



## Influence of dissolved organic matter character on mercury incorporation by planktonic organisms: An experimental study using oligotrophic water from Patagonian lakes

María C. Diéguez<sup>1,\*</sup>, Claudia P. Queimaliños<sup>1</sup>, Sergio Ribeiro Guevara<sup>2</sup>, Mark Marvin-DiPasquale<sup>3</sup>, Carolina Soto Cárdenas<sup>1</sup>, María A. Arribére<sup>2</sup>

1. Laboratorio de Fotobiología, INIBIOMA CONICET-UNComahue, Quintral 1250, Bariloche, Argentina

2. Laboratorio de Análisis por Activación Neutrónica, Centro Atómico Bariloche, CNEA, Bustillo km 9.5, 8400 Bariloche, Argentina

3. United States Geological Survey, 345 Middlefield Rd./MS 480, Menlo Park, CA 94025, USA

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### Abstract

Ligands present in dissolved organic matter (DOM) form complexes with inorganic divalent mercury ( $\text{Hg}^{2+}$ ) affecting its bioavailability in pelagic food webs. This investigation addresses the influence of a natural gradient of DOM present in Patagonian lakes on the bioaccumulation of  $\text{Hg}^{2+}$  (the prevailing mercury species in the water column of these lakes) by the algae *Cryptomonas erosa* and the zooplankters *Brachionus calyciflorus* and *Boeckella antiqua*.  $\text{Hg}^{2+}$  accumulation was studied through laboratory experiments using natural water of four oligotrophic Patagonian lakes amended with  $^{197}\text{Hg}^{2+}$ . The bioavailability of  $\text{Hg}^{2+}$  was affected by the concentration and character of DOM. The entrance of  $\text{Hg}^{2+}$  into pelagic food webs occurs mostly through passive and active accumulation. The incorporation of  $\text{Hg}^{2+}$  by *Cryptomonas*, up to 27% of the  $\text{Hg}^{2+}$  amended, was found to be rapid and dominated by passive adsorption, and was greatest when low molecular weight compounds with a humic or fulvic signature kept  $\text{Hg}^{2+}$  in the dissolved phase, resulting in the lowest  $\text{Hg}^{2+}$  accumulation in this algae. In *Brachionus* and *Boeckella* the direct incorporation of Hg from the aqueous phase was up to 3% of the  $\text{Hg}^{2+}$  amended. The dietary incorporation of  $\text{Hg}^{2+}$  by *Boeckella* exceeded the direct absorption of this metal in natural water, and was remarkably similar to the  $\text{Hg}^{2+}$  adsorbed in their prey. Overall, DOM concentration and character affected the adsorption of  $\text{Hg}^{2+}$  by algae through competitive binding, while the incorporation of  $\text{Hg}^{2+}$  into the zooplankton was dominated by trophic or dietary transfer.

**Key words:** mercury; dissolved organic matter; plankton; mercury incorporation; Patagonian lakes; Argentina

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### Introduction

Mercury (Hg) is a global pollutant that is found even in areas far distant from emission sources. This metal is present in different forms which possess differential toxicity, and move between the atmosphere, water, soil, sediment and organisms. In aquatic ecosystems, this element enters directly from the watershed and also by the deposition of material transported in the atmosphere (Driscoll et al., 2007). Depending on the prevailing physical, chemical and biological conditions in the aquatic system, Hg compounds can be interconverted mainly between elemental mercury ( $\text{Hg}^0$ ), the divalent form ( $\text{Hg}^{2+}$ ) and the neurotoxic methylmercury (MeHg). One key-role aspect of mercury cycle in

the water is its affinity with ligands present in the dissolved organic matter (DOM) (Driscoll et al., 1995; Lamborg et al., 2004). The binding of  $\text{Hg}^{2+}$  and MeHg to DOM allows the mobilization and transport of this metal within aquatic ecosystems and can influence its accumulation in bacteria and algae at the base of food webs (Luengen et al., 2012). The effect of DOM on the uptake of  $\text{Hg}^{2+}$  by microbes depends on the species composition of the microflora and is relevant because sulphate and iron reducing bacteria can transform  $\text{Hg}^{2+}$  into MeHg which readily biomagnifies in the food web. Conversely, complexes of  $\text{Hg}^{2+}$  can inhibit the methylation process, thus reducing the bioaccumulation and toxicity of this metal (Drexel et al., 2002; Moye et al., 2002; Haitzer et al., 2003; Gorski et al., 2008).

In general, organisms take Hg by direct aqueous accumulation and/or from consumed prey, being the phy-

\* Corresponding author. E-mail: [dieguezmc@gmail.com](mailto:dieguezmc@gmail.com)

toplankton at the base of the pelagic food web the trophic level with the highest potential for bioconcentration of both  $\text{Hg}^{2+}$  and MeHg (Mason et al., 1995; 1996; Pickhardt and Fisher, 2007; Gorski et al., 2008; Luengen et al., 2012). Strong ligands, largely present in high molecular weight (HMW) DOM are competitive binding sites for Hg and thus, can reduce the availability of  $\text{Hg}^{2+}$  and MeHg to phytoplankton and bacteria (Gorski et al., 2008; Gilmour et al., 2011; Luengen et al., 2012).

In shallow and deep lakes within the Nahuel Huapi National Park (Patagonia, Argentina), sediment records indicate that periodic disruptive events such as volcanic activity and forest fires, in addition to global transport and deposition, contribute to build up a variable but considerable mercury pool in sediments and aquatic organisms (Ribeiro Guevara et al., 2010; Rizzo et al., 2011). Recent measurements of sterile natural water samples from these oligotrophic lakes revealed that just 3% of the total dissolved Hg (TDHg) pool is MeHg (Arcagni et al., 2013) while 97% is attributable to dissolved inorganic  $\text{Hg}^{2+}$ . Then, in the water column the dissolved  $\text{Hg}^{2+}$  is the largest fraction accounting to the TDHg and a potential competitor of MeHg for complexation with natural DOM. In fact, in these lakes DOM concentrations ranging from low (< 5 mg/L) to very low (< 1.5 mg/L) have been found to influence at different extents the complexation of  $\text{Hg}^{2+}$  (Ribeiro Guevara et al., 2008).

In this investigation, we hypothesize that the amount and character of natural lake DOM regulate the incorporation of  $\text{Hg}^{2+}$  into the pelagic food webs of oligotrophic Andean Patagonian lakes. Therefore, different DOM conditions present in natural and photobleached water from four Andean lakes were applied to study experimentally the incorporation of  $\text{Hg}^{2+}$ , the mercury species most readily available in these lakes, in planktonic species. The photobleached treatments were performed in order to induce modifications in the DOM quality through the reduction of the molecular size of the organic molecules. In this context, the algae *Cryptomonas erosa*, the rotifer *Brachionus calyciflorus* and the copepod *Boeckella antiqua* were cultured in natural and photobleached sterile water

from the different lakes inoculated with  $\text{Hg}^{2+}$  ( $^{197}\text{Hg}^{2+}$ ) to analyse: (1) the mechanisms of passive and active incorporation of  $\text{Hg}^{2+}$  in algae; (2) the role of DOM on  $\text{Hg}^{2+}$  incorporation from the dissolved phase by different planktonic organisms, and (3) the relative importance of direct adsorption and dietary incorporation of  $\text{Hg}^{2+}$  in zooplanktonic organisms.

## 1 Materials and methods

### 1.1 Sampling location

The natural water used in this study was collected in four Andean lakes located in the Nahuel Huapi National Park (Northwestern Patagonia, Argentina). The lakes sampled are the deep ultraoligotrophic lake Moreno West and the oligotrophic shallow lakes El Trébol, Morenito and Escondido, which are comprised in a gradient of DOM concentration and bear different limnological features, including their water color (**Table 1**).

### 1.2 Sample collection and processing

Water samples were collected from each lake using a 12-L Schindler-Patalas trap and poured into acid-washed 20 L polycarbonate carboys. The water was transported to the laboratory within 1 hr, insulated and in darkness. Each water sample was pre-filtered through 0.7  $\mu\text{m}$  glass-fiber filters (Whatman GF/F) and then sterilized through 0.22  $\mu\text{m}$  nitrocellulose filters (Gamafil). The samples were put in UV transparent containers (UVT Plasmatic, Spain) and incubated under two artificial light regimes: dark (aluminum foil wrapped) and a combination of photosynthetic active radiation (PAR, ten PAR lamps Sanyo), ultraviolet radiation A (UV-A, four lamps Q-Panel 340) and ultraviolet radiation B (UV-B, one lamp QFS 40). The incubations were run for 72 hr at 14°C in an environmental chamber (Sanyo MLR5). Pre-incubated sterile water treatments were used as culture medium for laboratory experiments and will be referred to hereinafter as natural and photobleached water. The photobleached water treatment was purposely designed to change the properties of natural lake

**Table 1** Limnological features of the Andean-Patagonian lakes

	Lake Moreno West	Lake El Trébol	Lake Morenito	Lake Escondido
Surface area (km <sup>2</sup> )	6.1	0.3	0.4	0.1
Maximum depth (m)	90	12	12	8
Kd <sub>PAR</sub>	0.12–0.21 <sup>1,2,3</sup>	0.40–0.51 <sup>5,6</sup>	0.34–0.55 <sup>3,4,6</sup>	0.46–0.41 <sup>1,5,6</sup>
Euphotic zone (m)	22–38 <sup>1,2,3</sup>	9–14 <sup>5,6</sup>	8–12 <sup>3,4</sup>	3–8 <sup>5,6</sup>
Conductivity ( $\mu\text{S}/\text{cm}$ )	37–42 <sup>7,8</sup>	65–78 <sup>8,10</sup>	57–75 <sup>9</sup>	35–65 <sup>9,11</sup>
pH	6.8–7.5 <sup>7,8</sup>	7.3–7.6 <sup>9</sup>	6.8–7.8 <sup>9</sup>	6.7–7.8 <sup>9,11</sup>
Chlorophyll <i>a</i> ( $\mu\text{g}/\text{L}$ )	0.5–1.5 <sup>2,4,7</sup>	1.4–10.5 <sup>5,8,10</sup>	0.6–3.8 <sup>4,6,9</sup>	0.2–3.3 <sup>1,5,6,9,11</sup>
DOC (mg/L)	0.4–0.9 <sup>1,2,3</sup>	1.4–2.5 <sup>5,6</sup>	1.8–3.3 <sup>3,6</sup>	2.7–5.4 <sup>1,5,6</sup>
TP ( $\mu\text{g}/\text{L}$ )	1.5–3.5 <sup>2,4</sup>	8.6–11.8 <sup>5,6</sup>	8.9–17.1 <sup>4,6,9</sup>	2.8–12.2 <sup>5,6,9,11</sup>
TN ( $\mu\text{g}/\text{L}$ )	50–210 <sup>7,8</sup>	n.a.	280–420 <sup>9</sup>	120–440 <sup>9</sup>

Kd<sub>PAR</sub>: PAR diffuse attenuation coefficient; DOC: dissolved organic carbon; TP: total phosphorus concentration; TN: total nitrogen concentration.

<sup>1</sup> Morris et al., 1995; <sup>2</sup> Queimaliños et al., 1999; <sup>3</sup> Alonso et al., 2004; <sup>4</sup> Modenutti et al., 2000; <sup>5</sup> Pérez et al., 2010; <sup>6</sup> Bastidas Navarro et al., 2009; <sup>7</sup> Diaz et al., 2007; <sup>8</sup> Queimaliños et al., 2013; <sup>9</sup> Gereá, 2012; <sup>10</sup> Queimaliños et al., 1998; <sup>11</sup> Diaz and Pedrozo, 1993.

DOM, thereby creating a wider DOM context to analyze the accumulation of  $\text{Hg}^{2+}$  by the planktonic organisms.

### 1.3 Experimental procedures

The accumulation of  $\text{Hg}^{2+}$  by algae and by zooplankters, was analyzed amending  $^{197}\text{Hg}^{2+}$  with a final concentration ranging from 5 to 12 ng/L across all experiments. The high specific activity ( $t^{1/2} = 2.673$  day) of  $^{197}\text{Hg}^{2+}$  (hereinafter radiolabeled  $\text{Hg}^{2+}$ ) used in these experiments was produced by irradiation of a 2%  $\text{HNO}_3$  solution of Hg enriched to 51.6 % in the  $^{196}\text{Hg}^{2+}$  isotope in the nuclear research reactor RA-6 ( $\Phi_{\text{th}} = 1 \times 10^{13}$  n/( $\text{cm}^2 \cdot \text{sec}$ )), Centro Atómico Bariloche, Argentina. The final concentration of  $^{196}\text{Hg}^{2+}$  was 57  $\mu\text{g}/\text{mL}$  and all isotope amendments used in the experiments are relative to this value. Standards and working solutions were prepared using ASTM-1 water.

The planktonic species used in the experiments were the algae *Cryptomonas erosa*, and two zooplankters, the rotifer *Brachionus calyciflorus*, and the copepod *Boeckella antiqua* which were derived from laboratory cultures. The duration of experiments was established by means of preliminary trials in the same conditions than the experiments described below.

*Cryptomonas* cells were concentrated using a 20  $\mu\text{m}$  mesh size and rinsed thoroughly with ASTM-1 water to remove the culture medium. After rinsing, the algae were concentrated again in a batch and an aliquot from it was counted in a haemocytometer. Assuming that the concentrated algal batch would release dissolved organic substances to the medium (e.g. through cell lysis and/or the presence of secondary metabolites), a similar volume of this filtered-sterilized batch was used for the control treatment, which was devoid of algae.

To assess passive versus active incorporation of radiolabeled  $\text{Hg}^{2+}$  in the algae *Cryptomonas*, both live and heat-killed cells of *Cryptomonas* (3000 cell/mL) were incubated in triplicate batch cultures using natural water from lakes El Trébol and Moreno amended with radiolabeled  $\text{Hg}^{2+}$ . Controls consisted of similar volumes of natural water of each lake devoid of algae and also amended with radiolabeled  $\text{Hg}^{2+}$ . The assays lasted 30, 90 and 150 min and were conducted in 500 mL Erlenmeyer flasks filled with 250 mL of the natural water of each lake, at 14°C and in darkness to prevent algal production.

To analyse the effect of DOM on the incorporation of labelled  $\text{Hg}^{2+}$  by live cells of *Cryptomonas* incubated in natural and photobleached water from Lakes Moreno, El Trébol, Morenito, and Escondido. Algal treatments included three replicates with a final abundance of 3000 cells/mL, except in the case of Lake Escondido for which algal density was 4200 cells/mL. In this latter case, the final Hg accumulation was normalized to 3000 cells/mL. Triplicate controls consisting of sterile natural and photobleached water from each lake were set up to evaluate the retention of Hg by the filters. The assays were conducted in 500 mL

Erlenmeyer flasks filled with 200–250 mL of the natural or photobleached water, over 24 hr at 14°C and in the dark.

After the incubation period in the above mentioned experiments, all replicates of the algal treatments and controls were filtered through 2.7  $\mu\text{m}$  glass-fiber filters (Whatman GF/D). The filters were individually transferred into 4 mL glass tubes for the final assessment of the Hg radioactivity. To obtain the net Hg accumulation by *Cryptomonas*, the activity measured in the filters with algae was corrected by subtracting the filter retention of Hg in the controls without algae. Hg incorporation was calculated for each experimental unit based upon the initial amendment of radiolabeled  $\text{Hg}^{2+}$  compared to the final Hg activity. The concentration of total Hg was determined by evaluating X-ray and  $\gamma$ -ray emissions associated with the radiolabeled  $\text{Hg}^{2+}$  decay, using a well type High Purity Germanium (HPGe) detector (Ribeiro Guevara et al., 2007).

Moreover, the influence of DOM conditions in the accumulation of  $\text{Hg}^{2+}$  from the aqueous phase by the zooplankters *Brachionus* and *Boeckella* was evaluated. The animals were starved during the incubation to ensure  $\text{Hg}^{2+}$  adsorption from the water, preventing any contribution of  $\text{Hg}^{2+}$  through algal consumption. Each treatment was run in three replicates in 100 mL glass beakers filled with 80 mL of the corresponding culture medium, at 14°C and in the dark, for 19 hr. Each experimental unit of both experiments contained 50 *Brachionus* and 20 *Boeckella* individuals, respectively.

In addition, the direct adsorption of  $\text{Hg}^{2+}$  from the aqueous phase and the accumulation of  $\text{Hg}^{2+}$  in the copepod *Boeckella* through the consumption of *Cryptomonas* (dietary incorporation) were determined. Prior to starting this experiment, a culture of *Cryptomonas* was inoculated with radiolabeled  $\text{Hg}^{2+}$  (5 ng/L) and incubated for 24 hr in natural water from Lake El Trébol. Also, natural water from this lake devoid of algae was amended with radiolabeled  $\text{Hg}^{2+}$  at the same time and in the same proportion in order to assure similar conditions. Two treatments were set up, one was supplemented with the radiolabeled  $\text{Hg}^{2+}$ -bearing *Cryptomonas* conditioned as explained above, at 3000 cells/mL. The other treatment consisted of the amended natural water devoid of algae. In both treatments, which were run with four replicates, 20 *Boeckella* individuals were put in each experimental unit. The incubations were conducted over 22 hr, in 100 mL glass beakers filled with 80 mL of the corresponding culture medium, at 14°C and in the dark.

In the trials using zooplankton, the rotifers and copepods were recovered individually after incubation by means of a Pasteur pipette under a direct microscope. The zooplankters were rinsed with ASTM-1 water to ensure the elimination of any dissolved non-adsorbed  $\text{Hg}^{2+}$ . The concentration of total Hg in the zooplankters was determined as in the previous experiment.

## 1.4 DOM characterization

DOM was characterized via UV-Visible absorbance and fluorescence spectrometry of the filtered natural and photobleached water. We focused on the calculation of two optical and one fluorescence parameters:  $a_{440}$  as a proxy of the concentration of chromophoric DOM (CDOM) which is the light absorbing fraction of DOM (Reche et al., 1999); the spectral slope between 275 and 295 nm ( $S_{275-295}$ ) as a proxy of the DOM molecular weight (Helms et al., 2008), and the synchronous fluorescence spectra (SFS) for the identification of fluorophores (Lu and Jaffé, 2001; Lu et al., 2003). Absorbance was determined in natural and photobleached water with a Hewlett Packard 8453 diode array spectrophotometer, using a 10 cm quartz cuvette and measuring a full scan from 200 to 800 nm at 1 nm intervals. ASTM-1 water was the blank. The average absorbance from 700 to 800 nm was subtracted from each spectrum to correct for offsets due to several instrument baseline effects (Green and Blough, 1994; Helms et al., 2008). Then, absorbance units were converted to Napierian absorption coefficients ( $a$ ,  $m^{-1}$ ) as follows:

$$a = 2.303 \frac{A}{l} \quad (1)$$

where,  $A$  is the absorbance, and  $l$  (m) is the path length.

The spectral slope of the interval of 275–295 nm ( $S_{275-295}$ ) was applied as a surrogate of DOM molecular sizes, and was calculated by fitting a linear regression of the log-transformed spectra, and provided as a positive number according to a mathematical convention (Helms et al., 2008). In this context, slopes are inversely associated with DOM molecular size (Helms et al., 2008; Mladenov et al., 2011).

SFS were recorded from natural and photobleached water using ASTM-1 water blanks over the excitation wavelength range 250–550 nm. Measurements were performed with a Perkin Elmer LS55 spectrofluorometer equipped with a 150-W Xenon arc lamp and a Peltier temperature controller, using a 1 cm quartz fluorescence cell, at a constant offset value ( $\delta\lambda = 30$  nm) between excitation and emission wavelengths, with a slit width of 10 nm (Lu and Jaffé, 2001; Lu et al., 2003). The SFS were obtained at natural pH and at 20°C ( $\pm 0.1^\circ\text{C}$ ). Individual peaks in the SFS were used as proxies for fluorophores of different molecular sizes and structures, such as aromatic amino acids or humic substances in the bulk DOM (Ferrari and Mingazzini, 1995; Peuravuori et al., 2002; Barker et al., 2009). The SFS were processed with the software FL-WinLab and corrected for Raman spectral overlap and for the inner-filter effect using UV-Visible absorbance scans of the natural and photobleached water samples (Ferrari and Mingazzini, 1995). All fluorescence intensities were standardized relative to a quinine sulphate calibration curve and intensities were expressed in quinine sulphate units. In each water sample, the main fluorescence peaks

were determined and their magnitudes were compared as per Jaffé et al. (2004) and Breton et al. (2009).

## 1.5 Statistical analyses

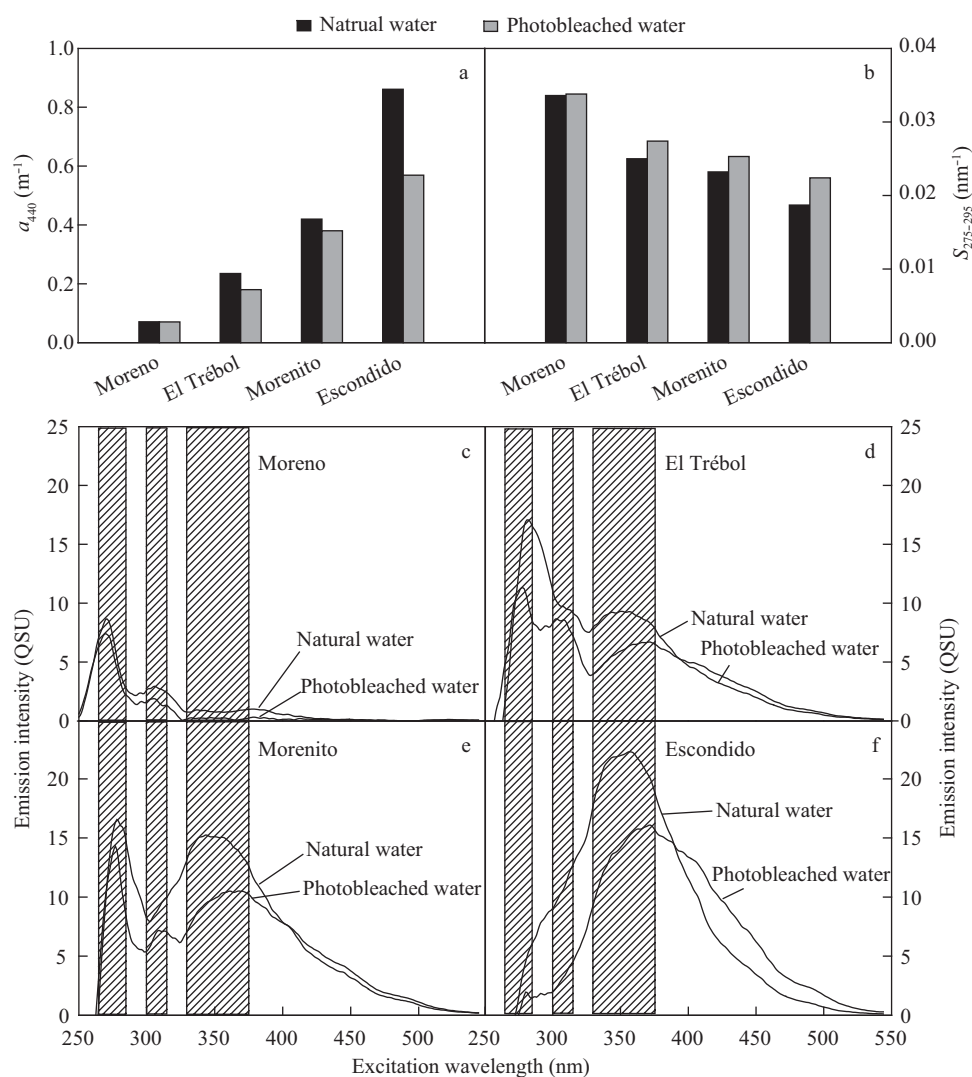
The results from the different experiments were tested for normality (Kolmogorov-Smirnoff, normality test) and homoscedasticity before performing the Analysis of Variance (One-way and Two-way ANOVA) to evaluate differences among treatments. Post hoc tests were applied to evaluate the effect of levels within factors resulting from significant ANOVA tests. Linear regression models were applied to fit the relationships between the retention of radiolabeled  $\text{Hg}^{2+}$  by filters and algae in relation to  $a_{440}$  and the intensity of Peak<sub>360</sub> of the SFS spectra.

## 2 Results and discussion

### 2.1 DOM characterization of natural and photobleached water of the four lakes

Absorption measurements at 440 nm (as proxies of chromophoric DOM concentrations) were lowest in Lake Moreno, followed by El Trébol, Morenito and Escondido. As expected, the photobleaching treatment caused a loss of absorbance in all water samples (Fig. 1a). The  $S_{275-295}$  values of natural water from all lakes ranged between 0.0187 and 0.0336  $\text{nm}^{-1}$ , with higher  $S_{275-295}$  values related to lower bulk molecular weight DOM according to Helms et al. (2008). The  $S_{275-295}$  was greatest in Lake Moreno followed by Lakes El Trébol, Morenito and Escondido (Fig. 1b). The differences in the slopes could be indicative of both different DOM molecular sizes and sources (Helms et al., 2008). In fact, the particular limnological features of these Patagonian lakes (Table 1) could account for the observed differences in the slopes and spectral absorbances. Furthermore, the  $S_{275-295}$  values were higher in photobleached water compared to natural water, suggesting that photobleaching induced a transformation of the organic molecules, likely towards a reduction in the molecular size of the DOM (Fig. 1b).

The SFS of both natural and photobleached water at the initial conditions, exhibited a generally consistent pattern with three fluorescence maxima corresponding to three different excitation intervals: 265–285 nm, 300–315 nm and 330–375 nm (Fig. 1c–f). These maxima were identified according to the mean wavelengths of each range as: Peak<sub>280</sub>, Peak<sub>310</sub> and Peak<sub>360</sub>, respectively. Peak<sub>280</sub> exhibited the largest intensity in Lake Moreno, followed by Lakes El Trébol and Morenito (Fig. 1c and e), whereas it was absent in natural water of Lake Escondido (Fig. 1f). Peak<sub>310</sub> was present in natural water from Lake Moreno and El Trébol (Fig. 1c–d), and in photobleached water of Lakes El Trébol and Morenito (Fig. 1d–e). Peak<sub>360</sub> was present in natural and photobleached water of all lakes, with the highest intensity in natural water of Lake Escondido, where it was also the only peak present



**Fig. 1** Optical and fluorescence characterization of the dissolved organic matter (DOM) in the natural and photobleached water of the four Andean lakes. (a) absorption coefficient at 440 nm (as proxy of chromophoric DOM concentration); (b) spectral slopes ( $S_{275-295}$ ) as proxy of DOM molecular size; (c) synchronous fluorescence spectra of natural and photobleached water of Lake Moreno, (d) Lake El Trébol, (e) Lake Morenito, (f) Lake Escondido. Shaded areas represent the different excitation intervals: 265–285 nm, 300–315 nm and 330–375 nm, where the fluorescence peaks are observed. QSU: Quinine sulfate Units.

(**Fig. 1f**). Two of the three peaks detected, Peak<sub>280</sub> and Peak<sub>360</sub>, coincided with peaks that have been reported in other studies as Peak I and Peak II (Lu and Jaffé, 2001; Jaffé et al., 2004), while Peak<sub>310</sub> has not been described previously. Peak<sub>280</sub> appears to relate with the presence of dissolved proteins or peptides of low molecular weight (LMW) (Ferrari and Mingazzini, 1995; Lu et al., 2003). The peak obtained in the excitation-emission matrices associated with the synchronous Peak I, called as T-peak, is constituted by proteinaceous materials and also small phenols from tannin degradation (Maie et al., 2007). On the other hand, the peaks at higher wavelengths reflect the presence of humic substances of higher molecular weight. For example, a peak between 330–375 nm indicates the presence of fulvic acids (Lu et al., 2003; Jaffé et al., 2004; Breton et al., 2009). In this context, Lake Moreno DOM is likely characterized by a high proportion of proteinaceous

and/or phenolic LMW compounds, while Lake Escondido DOM is dominated by HMW compounds like fulvic acids (**Fig. 1c** and **f**). The DOM of Lakes El Trébol and Morenito constitute intermediate cases, with particular proportions of both LMW and HMW compounds (**Fig. 1d** and **e**). The photobleaching conditioning induced a transformation of the DOM in the natural water of the four lakes decreasing the proportion of HMW compounds (**Fig. 1a–f**). The most striking transformation was observed in the case of Lake Escondido where photobleaching led to the break-down of prevalent HMW compounds increasing concomitantly the pool of LMW compounds. This was suggested by the increase of  $S_{275-295}$  and by the changes in the SFS, mostly the occurrence of a small Peak<sub>280</sub> (**Fig. 1b** and **f**), indicative of small phenols from tannin/lignin degradation (Maie et al., 2007). These LMW compounds produced by photobleaching of NW of Lake Escondido likely were

derived from the fulvic acids that dominate in this lake.

## 2.2 Passive vs. active accumulation of radiolabeled $\text{Hg}^{2+}$ by *Cryptomonas erosa*: a time trend analysis

The incorporation of radiolabeled  $\text{Hg}^{2+}$  was similar in live and heat-killed cells of *Cryptomonas* indicating that the predominant mechanism of  $\text{Hg}^{2+}$  incorporation is passive adsorption (Fig. 2). The  $\text{Hg}^{2+}$  adsorption values recorded in live and heat-killed cells of *Cryptomonas* indicate a similar capacity to bind  $\text{Hg}^{2+}$ . This Hg species has a greater reactivity for particulate cellular material and concentrates mostly in the outer cell membranes of the algae (Mason et al., 1996; Pickhardt and Fisher, 2007). Interestingly, these external structures act as barriers preventing the incorporation of the metals into the cytoplasm and, along with the production of binding factors and proteins, are known to act as cellular defenses to counteract metal toxicity (Rajamani et al., 2007).

Differences in the adsorption of radiolabeled  $\text{Hg}^{2+}$  by different organisms were observed between natural water of the lakes. In particular, the adsorption of  $\text{Hg}^{2+}$  by *Cryptomonas*, independently of the cell condition, was lower in natural water from Lake Moreno (4%–12% of  $\text{Hg}^{2+}$  amendment) compared to natural water from Lake El Trébol (15%–22% of  $\text{Hg}^{2+}$  amendment) (One Way ANOVA,  $F = 76.67$ ,  $p < 0.001$ ; Fig. 2). These results suggested that differences in water chemistry, including DOM concentration and character, influenced the absolute extent of metal adsorption by *Cryptomonas*. Recently, Luengen et al. (2012) confirmed that the accumulation of MeHg in *Cyclotella meneghiniana* decreased with the

addition of DOM, attributing this pattern to DOM complexing MeHg and competing with algal cells for this Hg compound. In contrast, in our study the higher adsorption of  $\text{Hg}^{2+}$  to *Cryptomonas* was recorded in natural water with comparatively higher DOC concentration (Table 1). In this line, a previous study by Pickhardt and Fisher (2007) showed that the adsorption of  $\text{Hg}^{2+}$  by the diatom *Cyclotella meneghiniana* was higher in natural high DOC water as compared to low DOC water. This pattern may indicate that besides DOM concentration, DOM character among other factors may influence the bioavailability of  $\text{Hg}^{2+}$  in natural water.

In natural water from Lake Moreno, the adsorption of  $\text{Hg}^{2+}$  to live algae steadily increased along the 150 min of exposure (One Way ANOVA,  $F = 117.70$ ,  $p = 0.001$ ; Fig. 2). In the case of the dead algae the incorporation was similar along the time trend (One Way ANOVA,  $p > 0.05$ ) suggesting a rapid adsorption within the first 30 min of incubation. In natural water from Lake El Trébol the adsorption was also rapid and occurred within the first 30 min, resulting in similar values of Hg incorporation throughout the incubations of both live and dead cells (Two Way ANOVA,  $p > 0.05$ ; Fig. 2). The kinetics of  $\text{Hg}^{2+}$  adsorption observed in *Cryptomonas* appears to be mediated by naturally occurring ligands, as has been reported in previous studies (Lamborg et al., 2004; Black et al., 2007; Miller et al., 2009). In this study, the differences observed in the pattern of  $\text{Hg}^{2+}$  adsorption by *Cryptomonas* among natural water of the different lakes suggest that water chemistry, particularly natural DOM, readily influenced the dynamics of this process. One conceptual model of how  $\text{Hg}^{2+}$  interacts with DOM suggests two general categories of  $\text{Hg}^{2+}$  binding sites on DOM macromolecules. The first represented by functional groups with a weak-to-moderate affinity to complex  $\text{Hg}^{2+}$  but that are high in abundance (e.g. carboxyls and amines). The second represented by functional groups with a strong affinity to bind  $\text{Hg}^{2+}$  that are in comparatively lower abundance (e.g. thiols) (Gasper et al., 2007). However, thiols alone might not account for all bindings, as it has been found that  $\text{Hg}^{2+}$  and MeHg strongly bind to DOM even when the DOM lacks sulfide (Gasper et al., 2007; Luengen et al., 2012). Thus, the presence and relative proportion of different DOM molecules in natural lake water create a particular affinity to  $\text{Hg}^{2+}$  which could reflect in the rate of  $\text{Hg}^{2+}$  adsorption to algae. Our results appear consistent with this conceptual model of Hg-DOM complexation kinetics.

## 2.3 $\text{Hg}^{2+}$ adsorption by *Cryptomonas erosa* in relation to DOM concentration and quality

As shown in Fig. 3 the net adsorption of radiolabeled  $\text{Hg}^{2+}$  by *Cryptomonas* was significantly different among natural water samples of the four lakes (Two Way ANOVA,  $F = 574.9$ ,  $p < 0.001$ ), with the highest adsorption observed in natural water of Lake El Trébol, followed by Lakes

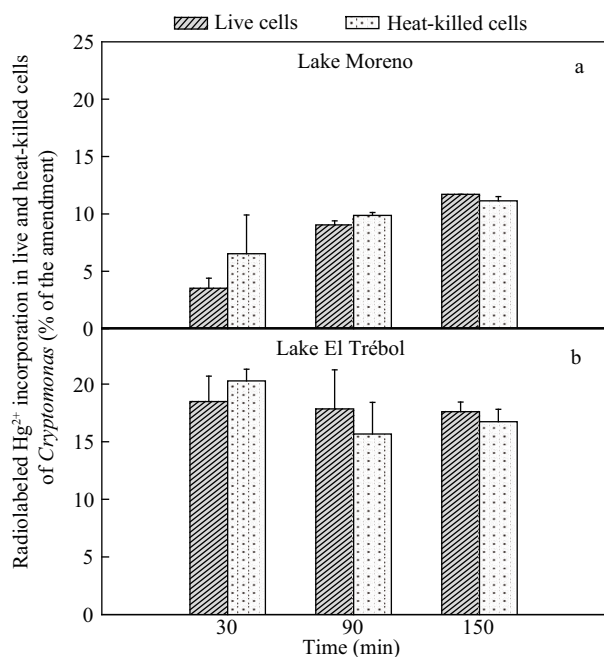
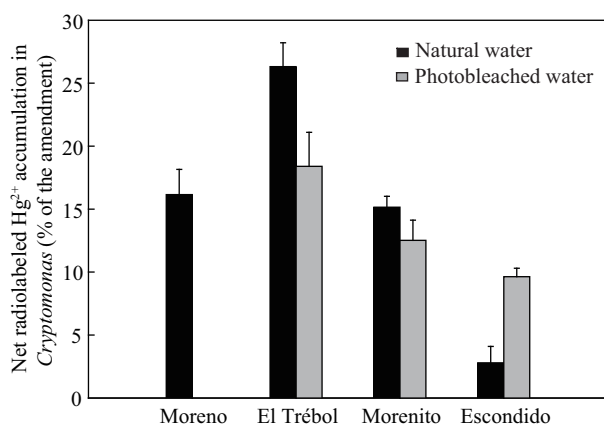


Fig. 2 Accumulation of radiolabeled  $\text{Hg}^{2+}$  in live and heat-killed cells of the algae *Cryptomonas erosa*, incubated for 30, 90 and 150 min in natural water from Lake Moreno and Lake El Trébol.



**Fig. 3** Accumulation of radiolabeled  $\text{Hg}^{2+}$  by the algae *Cryptomonas erosa* in natural and photobleached water from Lakes Moreno, El Trébol, Morenito and Escondido, after 24 hr of incubation. In the case of Lake Moreno the photobleached water treatment is lacking due to a failure in the manipulation of the replicates.

Morenito, Moreno, and Escondido (Holm-Sidák all pairwise Post-hoc multiple comparisons  $p < 0.02$ ).

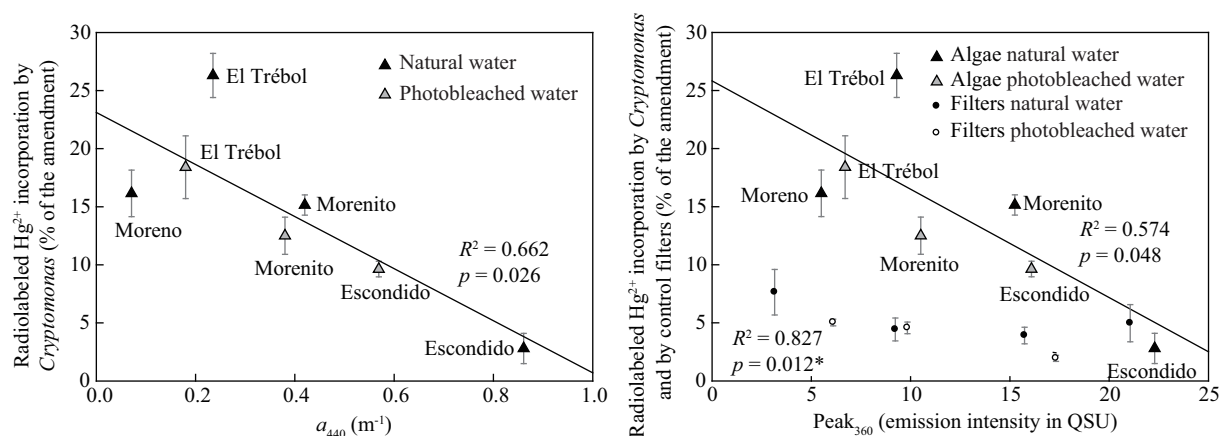
The effect of the photobleaching on the adsorption of radiolabeled  $\text{Hg}^{2+}$  by *Cryptomonas* was also significant both among lakes (Two Way ANOVA,  $F = 5.1$ ,  $p = 0.045$ ) and within lakes (Two Way ANOVA,  $F = 29.9$ ,  $p < 0.001$ ). However, the response to the photobleaching was not consistent, since it decreased the Hg adsorption to algae in water from Lakes Morenito and El Trébol ( $t = 2.2$  and  $6.7$ ,  $p < 0.05$ , respectively), while increased the adsorption in Lake Escondido ( $t = -5.5$ ,  $p = 0.001$ ).

We found a significant negative relationship between total absorption ( $a_{440}$ ) and Hg accumulation ( $R^2 = 0.618$ ,  $p < 0.001$ ), independently of the water treatment (natural water and photobleached water) (Fig. 4a). Absorption at 440 nm, used as indicator of CDOM is usually correlated positively with DOC concentration (Haitzer et al., 2002). This indirectly suggests that concentration may play a role in regulating  $\text{Hg}^{2+}$  adsorption to *Cryptomonas* through competition with DOM molecules for binding  $\text{Hg}^{2+}$ . Experimental evidence reported first by Gorski et al. (2008) and more recently by Luengen et al. (2012) has confirmed that DOM concentration is one of the most important factors determining algal bioaccumulation of Hg species. Our results evidenced that also DOM character may drive the accumulation of Hg by algae. We assessed the influence of DOM character on  $\text{Hg}^{2+}$  adsorption by algae under the different water conditions, utilizing the intensities of Peak<sub>360</sub> (Fig. 1c–f) as a proxy for HMW fulvic compounds. The results indicate that the net algal  $\text{Hg}^{2+}$  incorporation significantly decreased in water with increasing concentrations of HMW DOM (increasing Peak<sub>360</sub> intensity) ( $R^2 = 0.574$ ,  $p = 0.048$ ; Fig. 4b). This suggests that HMW DOM can effectively compete for  $\text{Hg}^{2+}$  producing Hg-DOM complexes keeping it in solution and off of the algae. Similarly, other studies have also reported that DOM quality plays a critical role

in the accumulation kinetics and that the availability of  $\text{Hg}^{2+}$  to phytoplankton is enhanced at low concentrations of DOM (Haitzer et al., 2002; Gorski et al., 2008; Miller et al., 2009). We assumed that the complexation was a fast process which occurred during the short period of incubation previous to filtration (e.g.  $< 30$  min). HMW organic compounds may be stronger ligands for  $\text{Hg}^{2+}$  than are LMW compounds, because they generally have higher reduced sulphur content (Ravichandran, 2004).

Likewise,  $\text{Hg}^{2+}$  retention in the control filters without algae showed a similar trend with the intensity of Peak<sub>360</sub>, although the range of accumulation in the filters was much lower (1.94%–7.46% of the radiolabeled  $\text{Hg}^{2+}$  amendment; Fig. 4b). These results suggested that the algae and the filters behaved similarly in terms of the  $\text{Hg}^{2+}$  retention pattern, even though the values were always higher in the algae. The exception were the control filters corresponding to natural water from Lake Escondido, in which the retention of  $\text{Hg}^{2+}$  was higher than in the algae (this point was out of the regression fit). Considering that this water was dominated by HMW compounds (Fig. 1f), it seems possible that the Hg-DOM complexes produced had a higher affinity to the glass fiber matrix compared to the algae and to those formed in the photobleached water treatment of the same lake. Also, HMW compounds may form macromolecules that could be retained in the filter matrix due to their size. The photobleaching process modified the molecular structure of the native DOM likely influencing its charge, size and distribution of functional groups and resulting in an increased affinity towards the glass fiber matrix. Indeed, adsorption of DOM to glass fiber filters has been reported in previous studies, and attributed to the relative polarity between the dissolved molecules and the fibers (Morán et al., 1999). Moreover, the fact that *Cryptomonas* showed a higher  $\text{Hg}^{2+}$  accumulation in the photobleached water rather than in natural water of Lake Escondido (Fig. 4b) could also be understood taking into account the differences in the molecular structure of the DOM in this treatment.

Overall, our results may be explained considering two kinds of affinities: first, the affinity between Hg and DOM which allows the formation of Hg-DOM complexes, and second, the differential affinity between these Hg-DOM complexes with the glass fiber matrix and/or the algal cell surface. In the latter case, the similar pattern of  $\text{Hg}^{2+}$  incorporation by *Cryptomonas* and by the filters points to DOM character as the factor regulating the binding of this metal to both biological and artificial membranes. The underlying mechanisms behind the differential accumulation of  $\text{Hg}^{2+}$  by algae in diverse DOM environments are not fully understood, and may involve changes in membrane permeability related to DOM features, such as molecular structure, polarity, functional groups distribution, etc. (Mason et al., 1996; Pickhardt and Fisher, 2007). However, complexes of  $\text{Hg}^{2+}$  with HMW DOM appear to be too



**Fig. 4** Relationships between the net accumulation of radiolabelled Hg<sup>2+</sup> by *Cryptomonas* cells and the retention of Hg<sup>2+</sup> in the control filters (incubation devoid of algae). (a) absorption at 440 nm as a proxy of chromophoric dissolved organic matter (CDOM); (b) intensity of the SFS Peak<sub>360</sub> expressed in QSU, in natural water and photobleached water from Lakes Moreno, El Trébol, Morenito and Escondido. The intensity of Peak<sub>360</sub> indicates the presence of high molecular weight organic (HMW) compounds. \* The regression was performed including six out of seven samples. Data of the natural water treatment of Lake Escondido was excluded from the regression.

large or hydrophilic to readily attach to the cell membrane (Vigneault et al., 2000; Boullemant et al., 2004; Gorski et al., 2008).

In water with lower levels of HMW compounds, such as water from Lakes Moreno and El Trébol, Hg<sup>2+</sup> was more available, as reflected by the comparatively higher levels of Hg<sup>2+</sup> adsorbed to the algae (16%–27%) (Fig. 4). This suggests a simple competitive binding with Hg being available to both chemical and biological interactions. In particular, as was discussed above, algal surfaces (mucilage, cell walls and membranes) can act as ligands for metal ions since they contain several functional groups with different metal affinities, which may behave similarly to DOM in terms of Hg<sup>2+</sup> complexation (Rajamani et al., 2007). For example, the amino acid cysteine (Cys) contains a sulphhydryl (thiol group) which is the site of metal binding. Cys-containing peptides such as glutathione are responsible for metal sequestration in living cells (Torres et al., 2008). In particular, the antioxidant enzyme glutathione-S-transferase (GTS) is released by cells stressed by metal toxicity, such as those produced by high Hg concentrations (Croot et al., 2002). Thus, we cannot rule out this process and the potential release of GTS in our experiments given the oxidative stress imposed upon the algal cells. Further, the net algal Hg<sup>2+</sup> accumulation and the Hg<sup>2+</sup> retention in the control filters increased with the proportion of LMW substances (R<sup>2</sup> = 0.630, p = 0.033 and R<sup>2</sup> = 0.919, p = 0.001, respectively), suggesting the presence of Hg-LMW DOM complexes readily bound to either algal surface and/or the glass fiber matrix of the filters. The complexation of Hg<sup>2+</sup> with LMW substances (essentially protein-like and phenols) appeared to favor Hg<sup>2+</sup> adsorption by *Cryptomonas* in the lakes where DOM was mainly characterized by LMW compounds.

Overall, our results suggest that the higher proportion of LMW compounds in solution and the presence of

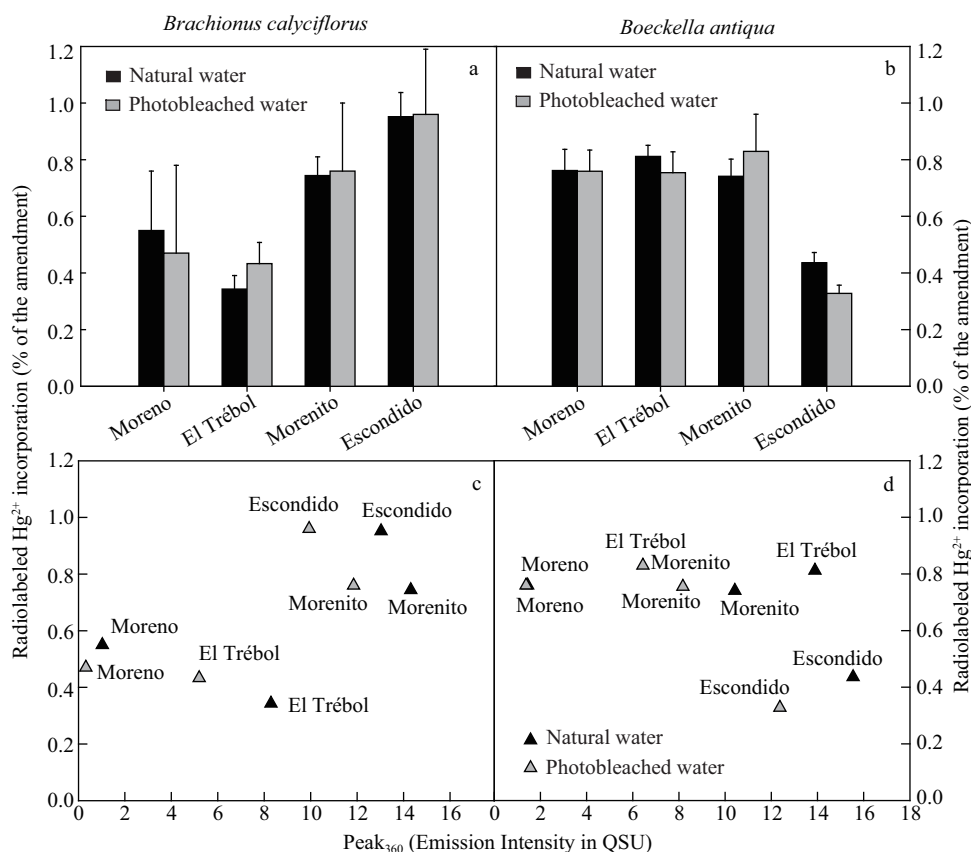
certain organic molecules associated to the algal surface could enhance adsorption of Hg to the cells or alter the permeability of cell membranes, as has been previously postulated (Vigneault et al., 2000; Boullemant et al., 2004). Pickhardt and Fisher (2007) proposed that at low DOC concentrations, these factors could dominate over the inhibitory influence of Hg binding to aqueous DOC, resulting in a higher Hg accumulation into phytoplankton in some systems, as observed in our study.

#### 2.4 Hg<sup>2+</sup> accumulation by zooplankton in relation to DOM character

We evaluated the incorporation of dissolved radiolabeled Hg<sup>2+</sup> by the rotifer *Brachionus calyciflorus* and the copepod *Boeckella antiqua*, in natural and photobleached water from the four Andean lakes. The effect of DOM quality on Hg<sup>2+</sup> accumulation by *B. calyciflorus* showed that the pattern of response was similar in natural and photobleached water of all lakes (Two Way ANOVA, p > 0.05). However, the accumulation of dissolved radiolabeled Hg<sup>2+</sup> by *Brachionus* differed significantly between lakes (Two Way ANOVA, F = 11.54, p < 0.001, Fig. 4a), with the highest values observed for Lake Escondido followed by Morenito, Moreno and El Trébol, regardless of the water treatment (Fig. 5a). The dissolved Hg<sup>2+</sup> accumulation in *Boeckella antiqua* was similar between natural and photobleached water (Two Way ANOVA, p > 0.05), but differed significantly among lakes (Two Way ANOVA, F = 23.03, p < 0.001). The incorporation of dissolved Hg<sup>2+</sup> by *Boeckella* was similar in magnitude for Lakes Morenito, El Trébol, and Moreno, but much lower in water from Lake Escondido (Fig. 5b).

In general, we found that in *Brachionus* and *Boeckella* the Hg<sup>2+</sup> incorporation (< 3% of dissolved radiolabeled Hg<sup>2+</sup> amended) was one order of magnitude lower than in *Cryptomonas*.





**Fig. 5** Incorporation of radiolabeled Hg<sup>2+</sup> in *Brachionus calyciflorus* (a), and *Boeckella antiqua* (b) in natural and photobleached water of the four Andean lakes, and the relationship between the mean radiolabeled Hg<sup>2+</sup> incorporation in the rotifer *Brachionus calyciflorus* (c) and the copepod *Boeckella antiqua* (d), versus the intensity of Peak<sub>360</sub> expressed in QSU, used to characterize the natural water and photobleached water DOM pool of Lakes Moreno, El Trébol, Morenito and Escondido.

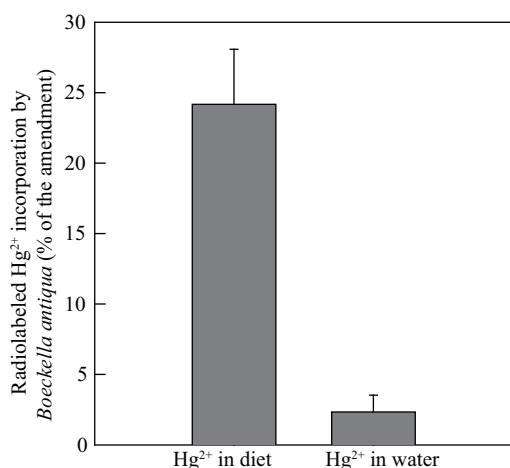
The accumulation of dissolved Hg<sup>2+</sup> in relation to DOM quality revealed differences between the rotifer and the copepod (**Fig. 5c** and **d**). *Brachionus* showed a positive tendency in its Hg<sup>2+</sup> accumulation with increasing HMW DOM concentrations, as reflected by increasing Peak<sub>360</sub> intensities, whereas the incorporation of *Boeckella* was lowest at the highest HMW DOM concentration. The differences in Hg<sup>2+</sup> accumulation observed between the two species may partially reflect differences in their feeding behaviors, filter-feeding in the case of the rotifer and particle capture in the case of the copepod. These feeding modes involve different contact with the medium, which is high between soft tissues of the filtration organ in the case of the filter-feeder *Brachionus* and likely much lower in the case of particle-feeders such as *Boeckella*. The lower Hg<sup>2+</sup> accumulation by *Boeckella* in water from Lake Escondido may indicate that the comparatively high CDOM concentration and high proportion of HMW compounds present in this lake, complex with Hg<sup>2+</sup> to a larger degree than in the other lakes, thus reducing the availability of Hg<sup>2+</sup> for incorporation onto body surfaces (**Fig. 5d**).

The incorporation of radiolabeled Hg<sup>2+</sup> by *Boeckella* directly from the dissolved phase and through a diet of *Cryptomonas* enriched with radiolabeled Hg<sup>2+</sup> was compared. The dietary accumulation of Hg<sup>2+</sup> was 12 fold

higher (24.2% ± 3.9% of the radiolabeled Hg<sup>2+</sup> amended) than the direct accumulation of dissolved Hg<sup>2+</sup> (2.3% ± 1.2%;  $t = -10.7$ ,  $p < 0.001$ ) (**Fig. 6**). Remarkably, the amount of dietary incorporation was similar to the adsorption of Hg in their prey. This result confirmed that Hg<sup>2+</sup> accumulation in this crustacean comes largely through the diet rather than directly from the water, as has been found in previous investigation (Fisher et al., 1991). In our experiment the accumulation of Hg<sup>2+</sup> by *Boeckella* likely out-balanced the elimination of this metal, given that the duration of the experiments involved at least three events of fecal pellet production (Fu et al., 2007). It is known in copepods that unassimilated metals get packaged into fecal pellets, being excretion one of the routes for the elimination of Hg (Wang, 2002).

### 3 Conclusions

Our results showed that the bioavailability of inorganic Hg<sup>2+</sup> is affected by DOM concentration, and the entrance to the pelagic food webs occurs mostly through two processes: passive and active accumulation. The accumulation of Hg<sup>2+</sup> by algae was found to be rapid and dominated by passive adsorption, while the incorporation of Hg<sup>2+</sup>



**Fig. 6** Radiolabeled Hg<sup>2+</sup> incorporation by the calanoid copepod *Boeckella antiqua* during 24 hr incubations in which the copepod was either fed *Cryptomonas* algal cells (3000 cells/mL) previously cultured (24 hr) with radiolabeled Hg<sup>2+</sup> (Hg<sup>2+</sup> in diet treatment) (a); or deprived of algal cells with incubation media amended with radiolabeled Hg<sup>2+</sup> (Hg<sup>2+</sup> in water treatment) (b).

into the zooplankton was dominated by trophic or dietary transfer. CDOM concentration and character affected the adsorption of Hg<sup>2+</sup> by algae through competitive binding. Higher concentrations of CDOM, and particularly HMW humic or fulvic compounds were related to lower Hg<sup>2+</sup> adsorption. This effect was carried through to the zooplankton. Although CDOM played little part in direct adsorption of Hg<sup>2+</sup> on zooplankton (< 1% of radiolabeled Hg<sup>2+</sup>), the dietary uptake of Hg<sup>2+</sup> by *Boeckella* (24%) was remarkably similar to the adsorption of Hg<sup>2+</sup> in their prey *Cryptomonas* (up to 27%). Collectively, our results help reveal the relative importance of DOM and its influence on the accumulation of Hg<sup>2+</sup> by planktonic organisms, and by extension on the fate of Hg in pelagic food webs of Andean Patagonian lakes.

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