $t_{24\,h}$	25 ppb	78	22	63	37
10 , $t_{24\,h}$	27 ppb	67	33	72	28
B.- 10 ppb CH_3HgCl					
Day 1 , $t_{0\,h}$	10 ppb	2	98	66	44
9 , $t_{24\,h}$	10 ppb	36	64	0	100

It thus appears that when the water is renewed and fishes not fed about 90 % of the $HgCl_2$ remains in solution and that this value drops to 20 and 30 % when large amounts of organic matter is present. In the case of CH_3HgCl in the same conditions, the amount in solution remains much higher. The distribution at the 2000 MW level varies with time and after a few days no CH_3HgCl can be found on molecules with a molecular weight higher than 2000 .

In conclusion the factor $t_{b\frac{1}{2}}$ seems to reflect not only the rate of elimination or excretion of the animal but also possible protective mechanisms reducing the uptake rate, as well as the competition between the fish and organic matter to trap the pollutant. At equal concentration,

CH_3HgCl will accumulate faster in fish than $HgCl_2$ because the affinity of the former for suspended organic matter and high molecular weight dissolved organic matter is lower. This means also that at equal total metal concentration water heavily loaded with organic matter is less toxic than water containing no organic detritus or dissolved organic substances.

3.4.- THE EFFECT OF SALINITY ON THE TOXICITY AND THE ACCUMULATION OF Hg IN THE CASE OF THE EURYHALINE CRAB *Eriocheir sinensis*

Bouquegneau and Hibaude (to be published) have used *Eriocheir sinensis*, animal capable of living either in fresh water or sea water, to investigate the influence of salinity on the direct accumulation of Hg and the corresponding toxicity.

The animals are adapted during 15 days to the chosen salinity : natural sea water (Atlantic, S = 33.6 ‰ , 0.07 ppb Hg), the same diluted twice with tap water, tap water. The intoxication experiments are carried out in polyethylene tanks [2.5 ℓ per crab, water being renewed every two days (100 ppb $HgCl_2$) or four days (10 ppm $HgCl_2$), 15 °C]. The animals were not fed during the tests.

Lethal toxicity tests show that at the 10 ppm level 100% mortality is attained after 24 h for fresh water (FW) adapted animals and after 40 days for sea water (SW) adapted ones. Further when SW adapted crabs are intoxicated in freshwater (hypoosmotic shock) 100% mortality is reached after 24 h , and when FW adapted crabs are intoxicated in sea water (hyperosmotic shock) 100% mortality is observed after 19 days. In absence of $HgCl_2$, there is no mortality during the same time lapses. In thus seems clear that at given $HgCl_2$ concentration and for the same animal, mercury is more toxic in freshwater than in sea water. Either the pollutant accumulates faster in freshwater, or the metal affects osmoregulation mechanisms (*Eriocheir sinensis* is in hyperosmotic conditions in diluted media, but isosmotic at high salinities : Schoffeniels and Gilles, 1970).

Both hypothesis have been tested in the folowing.

3.4.1.- Accumulation of Hg by *Eriocheir sinensis* in function of salinity.

The Hg load in gills (anterior and posterior), hepatopancreas, viscera, muscles, carapace and legs has been followed in time at two $HgCl_2$

concentrations 10 ppm and 100 ppb . Figure 6 shows the evolution of the total load of the animals with time at 10 ppm $HgCl_2$ in sea water (SW), SW/2 and fresh water (FW).

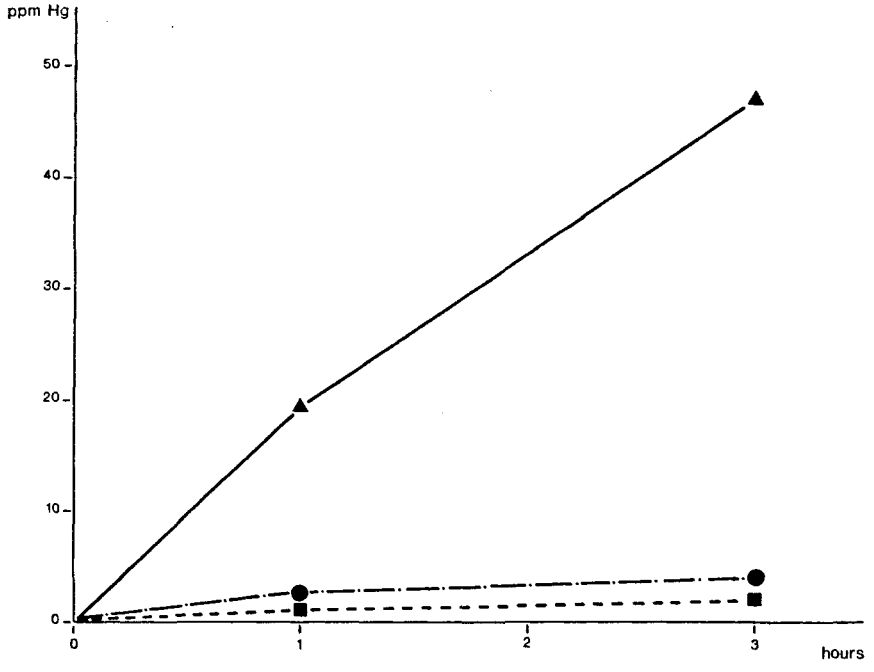

fig. 6.

Mercury uptake by *Eriocheir sinensis* living in various salinity ranges and intoxicated at the 10 ppm Hg level ($HgCl_2$).

▲ FW ● SW/2 ■ SW

Accumulation in FW is 20 times more important than in SW . The concentration factor in FW is 21 times that observed in SW and 2.5 times that obtained in SW/2 .

Figure 7 gives the kinetics of Hg accumulation at 100 ppb for the whole body load. In FW , accumulation is fast during the first day and ends in a plateau, mortality reading 100 % on the 6th day. In SW and SW/2 , accumulation is much retarded but increases continuously at levels higher than in FW although only 6 % mortality is attained after 16 days. The difference between the effects in FW and SW could be explained either by

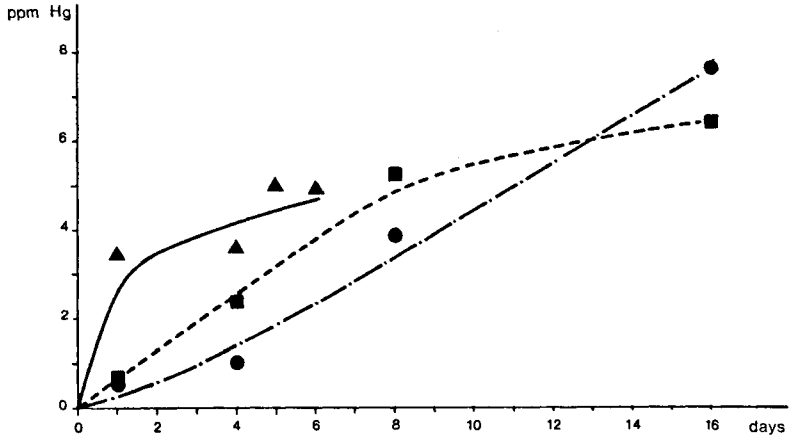

fig. 7.

Uptake kinetics of mercury by *Eriocheir sinensis* adapted to various salinity ranges and intoxicated in water containing 100 ppb Hg ($HgCl_2$)

▲ FW ● SW/2 ■ SW

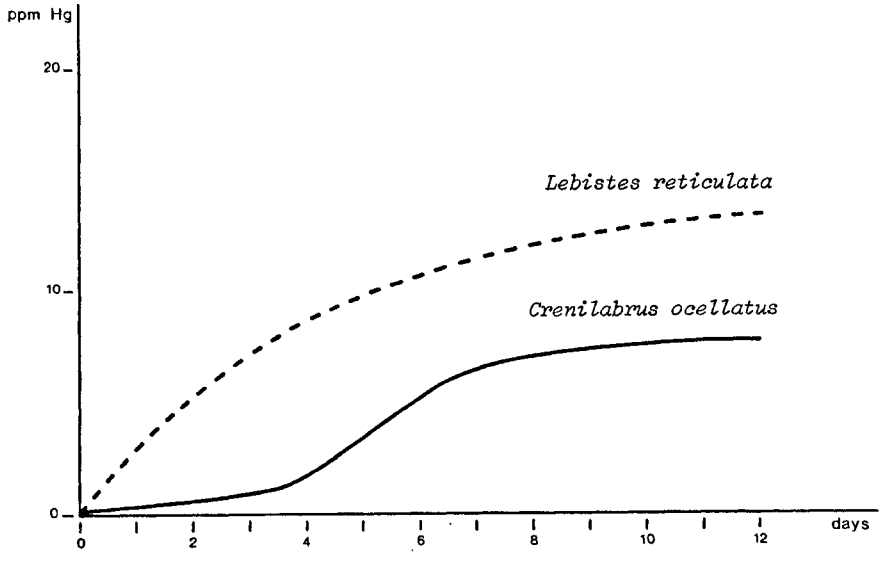

fig. 8.

Uptake kinetics of mercury by *Crenilabrus ocellatus* and by *Lebistes reticulata* living in water containing 50 ppb Hg ($HgCl_2$)

the fact that Hg loss is faster in FW than in SW but there is actually no experimental evidence to support this idea. On the other hand if one considers the results (figure 8) showing the difference between Hg accumulation in *Lebistes reticulata*, freshwater fish, and *Crenilabrus ocellatus*, seawater fish, similar differences in the accumulation curves do appear and have been explained by the appearance of metallothioneins in *Crenilabrus ocellatus*, proteins capable of retaining large amounts of Hg , reducing its toxicity. Extracts of crab organs (gills, hepatopancreas and muscles) passed on Aca 54 column show that in fresh water Hg is attached to proteins of molecular weight greater than 50 000 . The same observation is made for hepatopancreas and muscles of crabs adapted to sea water. However in the gills of the animals kept in sea water, 54 % of the Hg is bound to low molecular weight proteins, in the range of metallothioneins. It thus appears that, in sea water, mercury binds to low molecular weight proteins in the gills, which favours non toxic accumulation in these organs.

3.4.2.- Effect of Hg on osmoregulation in *Eriocheir sinensis* adapted to fresh water

Water content, Cl^- , Na^+ and K^+ concentrations have been followed in crabs living in fresh water containing 10 ppm Hg , that is in highly toxic conditions inducing death within 24 hours.

The results compared to observations on non-intoxicated animals can be summarized as follows :

- an important entry of water in the gills, with subsequent dilution of the interior of the animal; the extracellular internal space increases, the ion concentrations in the plasma decrease.
- an outflow of Na^+ through the gills; this, which perhaps initiates all other events, could be the consequences of a modified passive permeability for Na^+ or/and an inhibition of active transport [see Bouquegneau (1977)] at the level of the Na - K - ATPase.
- an outflow of Cl^- through the gills and parallel to the outflow of Na^+ .
- a diminution of the K^+ concentration in the gills, K^+ being lost to the exterior.

The system seems to evolve towards an osmotic equilibrium, the ionic pumps being blocked. During this process important changes of the cellular volume are induced and elevated concentrations of NaCl in the intracellular space of the gill epithelium can be observed. All these abnormal phenomena probably lead to the death of the animal, uncapable of maintaining its hyperosmoticity in diluted media.

These observations lead to the conclusion that the salinity might have to be considered carefully when dealing for instance with animals living in estuaries and adapted to withstand osmotic stresses. It looks as if they were more vulnerable in diluted sea-water or freshwater, which fits with observations made by Jones (1973). Species migrating from or to freshwater habitats to or from the sea might also be affected by abiotic effects of this type.

4.- Cadmium accumulation by marine animals; the role of metallothioneins

The natural cadmium concentration of sea water is below 0.1 ppb . Levels two orders of magnitude higher are however reported in coastal regions (Abdullah and Royle, 1974; Chan et al., 1974).

4.1.- TOXICITY OF CADMIUM

Cadmium toxicity is relatively well documented for mammals (Friberg et al., 1974) but few data are available for the other groups.

Measurements of acute toxicity of cadmium in the case of aquatic animals indicate lethal thresholds varying from less than 0.1 ppm to more than 50 ppm of cadmium in water (Eisler, 1971 ; Ashanullah, 1976). As a general rule, marine animals are much more resistant to cadmium than freshwater species. It can also be observed that fish are often more resistant than invertebrates. The toxic effects described for marine animals in the case of long-term exposure to cadmium also vary according to the species : effects on growth and reproduction (D'Agostino and Finney, 1974 ; Stebbing, 1976 ; Reish and Carr, 1978 ; Mirkes et al., 1978), on blood composition (Larsson, 1975), on skeleton formation (Bengtsson, 1975), ...

Toxic effects of cadmium result from its high affinity for organic substances with -SH groups (Vallee and Ulmer, 1972). By binding to proteins and specially to enzymes, cadmium disturbs thus many cellular mechanisms.

4.2.- BIOACCUMULATION OF CADMIUM

We shall report here some studies on the accumulation of cadmium by various marine organisms when exposed, naturally or experimentally, to high levels of this metal. In our experiments, cadmium was directly added to sea-water. Indeed, many data seem to indicate that uptake of this metal from water would be more important than from food (Kerfoot and Jacobs, 1974, cited by George et al., 1978 ; Berg and Weiss, 1975 ; Bouquegneau et al., 1976).

Direct uptake involves all surfaces in contact with water. In fish the gills seem the most important pathway. In the intestine, absorption, in the case of Cd is further limited by the existence, as we have shown in many fish species, of corpuscules rich in $CaCO_3$ and capable of adsorbing relatively large amounts of Cd and lowering the metal content of the water passing the digestive tract.

In fish exposed for long time to relatively high cadmium concentration in water (as in the experiment presented in table 5), cadmium is predominantly accumulated in the viscera (particularly in liver and kidneys) whereas muscles concentrations are always very low (generally below 1 ppm wet weight).

It must be noticed that in this case of drastic intoxication, the highest concentration is recorded in the liver but that at lower Cd levels, for example in the case of intoxications under field conditions, the maximum Cd concentration is attained in the kidneys.

In comparison with control fishes, the values presented in table 5 are exceptionally high. Indeed, under natural conditions, cadmium concentrations in fish tissues are generally below 1 ppm, except in the kidneys and in the liver where values of some ppm can sometimes be attained. Table 5 further shows that viscera are more exposed than muscles. After 180 days exposure ti 13 ppm Cd , the liver, in spite of its little weight fraction (1.2 % of the total body weight), contains more than 50 %

Table 5

Cadmium concentrations (mean ± standard error, µg/g wet weight or ppm wet weight) in various organs of sea-water adapted eels exposed during 180 days to 13 ppm Cd in sea water. (n = 3).

Organs	Cd concentration (µg/g)	Weight of organs (g)	Cd load	
			(µg)	% of total body load
Muscles	1,0 ± 0,1	76,1	76,1	9,1
Skin	3,1 ± 0,1	10,6	32,9	4,0
Bones	< 4,0	6,6	(6,6)	0,8
Digestive tract:				
oesophagus	23,8 ± 3,6			
stomach	19,8 ± 3,3	2,1	92,5	11,1
duodenum	72,6 ± 23,4			
intestine	60,0			
Liver	387,2 ± 41,5	1,2	464,6	56,0
Bile	4,5 ± 1,4	0,1	0,4	< 0,1
Kidneys	155,6 ± 34,7	0,7	108,9	13,1
Adipose tissue	11,6 ± 1,3	0,7	8,1	1,0
Plasma	1,4	0,6	0,8	< 0,1
Blood cells	7,8	0,3	2,3	0,3
Gills	31,3 ± 0,9	0,5	15,6	1,9
Spleen	74,2 ± 17,1	0,2	14,8	1,8
Air-bladder	6,5	0,2	1,3	0,2
Heart	35,0 ± 9,5	0,1	3,5	0,4
Brain	5,5	< 0,1	< 0,5	< 0,1
Total body*	8,29 ± 0.53	100	828,9	100

* The Cd load of each organ is calculated from its Cd concentration and its weight percentage [see also Noël-Lambot and Bouquegneau (1977)].

of the Cd body load; muscles, representing 76 % of the body weight, only contain 9 % of the total Cd load.

Owing to this very low Cd accumulation capacity of fish muscles, it is very improbable that sea pollution by Cd, to the contrary with Hg pollution, might ever lead to metal levels in fish dangerous for man. Therefore a control of the Cd concentration in fish caught for human consumption does not seem to be required. However, the control of the Cd content of the viscera would be useful in the case of small species eaten

whole and in the case of fish used to make flour. As it will be shown in the next pages, this control also seems to be very necessary for many invertebrates and especially for the molluscs.

Fig.9 shows the kinetics of accumulation and release of cadmium by the whole body of sea-water adapted eels. This figure illustrates two very important characteristics of cadmium bioaccumulation, not only in fish but also in some invertebrates (*vide infra*).

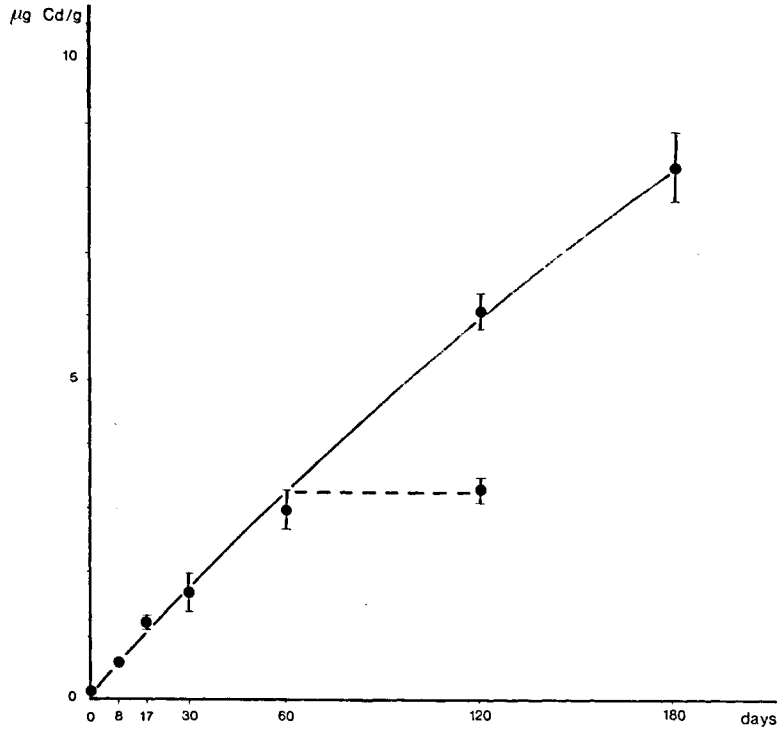

fig. 9.

Accumulation and release of cadmium (Cd concentration of the whole body) : continuous line.
Cadmium concentration of animals kept in non polluted water after an initial intoxication : broken line.
Each point represents the mean calculated for 2 or 5 specimens ± standard error.

1. Cadmium loading can occur over a very long period of time. In fig. 9, a steady state is not achieved within 180 days.
2. Cadmium is very slowly lost from the tissues when organisms previously intoxicated are returned to clean water. In many cases, binding of cadmium in the tissues may be considered as an almost irreversible phenomenon.

Identical characteristics of Cd accumulation are displayed by other marine organisms. It is the case of the limpets *Patella vulgata* and *Patella caerulea* (Noël-Lambot, 1979).

According to the evidence accumulated in our laboratory, most molluscs species seem to have an enormous capacity for Cd accumulation. When they are exposed for long time to very low levels of cadmium, they can thus accumulate very high quantities of this metal. It is specially the case of the species *Patella vulgata* (limpet) and *Purpura lapillus* (dog whelk) as shown in table 6. This table presents the Cd concentrations of several invertebrates collected from unpolluted waters and from

Table 6

Mean cadmium concentrations (in ppm wet weight) in some species caught off unpolluted or polluted waters (see in text)

	Unpolluted water	Polluted water
Molluscs		
Patella vulgata (shell length < 3 cm)	-	22.1
Patella vulgata (shell length > 3 cm)	0.9	53.9
Patella vulgata : muscle	0.6	38.6
Patella vulgata : viscera	1.4	78.3
Nucella lapillus	3.0	93.1
Littorina littorea	0.6	7.9
Littorina obtusata	-	16.7
Sepia officinalis : muscle	0.4	0.2 *
Sepia officinalis : liver	12.9	42.7 *
Hydrozoaires		
Actinia equina	< 0.5	0.5
Annelids		
Arenicola marina	-	6.0

* Specimen from Hinkley Point on Bristol Channel.

an area well known for its pollution by cadmium, the Bristol Channel (Portishead or Weston-super-Mare, England). Cadmium concentration in water of both these localities is near to 5 ppb (Preston, 1973 ; Abdullah and Royle, 1974). It can be seen that in molluscs, as in fish, Cd concentration is also higher in viscera than in muscle but muscles from molluscs nevertheless can present considerable Cd concentrations. In the case of *Patella vulgata*, a species with a high capacity for Cd bioaccumulation, it can also be observed that the old specimens show much higher Cd concentrations than the young ones. In fact, a direct relationship links Cd concentration and body size (Noël-Lambot et al., in preparation). This phenomenon is most probably a consequence of the very long exposure time and continuous accumulation of the metal by this species (see above).

From a practical point of view, this influence of the size of *Patella vulgata* on Cd concentration as also on concentrations of other metals such as Zn and Cu (for both elements we have observed an inverse relationship between concentration and body size) suggests that the use of organisms as indicators of pollution may present some problems. Indeed, differences in metal tissue concentrations between populations of a same species may reflect real differences in environmental concentrations but they also may be due to differences in body size. Moreover, it is known that environmental variables such as salinity, temperature, position of the organism in the water column, season, coexistence of several metals and presence of chelating agents also may affect the uptake of metals by organisms (Eisler and Gardner, 1973 ; Vernberg et al., 1974 ; Phillips, 1976 ; George and Coombs, 1977 ; George et al., 1978 ; this report page 12 and 14). It is essential to bear all this in mind when biological indicators are used to monitor environmental contamination by trace metals. We should also take in consideration the wide variety of responses already quoted but evident from table 6 which might mislead the experimentor so that one have to consider whether biological toxicity tests promoted by some are realistic. Anyway in the case of surveyance of marine food, molluscs should deserve special attention. Everything seems to converge to make these organisms often used as seafood have the highest Cd content compared to other animals :

1. the tissues of many molluscs have a high affinity for Cd,
2. commercially exploited molluscs live in coastal water, more exposed to Cd pollution,
3. the animals are generally eaten whole, that is viscera included, site of heavy accumulation.

As already quoted, the toxicity and bioaccumulation of the Cd^{++} ion result from its ability to form stable complexes with many cellular components, especially proteins where it attaches to -SH groups. We will study this problem in more detail in the next section.

4.3.- CADMIUM BINDING WITHIN THE CELLS : THE METALLOTHIONEINS

In several tissues from Cd-intoxicated animals, especially in those with high Cd concentrations such as liver, kidney and intestine in fish or in organs of the molluscs *Patella spp.* and *Nucella lapillus*, most of the metal accumulated is bound to metallothioneins which are soluble proteins with very particular properties (Kojima and Kägi, 1978). The presence of those proteins in the tissues can be shown by gel filtration technique. Fig.10 and 11 show the results for the soluble fraction of eel liver and of the whole of the soft tissues of limpet. On these figures, the chromatographic fraction called "MT" corresponds to metallothioneins. It can be seen that this fraction binds most of Cd present in the soluble extract and in some tissues such as liver, kidneys and digestive tract of fish, it also contains high amounts of Zn and Cu. Note that for many tissues, metallothioneins do not seem to be a normal constituent. Generally these proteins only appear in the cell after Cd administration. In some tissues such as in fish liver, they however are detected in the absence of any experimental intoxication (Noël-Lambot et al., 1978a) ; in these tissues, they probably play a role in the metabolism of Zn and Cu.

Isolation and characterization of metallothioneins can be performed as described in previous papers (Bouquegneau et al., 1975 ; Noël-Lambot et al., 1978a ; Frankenne et al., in preparation). The method includes the following steps : homogenization of the tissue followed by centrifugation, acetone fractionation of the supernatant, gel filtration on LKB Ultrogel AcA 54, ion exchange chromatography (DEAE Cellulose).

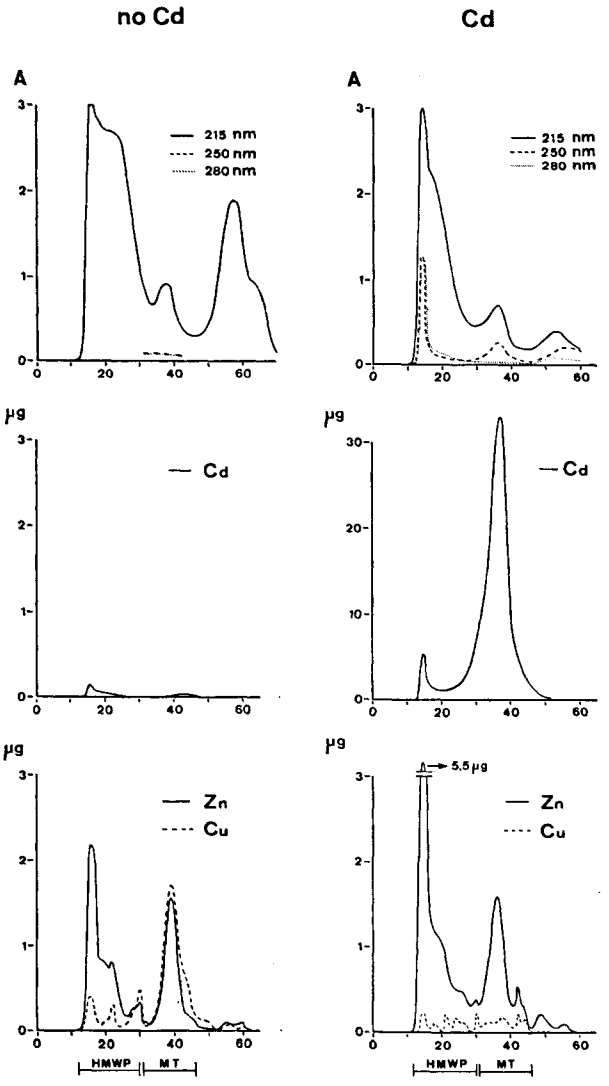

fig. 10.

Elution profiles on Sephadex G-75 column (76 × 3 cm) of liver soluble fraction obtained from non Cd-intoxicated eels or from eels intoxicated during 180 days in sea water containing 13 ppm Cd . Metals concentrations in the elution fractions are expressed in µg per fraction and per gram of organ. Fractions volume : 10 mℓ . [After Noël-Lambot et al. (1978a).]

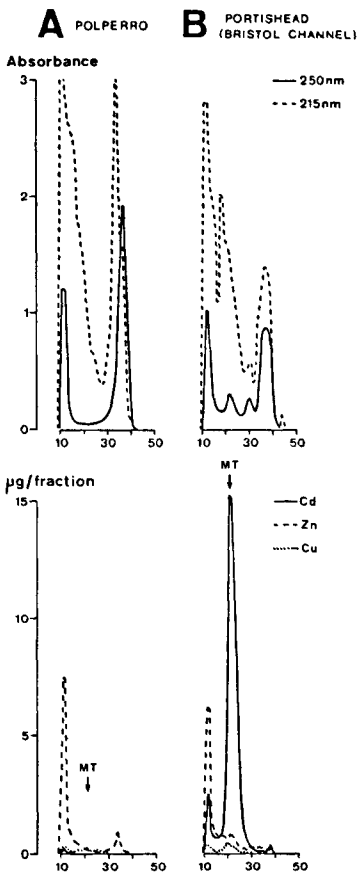

fig. 11.

Elution profiles on LKB Ultrogel AcA 54 column (2.6 × 33.5 cm) of the soluble extracts from limpets (*Patella vulgata*) collected from unpolluted water (A) or from Cd polluted water (B). Metals concentrations in the elution fractions are expressed in µg per fraction and per 1.2 g of tissue. Fraction volume : 5 mℓ .
[After Noël-Lambot et al. (1978b).]

The amino acid composition of the purified metallothioneins can be determined using the procedure of Benson and Patterson (1965). Metallothioneins also can be studied by spectroscopy and by electrophoresis on polyacrylamide gel electrophoresis.

The above methodology has been used for isolation and characterization of metallothioneins from the liver of the eel *Anguilla anguilla*, from the tissues of the mussel, *Mytilus edulis* and the limpet, *Patella vulgata*.

The metallothioneins isolated from all these different sources are not identical but they all are characterized by a low molecular weight (close to 10 000), a very high metal content (about 10% in weight), an unusual U.V. spectrum showing a maximum absorbance at 250 nm, a very particular amino acid composition with a high content in cysteine and the absence or a low content in aromatic amino acids (tyrosine, phenylalanine, tryptophan), histidine and arginine. Moreover, in all the tissues studied, we have observed the occurrence of two or more metallothioneins with electrical charges in the same experimental conditions (Noël-Lambot et al., 1978a ; Frankenne et al., in preparation).

For what regards all these properties, metallothioneins from the various aquatic animals studied here are very similar to those of mammals.

Metallothioneins, originally isolated and characterized from equine kidney by Margoshes and Vallee in 1957, have extensively been studied in mammals (for a literature review, see Webb, 1975 ; Bouquegneau and Noël-Lambot, in press), but very few informations are available about the other zoological groups and particularly about the invertebrates (Noël-Lambot, 1976 ; Howard and Nickless, 1977 ; Brown et al., 1977 ; Noël-Lambot et al., 1978b ; Overnell, personal communication). Until now, the studies of these proteins from an ecotoxicological point of view were very scarce (Brown et al., 1977 ; Noël-Lambot et al., 1978b).

In mammals, metallothioneins are generally considered to have a function in the metabolism of the essential metals Zn and Cu and in the detoxication of Cd and Hg. The metallothioneins of many aquatic animals display similar general properties and play a considerable role in the accumulation of both Hg and Cd.

4.3.1.- Metallothioneins and the accumulation of cadmium

An example of the part played by metallothioneins in Cd accumulation is given in fig.12. This figure has been drawn from data obtained from chromatographic analysis of eel liver samples. Cd was measured in the

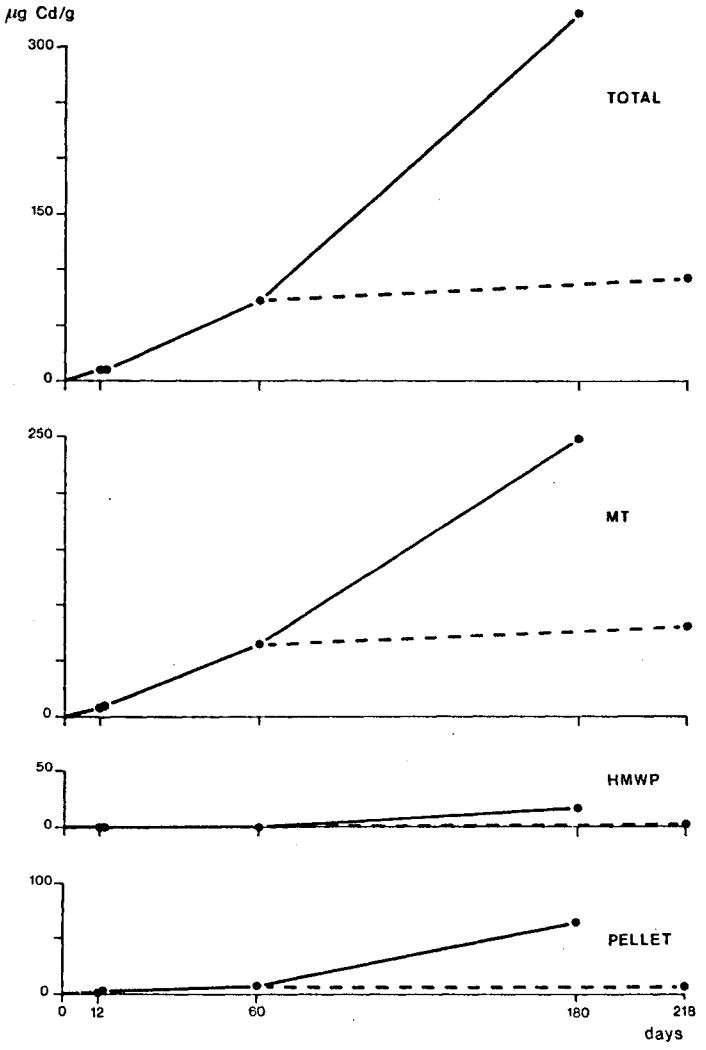

fig. 12.

Cd accumulation in the whole liver of the eel and various cellular fractions of this organ during a chronic intoxication in sea water containing 13 ppm of Cd .
MT = metallothioneins; HMWP = soluble proteins of high molecular weight.
All the concentrations are expressed in µg of Cd per g of wet tissue.
Broken line corresponds to animals transferred to unpolluted water after an initial intoxication.

centrifugation pellet and in the elution fraction obtained by chromatography of the supernatant (see above, fig.10).

As shown in fig.10, the Cd accumulated during a chronic intoxication is associated with two main fractions : the first one is quite heterogen and corresponds to the soluble proteins of high molecular weight (HMWP), the second one corresponds to metallothioneins (MT).

From fig.12 it is clear that the Cd loading of the liver occurs over a very long period of time. During the same time, Cd bound to metallothioneins (MT) raises to a considerable extent whereas Cd bound to the other soluble proteins (HMWP) and to the pellet is little affected. Therefore, the Cd-thionein complex represents the major form in which the metal is present in livers with heavy Cd load.

Owing to the chemical properties of metallothioneins, the amount of Cd bound to these proteins may be considered as a good indicator of their abundance so that the increase with time of the Cd contained in the MT fraction (fig.12) corresponds in fact to an increase in metallothioneins concentration (Noël-Lambot et al., 1978a).

It seems evident that the increase of the amount of metallothioneins with time corresponds to an equivalent increase of Cd-binding sites and thus to a more and more extensive capacity of Cd storage.

Quite similar results can be obtained with limpets naturally or experimentally exposed to Cd (Noël-Lambot et al., 1979).

The induced production of metallothioneins might thus be if not the main, one of the most important mechanisms responsible for the continuous accumulation of Cd in marine organisms, at least in certain of their organs.

We have already indicated that the total Cd concentration of many tissues does not decrease, or only very little when the animals are exposed to clean water again. Fig.12 shows that the intracellular distribution of the metal does not change either. The largest part of tissular Cd thus persists as Cd-thionein, although the cause of their formation has disappeared. The persistance of Cd-thioneins would however be linked to a continuous important turnover of the apoprotein as shown by Chen et al. (1975).

Metallothioneins can be considered as very efficient traps for Cd, screening other proteins from its toxic effects as is for Hg (Bouquegneau

et al., 1975). They do not disappear when intoxication ends but are rapidly distroyed after death (probably by bacteria or simply by proteolytic enzymes) as indicated in preliminary results. Cd is then returned to the water column either as free ionic species or complexed ones with renewed potential toxicity.

All tissues are not capable of producing metallothioneins and if they possess the capacity to do so a certain threshold of intoxication must be reached before these proteins do appear.

This threshold varies from one tissue to another, the tissues with the lowest threshold being also those storing the greatest amount of metal.

These tissues (viscera, in particular liver and kidney from fish and other vertebrates, soft parts of molluscs) should be used for surveyance work. They can be considered as good indicators of Cd pollution in natural conditions where it is difficult to estimate since very little is known about the effect of speciation of Cd on its toxicity, besides the effects of suspended matter and abiotic factors as discussed in the first part of this work for mercury.

We have mentioned that, as in mammals, metallothioneins in fish not only associate with Cd (or Hg) but also with various amounts of Cu and Zn. This problem will be discussed next.

4.3.2.- Metallothioneins and the metabolism of Zn and Cu

We have observed that under natural conditions, the Zn and Cu concentrations in the eel liver are very variable but closely related to each other. The study of the distribution of these metals in the soluble fraction of liver extracts shows that the amounts of Zn and Cu associated with metallothioneins, and also the concentration of these proteins, are directly dependent on the Zn and Cu concentrations in the whole liver. On the other hand, the metal content of the other hepatic components are little affected by these variations. These results suggest that metallothioneins have an important role in storage of excess Zn and Cu in fish. It must be quoted that this intervention of metallothioneins in the metabolism of the essential metals Zn and Cu seems to be limited to vertebrates.

Indeed, molluscs metallothioneins never bind important amounts of Zn or Cu , even in animals exposed to high amounts of both metals. In molluscs as in some other invertebrates there exists an other storage and detoxication mechanism for Zn and Cu . The metals are inclosed in vesicles within the cell. These vesicles act, as metallothioneins do in way for Cd and Hg , by preventing contact of excess metal with vital constituents (Coombs and George, 1978).

4.3.3.- Metallothioneins and the adaptation to cadmium during a long-term intoxication

Fig.13 compares the concentration and the distribution of Cd in tissues of animals intoxicated either at a lethal dose or a sublethal one (chronic intoxication). These data show that animals can die from Cd intoxication although the Cd concentrations in their tissues are far below the one observed after long periods of chronic intoxication during which the animals adapt. In the first case, the then lethal concentration is reached in a few hours. The toxic effect of Cd is thus not necessarily linked to very high tissue concentrations.

In animals adapted at low external Cd concentrations, Cd binds to metallothioneins and as already said becomes toxically inert : figure 13 shows that the Cd concentration in other cellular compartments remain relatively low. This examplifies the screening effect of metallothioneins. The drawback is that this protective mechanism is also the cause of an important bioaccumulation. The heavily loaded animals are a potential danger for others. Fortunately in aquatic food chains it seems that transfer through predators remains small. Nevertheless by continuous absorption of contaminated food, chronic intoxication only displaces Cd from one metallothionein to another and the metal will be returned to the environment at the death of either prey and predator.

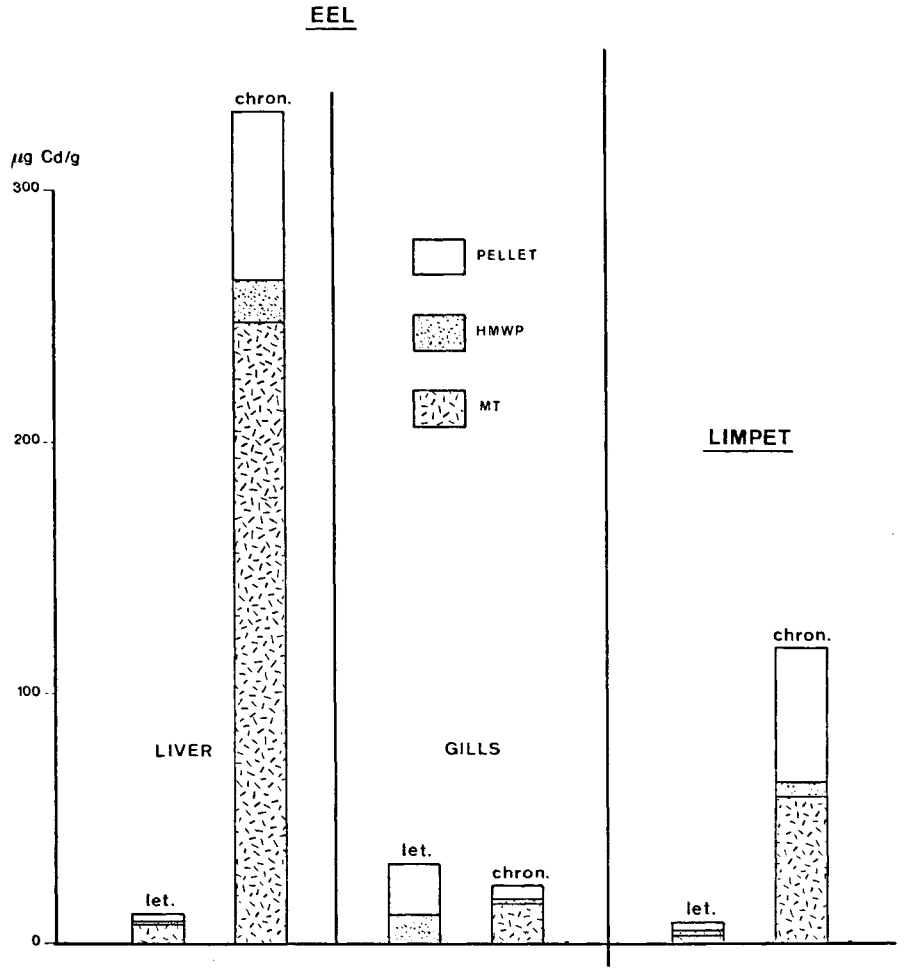

fig. 13.

Intracellular distribution of the cadmium accumulated during a lethal (let.) or chronic (chron.) intoxication. Cd concentrations (in µg Cd per g tissue) in the pellet, the soluble proteins of high molecular weight (HMWP) and the metallothioneins (MT).
Experimental conditions :
eel liver : 5 hours in 200 ppm Cd (n = 3) or 180 days in 13 ppm Cd (n = 2)
eel gills : 5 hours in 200 ppm Cd (n = 1) or 68 days in 13 ppm Cd (n = 2)
Patella vulgata : 48 hours in 2 ppm Cd (n = 3) or *in situ* intoxication (Portishead) n = 1 .
Note that the animals exposed to short time lethal Cd concentrations were still living when collected.

Conclusion

Going back to the introduction to this paper, one might ask in how far some of the questions have been answered. Regarding Hg^{++}, Cd^{++} and also Cu^{++} and Zn^{++}, transfer through the food chain is much less efficient than direct uptake from sea water. Only a few percentage of the metal ingested in food is assimilated except for CH_3HgCl because of its high solubility in lipids.

A relationship with carbon and nitrogen fluxes is only apparent because direct intoxication is repeated at each step of the food chain and increases with the age of animal at constant exposure. Practically, as much metal ingested with food is excreted. The direct route of entry shows kinetic differences between species and is modulated by abiotic effects (salinity, partition of metal between suspended matter and living matter, chemical speciation, etc). The significance of biological half-times calculated from uptake curves versus time is to be questioned because these other factors. The fact that some marine organisms if not all possess the capacity to fix heavy metals like Hg^{++} or Cd^{++} on metallothioneins which are continuously produced in chronically intoxicated animals leads to large accumulations with little or unknown effects on physiology but long retention times. It thus finally looks as if the heavy metal concentrations in the sea were under biological control as it is for many other systems (CO_2 and related substances, SiO_2, aluminium, etc.). If living matter or detritus resulting from living matter is the important sink for heavy metals where their trapped life span or residence time depends on the age of the animal and the time it takes to return the metals to soluble forms eventually toxic, then this will either affect the marine foodweb because of different resistance levels for different species, or it will not. On the other hand if we consume seafood we will in some extent eat what we tried to throw away. It is nice to know that man in some countries eats between 50 and 150 µg Cd per day (Karhausen, 1973; Friberg et al., 1974) from which 1.6 to 3 µg are really assimilated. This corresponds to eating one gram of limpets intoxicated in heavily Cd contamined (5 ppb in water) coastal zones. But who would eat limpets? If heavy metals like Cd or Hg distribute between sea living matter and detritus containing also land matter, the knowledge of the distribution of the metals between these compartments including species soluble in

water is of importance ; could one show for instance that detritus compared to living cells bind more metals, or that organic complexes are less or more toxic than ions ? Any evaluation to predict the fate of heavy metals in the marine environment either in a steady state or not would have to answer that question as well as how fast coastal waters diffuse to open oceans, deep currents and how fast chemicals are returned to the surface. This looks more important to the authors than to try and find model reduced food chains or single animals or plants to be used as unfallible tests for water quality. They believe it urgent to design more and more realistic laboratory experiments and to look at the ecosystem in a global way including its physics, its chemistry and biology, however crude the approach and to define the general rules of the game between it and the increasing amount of heavy metals dumped in the sea. We have little or no time to go in the details of species responses and far more detailed biochemical or physiological events. It might for instance be better to realize that heavy mortality in phytoplankton because of release of large amounts of nitrates and phosphates from land, because of agriculture, is in favour of a less toxic environment for fish and other animals regarding heavy metal potential toxicity and to ponder what would happen if this release was stopped and not the heavy metals release linked to industrial waste.

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