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HOW WATER OXYGENATION LEVEL INFLUENCES CADMIUM ACCUMULATION PATTERN IN THE ASIATIC CLAM *CORBICULA FLUMINEA*: A LABORATORY AND FIELD STUDY

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Abstract—The level of O_2 in water is highly variable in the aquatic environment and is a major ventilatory drive in all animals breathing water. Low O_2 partial pressure (P_{O_2}) strongly stimulates ventilatory activity compared to air-equilibrated or O_2-enriched water. We studied the influence of ventilatory activity on the bioaccumulation rate of Cd in the freshwater Asiatic clam *Corbicula fluminea* for P_{O_2} ranging from 4 to 40 kPa (2–20 mg/L at 15°C) during steady-state exposure to controlled concentrations of Cd of approximately 2 or 0.5 μg/L under both laboratory and field conditions. The concentration of Cd in the expired water and its apparent extraction coefficient (Ew_{Cd}) from the ventilated water were calculated. Results show that a low P_{O_2} strongly enhanced Cd bioaccumulation rate in the whole soft body and modified the distribution pattern and the relative burden in the organs. Whatever the water P_{O_2}, values for the concentration of Cd in the expired water remained close to the Cd concentration in the inspired water and Ew_{Cd} varied from 2 to 12%. Because the field results conformed to the laboratory analysis, the suggestion is made that the influence of O_2 on bioaccumulation patterns of metals in water-breathers should be classified as of primary importance.

Keywords—Oxygen Cadmium Ventilation *Corbicula fluminea*

INTRODUCTION

In aquatic ecotoxicology, the use of water-breathers, such as molluscs, as bioindicators is becoming more and more common because they are able to accumulate contaminants and facilitate their detection, even when minute amounts of trace contaminant are present. The two main routes as sources for metal uptake are food via the ingestion of contaminated material and water renewal at the gill level. Regarding the latter, the rationale is largely based on the fact that animals continuously breathe relatively large volumes of water, which expose their gills and the blood flowing in them to the contaminant.

In the aquatic environment water oxygenation is well known to be largely variable. It can follow seasonal and daily rhythms, varying from site to site according to the eutrophication status and, even when the bulk water is relatively saturated, the O_2 level can change throughout the water column with low values at the sediment level where burrowing species live [1–6]. Surprisingly, although numerous studies of respiratory physiology have demonstrated the major role of O_2 as a ventilatory drive in water-breathers [7,8], few works have taken into account this factor in studying the metal contamination process [9]. This was especially surprising because ventilation in fish, crustaceans, and molluscs is now well known to be stimulated strongly in low-oxygenated water (hypoxia) and decreased in hyperoxygenated water (hyperoxia) to maintain a constant O_2 supply for the organism [10–15].

Here we analyze the influence of these O_2-induced respiratory adaptations on the accumulation pattern of Cd, in a combined laboratory and field study with the Asiatic clam *Corbicula fluminea.* This freshwater bivalve is a burrowing species living in the upper layers of lake and stream sediments [16]. The study was performed at constant and low concentrations of dissolved Cd ([Cd]w = 2 and 0.5 μg/L), with various partial pressures of O_2 (P_{O_2}) ranging from 4 to 40 kPa (corresponding to O_2 concentrations of 2–20 mg/L at 15°C). In this inspired water P_{O_2} range, the ability of *Corbicula* to maintain a constant O_2 consumption is essentially based on ventilatory adjustments and not on the rate of blood flow [15]. Analysis of our data demonstrated that a decrease in water oxygenation stimulates ventilatory activity over a long period of time; greatly increases Cd accumulation in the whole soft body; and strongly modifies the Cd burdens in gills and visceral mass but not in the mantle, the foot, and the adductor muscle. Because some of these effects are of the same order of magnitude as the well-described influence of temperature, we consequently suggest that in any study based on the accumulation of Cd (and possibly other metals) in water-breathers, one must take into account the O_2-induced changes in ventilatory activity when comparisons must be made between sites. This is probably especially important for benthic organisms that inhabit the interface between water and sediment where a low-oxygenated microenvironment can exist.

MATERIALS AND METHODS

Animals and ambient conditions

Experiments were performed on 110 freshwater Asiatic clams *Corbicula fluminea* weighing 1.85 ± 0.21 g fresh weight (mean ± 1 standard error, n = 110). Note that all data are expressed on the basis of the wet flesh, fresh weight (excluding shell), which can be converted to a 70°C weight-specific dry-flesh basis by multiplying by 6.66. The clams were collected locally, from a noncontaminated site (Cazaux-Sanguinet Lake, France) and kept in large tanks supplied with aerated tap water (Table 1) at 15°C before any experiment. The bottom of the

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Table 1. Influence of water oxygenation level on the ventilatory flow rate (ventilated water flow rate) and on the corresponding quantity of Cd passing over the gills (ventilated Cd flow rate) in the freshwater clam *Corbicula fluminea* exposed to a constant concentration of Cd = 2.0 ± 0.3 μg/L during various periods of time. Temperature was 15 ± 0.5°C. Values are means ± SE. The ventilatory flow rate values are from Tran et al. [15]

Water P_{O_2}[a] (kPa)	Ventilated water flow rate (ml/h/g fresh wt)	Ventilated Cd flow rate (μg/h/g fresh wt)				
		1 h	2 d	6 d	9 d	15 d
40	6.5 ± 1.5	0.013 ± 0.003	0.6 ± 0.1	1.9 ± 0.4	2.8 ± 0.6	4.7 ± 1.1
21	16 ± 4	0.032 ± 0.008	1.5 ± 0.3	4.6 ± 1.2	6.9 ± 1.7	11.5 ± 2.9
4	65 ± 10	0.130 ± 0.020	6.2 ± 0.9	18.7 ± 2.8	28.1 ± 4.3	46.8 ± 7.1

[a] P_{O_2} = partial pressure of oxygen.

tanks was covered with a 1:1 mixture of quartz sand (grain size: 0.8–1.4 mm) and natural sediment from the Garonne River, France.

For reference 1 kPa = 7.5 Torr or mm Hg and in water, P_{O_2} = 1 kPa corresponds to an O_2 fraction of ≈1% and an O_2 concentration of ≈0.5 mg/L at 15°C (in water equilibrated with air, the O_2 fraction is 21% and P_{O_2} ≈ 21 kPa).

Laboratory experiments

Before any experiments, animals were acclimated in the laboratory for at least three weeks to adapt to indoor conditions and stabilize their resting respiratory activity. The bottom of the experimental tanks was covered with quartz sand and water conditions were: temperature = 15.0 ± 0.5°C; PO_2 = 20 to 21 kPa; pH ≈ 7.80 ± 0.10, controlled by a pH-CO_2 stat (Consort R 301); water alkalinity by titration= 1.85 ± 0.10 mmol/L; [Cd]w ≈ 2 ± 0.3 (n = 9) or 0.5 ± 0.1 μg/L (n = 6; 18.6 ± 2.8 or 4.6 ± 0.9 nmol/L). All experiments were performed from January to July under natural light conditions. During experiments, tanks were isolated from laboratory vibrations with antivibrating benches to minimize external disturbance. The animals were fed twice a week with an algae suspension of *Scenedesmus subspicatus.*

Ventilatory flow rate measurement

Seven *C. fluminea* were used in an experiment performed from January to March. Its aim was to study the constancy of the ventilatory flow rate at a given water P_{O_2} and its ability to return to a reference value after a change in the P_{O_2}. This experiment was performed during a 26-d period. Each animal was exposed successively to three selected and maintained levels of water P_{O_2} (40, 20, and 4 kPa). The N_2–O_2–CO_2 gas mixture was obtained via a laboratory constructed mixing unit (rotameters and valves from Brooks Company, Hatfield, PA, USA) and water P_{O_2} values were measured with a Radiometer (Copenhagen, Denmark) polarographic electrode thermostated at 15°C. The succession of inspired P_{O_2} was 20, 4, 20, 40, and 20 kPa. The time required to reach a plateau value of water P_{O_2} was 45 to 60 min. The ventilatory flow rate measurement, based on the determination of a volume of water cleared of algae per unit of time in a transiently closed system [17,18], was performed on seven animals separately held in individual chambers (volume 100 ml) half-filled with quartz sand. The duration of each exposure ranged from 3 to 8 d (Fig. 1) and the measurements were systematically performed from 11:00 AM to 5:00 PM. Importantly, the present protocol was characterized by the maintenance of the clams in open-flow systems held at constant water P_{O_2} and algae concentration (4–6 ± 0.5 × 10^5 algae/ml), and their confinement during 1-h measurement periods. During maintenance, each chamber was constantly supplied with preoxygenated water at constant flow (400 ml/h) via gravity and an algae solution of *S. subspicatus* via a water renewal pump (Gilson, Villiers-Le-Bel, France). The gas mixtures bubbled both through the reservoir feeding the chambers and, at low rate, in each individual chamber where the gas mixtures improved water homogenization and gas equilibration, especially during the confinement periods. During these confinement periods the water flow through the chamber was stopped and, at constant water P_{O_2}, the mean decrease in algae concentration was 1 to 3 × 10^5 algae/ml, depending on the algae concentration, the water P_{O_2}, and the ventilatory activity of each individual. The density of algae was measured by optic density at 750 nm with a spectrophotometer (UV-1601 Shimadzu, Columbia, MD, USA) regularly calibrated by counting cell density with a Malassez (Poly Labs, Strasbourg, France) cell using light microscopy. The Jorgensen equation [17] was used to calculate the ventilatory flow rate of the clams ($\dot{V}w$)

$$\dot{V}w = V\frac{(ln(do) - ln(df))}{(ut \cdot M)} - V\frac{(ln(do') - ln(df'))}{(t \cdot M)} \quad (1)$$

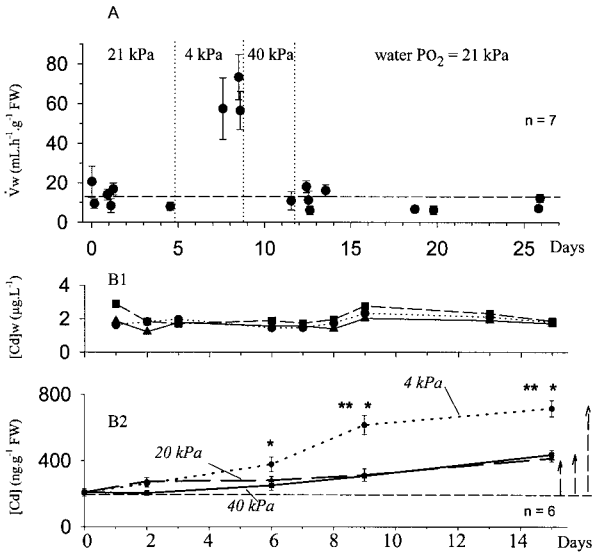

Fig. 1. (**A**) Changes of ventilatory flow rate ($\dot{V}w$) in *Corbicula fluminea* during long-term exposures to selected steady levels of water partial pressures of O_2 (Po_2) as a function of time. (**B1**) Changes in concentration of dissolved Cd ([Cd]w) expressed as average between nominal and actual value (measured at the end of 24-h cycles) as a function of time in the three experimental conditions, water Po_2 = 40, 20, and 4 kPa. (**B2**) [Cd] in whole soft bodies. Asterisks indicate significant differences from water Po_2 = 40* and 20 kPa**, respectively.

where $\dot{V}w$ is the ventilatory flow rate (ml/h/g fresh weight); V is the volume of water in each chamber (ml); $ln(do)$ and $ln(df)$ are the density of algae at the beginning and the end of the measurement period (algae/ml); $ln(do')$ and $ln(df')$ are the control density of algae at the beginning and the end of the measurement period in the control chamber without clam (algae/ml); t is duration of each exposure period, which was consistently ≈60 min; and M is body mass, fresh weight (g). Importantly, note that four assumptions are associated with the use of Equation 1 and subsequent Equations 2, 3, and 4: the water concentration of algae is homogeneous as experimentally measured in the control experiment; the decrease in algae concentration is only due to animal filtration as confirmed by the absence of change within an empty control chamber; all the algae passing over the gills are retained; and the $\dot{V}$ is constant during the entire measurement period (no transient or systematic periods of valve closure activity was observed in the present experimental conditions as shown by visual inspection, recording of valve activity, or both [D. Tran et al. in preparation]).

Cadmium contamination

All exposures were performed at controlled constant Cd concentration in the water. Two experimental series were performed with a total of 48 animals. The actual concentration of [Cd]w was measured daily and any deviation from the nominal value was systematically compensated. The decrease of [Cd]w was ≈30% per day. The compensation technique consisted of adding twice the difference between the actual and nominal concentrations at the end of each daily cycle. Metal addition was made with $CdCl_2$ (Merck, Darmstadt, Germany) using a stock aqueous solution containing 10 mg Cd/L. At the water pH we used, thermodynamic speciation calculation indicated that 76% of the metal is as the free ion species Cd^{2+} (MINEQL$^+$ [19]). In series 1 (15 d) the concentration was maintained at 2 ± 0.3 μg/L (range 1.22–2.78 μg/L, n = 9) and in series 2 (6 d) at 0.5 ± 0.1 μg/L (0.3–0.8 μg/L, n = 6).

Analysis of Cd accumulation in various tissues

The Cd levels in water, whole soft body, and organs (gills, visceral mass, mantle, foot and adductor muscle) were measured by atomic absorption spectrophotometry (AA 40, Varian, Mulgrave, Australia; Zeeman effect) equipped with a graphite tube atomizer (model GTA 96, Varian). The detection limit was 0.1 μg/L of Cd. The calibration was performed using a standard biological reference material (TORT-2, lobster hepatopancreas tissue, from the National Research Council, Ottawa, ON, Canada). The biological samples were digested by nitric acid (3 ml of HNO_3, 65%, Merck) in a pressurized chamber (borosilicate glass tube) at 95°C during 3 h. Then, the samples were diluted up to 20 ml with ultrapure water (MilliQ, Bedford, MA, USA). For each Cd determination a sample of 10 μl was taken and mixed before atomization with 4 μl of a mixture of paladium:$Mg(NO_3)_2$ (50:50) to eliminate the matrix effect. The water samples were directly measured after acidification with HNO_3. More details can be found in Baudrimont et al. [20,21].

Apparent extraction coefficient of Cd from the ventilated water and concentration of Cd in the expired water

This experiment was performed on 24 clams. Based on the $\dot{V}w$ determined in the former experiment and the corresponding [Cd]w, we estimated the total amount of Cd that passed on the gills during the 15-d exposure period. Then, the comparison with the total amount of Cd accumulated in the soft body during the same period gave an apparent extraction coefficient (Ew_{Cd}), calculated for each water P_{O_2} as

$$Ew_{Cd} = (\text{total amount of ventilated Cd} - \text{total amount of accumulated Cd}) \div (\text{total amount of ventilated Cd}) \quad (2)$$

The Fick principle was then used to calculate the concentrations of Cd in the expired water

$$\dot{M}_{Cd} = \dot{V}w(\text{inspired [Cd]w} - \text{expired [Cd]w}) \quad (3)$$

where $\dot{M}_{Cd}$ is the Cd accumulation during a 15-d period (μg/g fresh weight); $\dot{V}w$ is the ventilatory flow rate during the same period of time (L); and inspired [Cd]w and expired [Cd]w are the concentrations of Cd in the inspired and expired water (μg/L).

Visualization of the water flow pattern in the palleal cavity

These experiments were performed on 20 animals by placing each individual in a 250-ml beaker filled with a solution of neutral red (0.1–1 mg/L) either equilibrated with a gas mixture at P_{O_2} = 4 or 20–21 kPa during 1- to 3-d exposure periods. The use of neutral red as colorant was based on a preliminary study [22] that reported an absence of ventilatory reactions with this specific dye. At the end of exposure, the animals were immediately killed in liquid nitrogen to instantaneously interrupt ventilatory activity and avoid any diffusion of colorant in the palleal cavity. The distribution of colorant was then studied by visual inspection using a binocular microscope (Nikon, Melville, NY, USA). Pictures were taken of unfrozen material.

Field experiment (caging)

To test if the laboratory experiments (performed in well-controlled but simplified conditions) were representative of the in situ conditions, a field experiment of Cd accumulation was also performed at a location and at a water temperature that allowed a direct comparison between both approaches. A site on the Lot River (southwestern France, see Bouillac I in Andres et al. [23]) was selected because it is contaminated with a concentration of dissolved [Cd]w of approximately 1 to 2 μg/L [23]; experiments were performed when the temperature (T) was ≈15°C (autumn 1999) and in a location where the water was well equilibrated with air (P_{O_2} = 20–21 kPa and pH = 7.6–7.8) and the current velocity was minimal. Two groups of 12 clams were placed in two weighted bow-nets (35 × 25 × 15 cm, mesh size 1 cm) that were partly buried in a soft sediment to allow a natural positioning of the animals.

Statistics

Results are given as mean values ± 1 standard error. Statistical significance of the differences were evaluated using one-way analysis of variance (ANOVA), Mann–Whitney test, Wilcoxon test, Fisher least significant difference (LSD) methods, or a combination of these. A value of $p < 0.05$ was taken as the reference limit of significance.

RESULTS

The reversible and long-lasting influence of the water oxygenation level on the $\dot{V}w$ of *C. fluminea* is illustrated in Figure

1A. Clams were placed in the experimental set up at t_0. Starting from a reference water P_{O_2} of 21 kPa (normoxia, O_2 concentration 10 mg/L) where the $\dot{V}w$ was in the range 10 to 20 ml/h/g fresh weight, the water P_{O_2} value was varied from 4 to 40 kPa with transient recovery periods in normoxic water. Note first that during each of these recoveries the change of ventilatory activity either induced by the exposure to hypoxia (P_{O_2} = 4 kPa, 2 mg/L) or hyperoxia (P_{O_2} = 40 kPa, 20 mg/L) was perfectly reversible during the 26-d exposure period. Second, in hypoxia, the ventilation increased up to 60 to 70 ml/hg fresh weight (significantly different from the reference value, $p < 0.05$) and then it decreased slightly, although not significantly, in hyperoxia down to less than 10 ml/h/g fresh weight (one-way ANOVA, Mann–Whitney test, Fisher LSD methods). Thus, in hypoxia ventilation was about 10 times larger that in hyperoxia.

The consequences of these O_2-induced ventilatory changes on the flux of metal flowing through the branchial cavity of *Corbicula* exposed to a fixed concentration of dissolved Cd in the water ([Cd]w = 2 μg/L or 18.6 nmol/L) are summarized in Table 1. Taking as an example the amount of Cd that passed over the gills after 15 d, the results clearly show that during hyperoxia the quantity of Cd was 4.7 ± 1.1 μg/g fresh weight (n = 6) and during hypoxia it rose to 46.8 ± 7.1 μg/g fresh weight (n = 6) at water P_{O_2} = 4 kPa (significantly different, $p < 0.05$, one-way ANOVA, Wilcoxon test, Fisher LSD-methods). The consequences of these changes on the total amount of Cd accumulated in the whole soft body are presented in Figure 1B1 and B2. Figure 1B1 shows first the daily controlled pattern of dissolved [Cd]w in the three experimental units. No significant difference was found among units and the [Cd]w remained well controlled around 2 μg/L, independent of the water oxygenation status. Figure 1B2 presents the corresponding consequences for the bioaccumulation rate of Cd in the whole soft body of *Corbicula*. Interestingly, already by day 6 the total amount of accumulated Cd was higher under hypoxia than under normoxia or under hyperoxia ($p < 0.05$, one-way ANOVA, Mann–Whitney test, Fisher LSD methods). At day 15, the accumulation in the animals whose ventilation was continuously stimulated was twice the value in the two other conditions. This accumulation corresponds to a change in the bioconcentration factor (animal–water) from 200 under normoxia and hyperoxia to 360 under hypoxia. Under all conditions, a linear model yielded a good fit to the Cd accumulation (R^2 = 0.94, 0.93, and 0.95 at 40, 21, and 4 kPa, respectively), indicating a linear accumulation rate and an absence of saturation during the 15-d exposure period. We then measured the corresponding Cd accumulation rates, defined as the net balance between influx and outflux in the whole body. The corresponding values at 40, 21, and 4 kPa were respectively 1.21 ± 0.07, 1.15 ± 0.06, and 1.99 ± 0.13 ng/h/g fresh weight (10.8 ± 0.6, 10.2 ± 0.5, and 17.7 ± 1.2 × 10^{-3} nmol/h/g fresh weight). The above-reported data are thus the first indications of an O_2-induced ventilatory effect on these processes of accumulation. To gain more insights into the mechanisms of Cd uptake we then calculated (Eqn. 2) the fraction of Cd that was retained after 15 d by the animals based on the ratio between the quantity of metal accumulated (Fig. 1B2) and the quantity of Cd that flowed through the palleal cavity at each P_{O_2} condition (Table 1). Figure 2A shows that this fraction was smaller when the ventilation was enhanced by hypoxia. Indeed, the apparent extraction coefficient varied from 2% at P_{O_2} = 4 kPa to 12% at P_{O_2} = 40 kPa, which

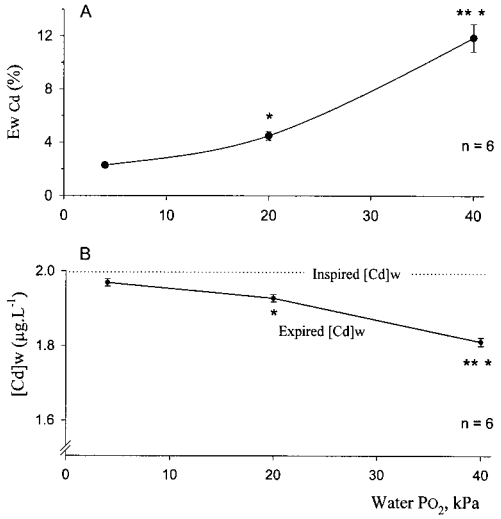

Fig. 2. (**A**) Relationship between apparent extraction coefficient of Cd from ventilated water (Ew_{Cd}) and water partial pressure of O_2 (Po_2). (**B**) Change in Cd concentration ([Cd]) in the expired water as a function of the inspired water Po_2. Asterisks indicate significant differences from water P_{O_2} = 4* and 20 kPa**, respectively.

means that the efficiency of the retention was lowered when the transit time within the palleal cavity was shortened. We then calculated the concentration of Cd present in the expired water at the outlet of the gills, that is, in the exhalent channel (Eqn. 3). Interestingly, Figure 2B confirms that under hyperoxia, when ventilation was minimum, the concentration of Cd in the expired water was lower (1.81 ± 0.01 μg/L, n = 6) than under normoxia (1.93 ± 0.01μg/L, n = 6) and under hypoxia (1.97 ± 0.01 μg/L, n = 6, all values statistically different among themselves, $p < 0.05$, one-way ANOVA, Mann–Whitney test, Fisher LSD methods). Importantly, also note that, whatever the ventilatory activity, the concentration of Cd in the expired water remained quite high, for example, under hyperoxia, it only decreased from 2 μg/L in the inspired water down to 1.8 μg/L. Analysis of the above set of data consequently demonstrated a net influence of the water P_{O_2} on the global uptake of Cd. We next addressed the issue regarding a possible O_2-induced action on the Cd distribution in the organism and the various amounts accumulated in the organs.

The results of this complementary analysis are presented in Figure 3A, B1, and B2. It is clear that experimental manipulation of the water oxygenation influenced Cd concentration and body burden in a nonuniform manner. Two behaviors were observed. First, in the gills and visceral mass the hypoxia-induced hyperventilation significantly stimulated accumulation rates but, obviously, no significant interaction occurred in the mantle, the foot, and the adductor muscle ($p < 0.05$, one-way ANOVA, Mann–Whitney test, Fisher LSD methods). Second, in the gills and the visceral mass, respectively, the accumulation rates at day 15 were multiplied by a factor ≈ 3 and 2 in comparison to the normoxic or hyperoxic references ($p < 0.05$). In the gills this corresponds to a change in bioconcentration factors (organ–water) from 650 under hyperoxia to 800 under normoxia and 2,000 under hypoxia. Finally, in addition to these effects on the metal distribution based on the concentration criterion, P_{O_2} also influenced the relative burdens in the organs. The changes that occurred among the different soft body compartments after 15 d are presented in

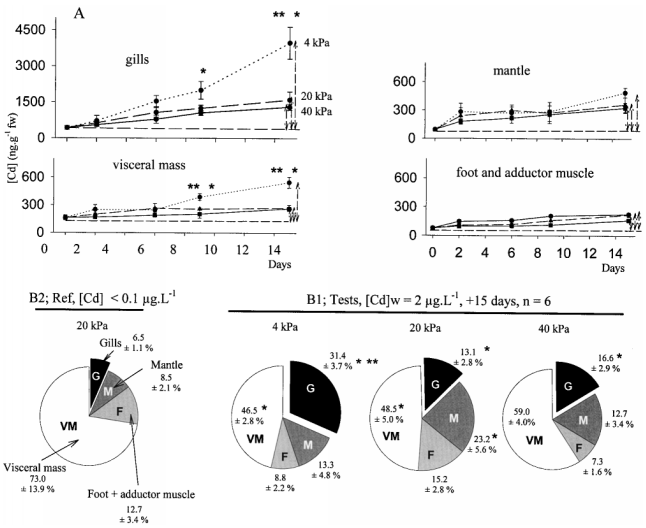

Fig. 3. (**A**) Change in Cd concentration ([Cd]) as a function of time and water partial pressure of O_2 (Po_2) in various organs of *Corbicula fluminea* exposed to concentration of dissolved Cd ([Cd]w) = 2 ± 0.3 µg/L during a 15-d exposure period. Asterisks indicate significant differences from water Po_2 = 40* and 20 kPa**, respectively. (**B1** and **B2**) Relative Cd burdens as a function of (**B1**) water Po_2 = 20 kPa, and water [Cd] < 0.1 µg/L; and (**B2**) water Po_2 = 4, 20, and 40 kPa and [Cd]w = 2 ± 0.3 µg/L. Asterisks indicate significant differences from reference* and from water Po_2 = 20 and 40 kPa**.

Figure 3B. These changes were especially impressive under hypoxia, where the percentage of Cd accumulated in the gills was significantly larger than under normoxia and hyperoxia ($p < 0.05$, one-way ANOVA, Mann–Whitney test, Fisher LSD methods). This Cd accumulated in the gills represents 30% of the total amount fixed in the whole body, which was twice the value at Po_2 = 21 and 40 kPa. Again, the differences between the normoxic status and hyperoxic status were minor or null (no statistical differences, $p < 0.05$, one-way ANOVA, Mann–Whitney test, Fisher LSD methods).

In addition to the already well-described central role played by the gills in regard to metal uptake and accumulation, a surprising finding was the absence of any significant effect on the mantle (Fig. 3). Specifically, the changes of $\dot{V}w$ in the palleal cavity, induced by the ventilatory activity of the gill cilia, seemingly did not reach the mantle in our experimental conditions. This was contrary to previous studies [24,25] because the mantle is often reported to participate in gas exchange and the existence of a very important physiologic dead space in the palleal cavity has been suggested. To determine if the mantle was indeed not significantly ventilated, we exposed the clams (n = 20) for periods ranging from 1 to 3 d to low fixed concentrations of neutral red dissolved in the water column. The experiments were performed at two water Po_2s, 20 and 4 kPa (Fig. 4A and B) and repeated nine times to empirically adjust the colorant dilution to assure that an excess could induce a saturation of the gill tissue coloration in normoxia and vice versa. Importantly, in all runs the striking observation was that coloration of the mantle was never visible, as shown in Figure 4A and B. Only when the concentration of colorant was too high did the edge of the mantle, directly at the contact of the bulk water at the shell opening, become colored, as did the internal side of the inhalant siphon. Figure 4C demonstrates that when a drop of colorant was directly added onto the mantle tissue of an opened clam and rinsed with clear water, the staining was instantaneous.

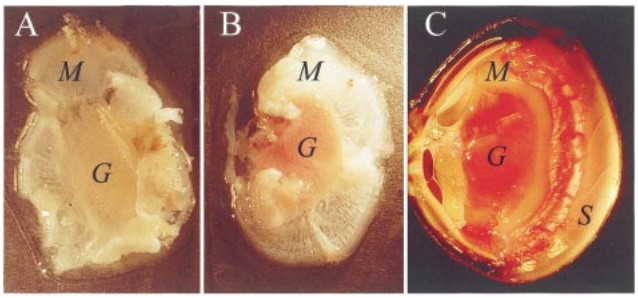

Fig. 4. Visualization of the ventilatory flow pathway in *Corbicula fluminea.* Animals were bathed in a solution of neutral red at water partial pressure of O_2 (Po_2) = 20 (**A**) or 4 kPa (**B**) during a 3-d exposure period. (**C**) Control experiment showing the effect of a direct addition of dye. G = gills; M = mantle; S = shell.

The above results, performed at constant Cd concentration in the water, demonstrated the fundamental role played by ventilatory adjustments in the modulation of the metal accumulation process. To further assess whether these O_2-induced changes can to amplify accumulation at lower Cd concentrations, we examined the effect of an exposure to [Cd]w = 0.5 ± 0.1 µg/L at water Po_2 = 4 kPa in comparison to an exposure to [Cd]w = 2 ± 0.1 µg/L at 20 kPa during 6 d. Interestingly, Figure 5A shows that under both conditions the accumulation was statistically similar ($p < 0.05$, one-way ANOVA, Mann–Whitney test, Fisher LSD methods). Thus, living at low Po_2 partial pressure can, even in a system with low contamination levels, induce a measurable and significant increase in metal accumulation.

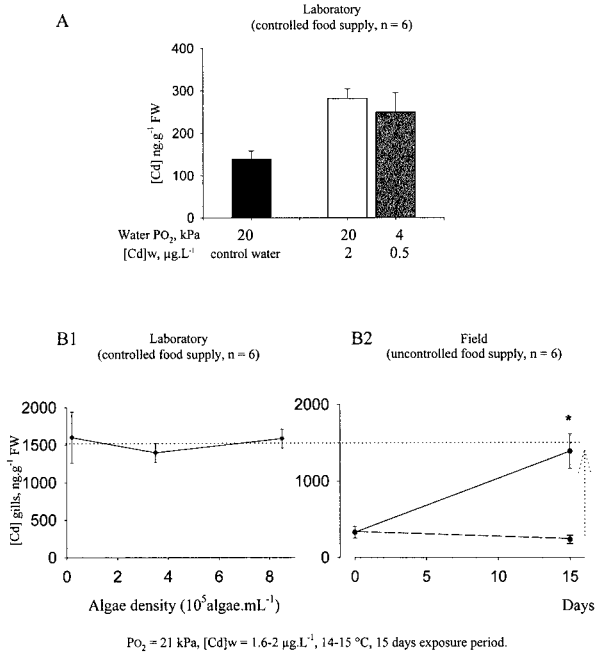

Fig. 5. Importance of the effect of O_2. (**A**) Comparative effect between concentration of dissolved Cd ([Cd]w) = 0.5 ± 0.1 µg/L at water partial pressure of O_2 (Po_2) = 4 kPa and [Cd]w = 2 ± 0.3 µg/L at 20 kPa during a 6-d exposure period on whole soft body accumulation pattern. The effect of O_2 counterbalanced the [Cd] difference. (**B1** and **B2**) Field versus laboratory study of Cd accumulation in the gills of *Corbicula fluminea.* (**B1**) Control laboratory experiment. (**B2**) Field experiment. The patterns of Cd accumulation were not different between field and laboratory conditions and were independent of the concentration of plankton in the studied range. An asterisk indicates significantly different from reference value.

All these experiments were performed under well-controlled laboratory conditions where great care was taken to reduce any mechanical stimulation (produced artificially by laboratory activity) that could stimulate the animal's ventilatory activity. To test if the animal status we obtained was representative of field conditions we then turned to a 15-d in situ caging experiment performed in the Lot River at a season and in a location where the physicochemistry was comparable to that in the laboratory. At the beginning and the end of the experiment the following parameters were as follows: T = 13.6 and 13.8°C, dissolved [Cd] = 1.74 and 1.63 μg/L, water pH = 7.62 and 7.60; and water P_{O_2} = 20.9 kPa. In addition to the presence of natural mechanical stimulus, a major difference was the uncontrolled presence of food in the field and the interaction it can induce as a contaminant factor. To estimate the role feeding could play, we thus examined in the laboratory the effect of different fixed concentrations of plankton on the accumulation rate of Cd at constant dissolved [Cd]w. First of all, note that the mean concentrations of bound Cd in the algae for the 15-d exposure period were respectively 98.9 ± 15.1 μg/g at 3 to 4 × 10^5 algae/ml and 82.0 ± 14.1 μg/g at 8 to 9 × 10^5 algae/ml; and that those concentrations were reached by day 4. The results are presented in Figure 5B1. They show, at least in our experiment under simplified and well-controlled conditions, that [Cd] in the gills is independent of algae density from 0.1 to 0.2 to 8 to 9 × 10^5 algae/ml (oligotrophic to eutrophic condition). Because this strongly suggested that gill contamination was mainly dependent on the dissolved [Cd], we focused our attention on this organ for the field experiment. Figure 5B2 presents the results of the caging experiment by comparing results to a control group that was held in the laboratory at [Cd] < 0.1 μg/L. Remarkably, the in situ rate of accumulation was very close to that obtained in the laboratory, which strengthens the relevance of the present experimental results both in terms of field and ventilatory activity and absolute values of the accumulation process.

DISCUSSION

In rivers, lakes and ponds, the partial pressures and concentrations of O_2 are highly variable, especially when considering eutrophic aquatic ecosystems enriched in plant nutrient minerals and organisms. Because ventilation in water-breathers is largely influenced by the level of oxygenation, we investigated in this study how and to what extent these changes of O_2-induced ventilatory activity can influence the rate of Cd accumulation and the metal body burden in the Asiatic clam *C. fluminea*. Importantly, the present study was performed during the first 15 d of exposure to moderate concentrations of Cd ([Cd]w = 0.5 and 2 μg/L), which allowed us to study the loading characteristics of the animals. Specifically, we demonstrated in the laboratory that when the P_{O_2} in the water is 4 kPa (it is 21 kPa in a well-aerated water), the ventilation of *Corbicula* is multiplied by a factor four and the amount of Cd accumulated in the whole soft body is multiplied by a factor two. The effect of amplification is such that even in the presence of only 0.5 μg/L of metal, a very significant amount of accumulation was measurable in animals breathing in hypoxic water. Based on the quantity of Cd accumulated during a 15-d exposure period and of Cd that passed through the palleal cavity with the ventilatory flow current during the same period, we have shown that the mean apparent extraction coefficient of Cd from water ranged from 2 to 12% for water P_{O_2} varying respectively from 4 to 40 kPa. This is of the same order of magnitude as for the extraction of O_2 in the ventilated water in the same species (4% independent of the inspired P_{O_2} [15]). We have similarly shown that body burdens are also under the influence of the water P_{O_2} and the associated ventilatory changes. Indeed, the accumulations in the gills and the visceral mass were dramatically influenced by the water P_{O_2} value, but accumulations in the mantle, foot, and adductor muscle were not influenced. The above conclusions based on laboratory studies were supported by a field exposure study performed in a location that was especially chosen for its water oxygenation, temperature, and Cd concentration status that were directly comparable to the laboratory conditions.

Note finally that the present data analyses do not demonstrate whether differences would be found in steady-state accumulation values. Whether a final metal tissue accumulation state can be influenced by the mechanism we demonstrated remains to be studied. However, as they stand, our data highlight the importance of the water O_2 factor during short-term caging experiments.

Comparison with previous data

The present work demonstrated that a decrease in water oxygenation enhances the biological availability for metal in the Asiatic clam *C. fluminea* by means of an O_2-induced ventilatory stimulation. To our knowledge this specific question has rarely been addressed in the literature [9]. In aquatic insects, the toxicity of Cd was reported to increase as O_2 concentration increased [26]. Karbe et al. [2], based on a field study in the Elbe River (eastern Germany), suggested the possibility of a relationship in mussels (*Dreissena polymorpha, Unio tumidus,* and *Anodonta anatina*) between O_2 saturation deficit and mercury concentrations. Decreasing the water P_{O_2} from 21 to 4 kPa resulted in a two- to fourfold increase in the accumulation of Cd by *C. fluminea* in the present experiment (twofold in the whole body and fourfold in the gills; Figs. 1B2 and 3A). Importantly, this O_2-induced amplification is comparable to the well-recognized effect of temperature. For example, Graney et al. [27] found that a temperature increase from 9 to 21°C increased by a factor of two to three the accumulation of Cd in *Corbicula.* Similarly, a temperature change from 10 to 20°C also doubled Cd accumulation in the marine bivalves *Mya arenaria* and *Mulinua lateralis* [28]. Thus, because water P_{O_2} as low as 4 kPa can be observed in eutrophic water bodies at the sediment surface (where burrowing animals live), our results indicated that O_2, as well as temperature, may be a major controlling factor of Cd accumulation, at least in *Corbicula.* Note also that the present experiments were performed at Cd concentrations that did not impair or stimulate ventilatory activity. The Cd concentrations required for such stimulation are indeed 25 to 50 times higher that the present ones. In the zebra mussel (*D. polymorpha*) [29] and in *Mytilus edulis* [30] an increase in filtration activity was reported at [Cd]w > 100 μg/L. In *Viviparus georgianus* and *Elliptio complanata,* a ventilatory stimulation was observed at [Cd]w > 50 μg/L [31]. For comparison, Graney et al. [27] also used a Cd concentration of 50 μg/L in their study on the influence of substrate, pH, diet, and temperature upon Cd accumulation.

The role of the mantle and the gills revisited

When the water oxygenation level was experimentally manipulated, the Cd accumulation rates in the gills and the visceral mass were significantly modified but surprisingly were

not modified in the mantle (Fig. 3A and B). Moreover, the total amount of Cd accumulated in the mantle after 15 d at water P_{O_2} = 4 kPa was only ≈300 ng/g fresh weight, whereas Cd accumulation was ≈4,500 ng/g fresh weight in the gills. This supported the idea that the gills were the primary site for uptake from the direct route of exposure and that the water renewal rate in the vicinity of the mantle was considerably reduced. The existence of such an important ventilatory dead space in the palleal cavity was confirmed by the experiment, which consisted of bathing the clams in a solution of neutral red; this clearly demonstrated an absence of mantle coloration, even under hypoxia. We propose then that, at least in *Corbicula* and contrary to what is often suggested, the mantle is not a major route of Cd uptake by direct transfer from water to the organism. Then, the metal accumulation reported here in the mantle very likely was the result of a minor uptake from water and a major transfer by blood transport. Note finally that this observation sheds a new light into a very classical statement in bivalve respiratory physiology about the respective role of the gills and the mantle in regard to O_2 uptake [24,25,32]. Specifically, Schmidt-Nielsen [33] proposed that gills have ''a primary function in food uptake but that their role in gas exchange is less certain'' and ''that the bivalve mantle could be sufficient to provide the required gas exchange.'' Based on the present experimental evidence, we suggest on the contrary that the mantle routinely should play a minor role in respiratory gas exchange because it is obviously not a well-ventilated organ. Note finally we used the gills as a key organ for testing Cd accumulation rates from the water in the field because Cd accumulation in the gills seemed mainly influenced by a direct uptake from water with little to no influence by food uptake under our experimental conditions (Fig. 5B1).

Insights into the contamination process

The discussion above argues on the central role played by the gills and not the mantle in regard to Cd uptake from water. Based on calculations performed with the 15-d accumulation data in the whole body and the quantity of Cd that passed with the ventilatory flow in the palleal cavity, we determined that 4% of the ventilated Cd was fixed in the animal in air-equilibrated water. This accumulation of Cd increased up to 12%, when ventilation slowed and the transit time of water was increased under hyperoxia, and it decreased to 2% under hypoxia (4 kPa), when the water velocity was increased. To our knowledge, this is the first report quantifying the relative importance of Cd extraction from water in a mollusc and possibly in a water-breather over such a long-term period. Using the same exposure period we also measured the accumulation rates of Cd in *Corbicula* and we showed that it remained constant from day 1 to day 15 (Fig. 3A). Note that these calculations correspond to the net result of a balance between uptake and elimination and that they must be clearly distinguished from studies of metal uptake based on short time exposure where the effluxes are quantified. However, it is worthwhile noticing that the accumulation rate we determined under normoxia was 1.21 ± 0.07 ng/h/g fresh weight (T = 15°C), which is not fundamentally different from the ≈ 1.1 to 2.2 ng/h/g fresh weight measured in juvenile carp (*Cyprinus carpio*) at T = 25°C and pH = 7.6 to 8.0 [34]. However, any additional comparison is purely speculative because of the differences in species, method, and temperature but the reasons for such an observation should be clarified. More specifically, it should be quite interesting to quantify the efflux rates for various species under well-controlled O_2 conditions because the above comparison suggests that during the loading period studied here the efflux could be small in *Corbicula.*

Finally, we also calculated the concentration of Cd in the expired water of *Corbicula.* This calculation shows that after having flowed through the gills, the Cd concentration remained nearly constant (in the range 1.81–1.97 μg/L) and quite close to the nominal concentration in the inspired water (2 μg/L), independent of the water oxygenation and the $\dot{V}w$. Thus, a global analysis simply based on a Cd concentration difference between bulk water and blood cannot explain by itself the observed changes in accumulation rates. Note also that the same comment is valid for the relationship between the quantity of Cd that flowed over the gills per unit of time and the accumulation rate; no direct, that is, 1:1, proportionality was found between $\dot{V}w$ and Cd accumulation (Figs. 1B2 and 3A). Several alternative, but not mutually exclusive, explanations are possible. These could include first the existence of changes of the gill-ventilated surface, that is, of the shunted gill area. Thus, in accordance to the modification of $\dot{V}w$, the exchange area could increase or decrease and consequently modify the processes of Cd adsorption and absorption. Another possibility could be the occurrence at high water flow rate of a thinning of the unstirred layers at the interface between the palleal cavity medium and the gill epithelium, which could reduce to a more or less large extent the metal gradients at the proximity of the gill epithelial cells. However, the study of these mechanisms was outside the scope of this paper and remains to be studied.

CONCLUSION

Chemical speciation is now clearly established to be an important factor controlling bioavailability of metals to organisms in the aquatic environment. However, it is well known that chemical speciation alone cannot explain all changes in the metal accumulation process and that biological factors must be included [35]. Up to now salinity, season, temperature, weight, growth rate, and diet were reported to be among these factors [27,28]. To our knowledge, an O_2-mediated influence in the normal in situ range where the animals are able to maintain their O_2 consumption has not been reported, although the widespread variability of O_2 in the field is a well-known phenomenon. Present results illustrate this potential O_2 influence and we suggest that classifying it should be of primary importance.

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