

## **Role of prey subcellular distribution on the bioaccumulation of yttrium (Y) in the rainbow trout.**

Authors: Pierre-Yves Cardon<sup>1</sup>, Olivier Roques<sup>2</sup>, Antoine Caron<sup>1</sup>, Maikel Rosabal<sup>3</sup>, Claude Fortin<sup>4</sup>, and Marc Amyot<sup>1\*</sup>

<sup>1</sup> GRIL, Université de Montréal (UdeM), Département de sciences biologiques, Complexe des sciences, C.P. 6128, succ. Centre-Ville, Montréal (Québec), Canada, H3C 3J7

<sup>2</sup> Université de La Rochelle, Faculté des sciences et technologies, avenue Michel Crépeau, La Rochelle, France, 17042

<sup>3</sup> GRIL, Université du Québec à Montréal (UQAM), Département des sciences biologiques, 141 avenue du Président-Kennedy, Montréal (Québec), Canada, H2X 1Y4

<sup>4</sup> Institut National de la Recherche Scientifique, Centre Eau Terre Environnement (INRS-ETE), 490 rue de la Couronne, Québec (Québec), Canada, G1K 9A9

\*Corresponding author: e-mail address: [m.amyot@umontreal.ca](mailto:m.amyot@umontreal.ca); Phone 1-514-343-7496.

GRIL, Université de Montréal (UdeM), Département de sciences biologiques, Complexe des sciences, C.P. 6128, succ. Centre-Ville, Montréal (Québec), Canada, H3C 3J7

Authors' e-mail addresses: [pierre-yves.cardon@umontreal.ca](mailto:pierre-yves.cardon@umontreal.ca);

[olivier.roques@etudiant.univ-lr.fr](mailto:olivier.roques@etudiant.univ-lr.fr); [antoine.caron@environnement.gouv.qc.ca](mailto:antoine.caron@environnement.gouv.qc.ca);

[rosabal.maikel@uqam.ca](mailto:rosabal.maikel@uqam.ca); [claud.fortin@ete.inrs.ca](mailto:claud.fortin@ete.inrs.ca)

## Abstract

Our knowledge of the processes leading to the bioaccumulation of rare earth elements (REE) in aquatic biota is limited. As the contamination of freshwater ecosystems by anthropogenic REE have recently been reported, it becomes increasingly urgent to understand how these metals are transferred to freshwater organisms in order to develop appropriate guidelines. We exposed rainbow trout (*Oncorhynchus mykiss*) to an REE, yttrium (Y), to either a range of Y-contaminated prey (*Daphnia magna*) or a range of Y-contaminated water. For the feeding experiment, the relationship between the Y assimilation by *O. mykiss* and the Y subcellular fractionation in *D. magna* was evaluated. Assimilation efficiency of Y by *O. mykiss* was low, ranging from 0.8 to 3%. These values were close to the proportion of Y accumulated in *D. magna* cytosol, 0.6-2%, a theoretical trophically available fraction. Moreover, under our laboratory conditions, water appeared as a poor source of Y transfer to *O. mykiss*. Regardless of the source of contamination, a similar pattern of Y bioaccumulation among *O. mykiss* tissues was revealed: muscles < liver < gills < intestine. We conclude that the trophic transfer potential of Y is low and the evaluation of Y burden in prey cytosol appears to be a relevant predictor of Y assimilation by their consumers.

Keywords: yttrium; subcellular partitioning; trophic transfer; metal; fish; rare earth elements

Capsule:

The trophic transfer potential of the rare earth element yttrium is low and the evaluation of Y burden in prey cytosol is a relevant predictor of Y assimilation by their consumers.

## Introduction

Rare earth elements (REE) find applications in the electrification of transport, in wind energy production, in medical imaging, in agriculture and in many new technologies (Gwenzi et al., 2018). In recent years, the environmental risks associated with REE have received increasing attention (González et al., 2015). Indeed, as a direct consequence of our high consumption of REE (Alonso et al., 2012), cases of natural freshwater aquatic ecosystems contaminated by these metals as a result of human activity have been reported (Kulaksız and Bau, 2011; Ma et al., 2018). These elements are often divided according to their mass into two groups, the light (LREE) and the heavy (HREE) REE, with the heavier ones considered by some authors as most toxic (González et al., 2015). Among the HREE, yttrium (Y) is the most abundant in the earth's crust but studies on Y behaviour and toxicity in freshwater ecosystems are scarce. It is therefore urgent to improve our knowledge of the risk associated with Y and HREE in general. Understanding how Y is internalised into freshwater organisms commonly consumed by humans, such as fish, is particularly important for the future assessment of the environmental and health risks of HREE.

Fish acquire metals through two uptake routes: water and food. Although water is often a significant source of metal assimilation (Ahlf et al., 2009), it is well established that food can also be an important source for some metals, such as selenium (Økelsrud et al., 2016), cadmium (Croteau et al., 2005), and methylmercury (Hall et al., 1997).

The gills and the gastrointestinal tract are the main sites of metal uptake in fish. Waterborne metal uptake in particular occurs largely across the gills. From this entry

point, metals can then be biodistributed in different organs. Models, such as the gill site interaction model (GSIM), the free ion activity model (FIAM) and the biotic ligand model (BLM), involving these different factors, have been developed for assessing this uptake (Pagenkopf, 1983; Campbell, 1995; Di Toro et al., 2001) .

Metals may also enter the body through diet. If, for organic contaminants, the lipophilicity of the contaminant seems to be a key parameter (Arnot and Gobas, 2006), for inorganic contaminants such as metals no consensus as to the physical or chemical factor controlling trophic transfer has yet been found. However, analysis of the subcellular fractionation of the metal considered in the prey could give an indication of its assimilative capacity by the predator. Indeed, several studies observed a correlation between the proportion of metal accumulated in the most soluble components of prey cells (e.g. cytosolic proteins, microsomes, etc.) and the assimilation efficiency of this metal by the predator (Reinfelder et al., 1994; Dubois and Hare, 2009a; Wallace and Luoma, 2003). Thus, it could be possible to predict the trophic transfer potential of a metal from the analysis of its subcellular fractionation in prey.

In a recent study (Cardon et al., 2019), we established that more than 75% of Y is accumulated in insoluble fractions (i.e. cellular debris and NaOH-resistant fraction) in *Daphnia magna* and *Chironomus riparius* which are typical trout prey. This Y accumulation in putative trophically unavailable fractions suggests a weak potential of trophic transfer for this metal. In addition, measurements of REE along trophic webs of temperate (Amyot et al., 2017) and arctic (MacMillan et al., 2017) freshwater ecosystems, demonstrate a biodilution of these metals with increasing trophic levels rather than a biomagnification.

This paper aims to: 1) compare the relative importance of diet and water as uptake pathways for Y in fish; 2) study Y biodistribution; 3) establish the potential for trophic transfer for Y under controlled laboratory conditions; and 4) assess whether the analysis of Y subcellular fractionation in prey can be used to predict its trophic transfer. For this purpose, feeding experiments on the rainbow trout (*Oncorhynchus mykiss*), a freshwater fish commonly used in ecotoxicology and consumed by humans, were performed with a range of Y-laden *D. magna* as prey. In parallel, exposures of *O. mykiss* to Y-spiked water, free of food, were set up to compare the potential of Y transfer from water and from food. Finally, Y partitioning between different tissues of *O. mykiss* (i.e., muscles, liver, intestine and gills) was assessed in each experiment to determine the main organs where this metal accumulates.

## Materials and methods

### **Sources of animals**

*Daphnia magna* was cultured at the *Centre d'expertise en analyse environnementale du Québec* (CEAEQ, Quebec City, QC, Canada). *Oncorhynchus mykiss* individuals were purchased from the *Pisciculture des Arpents Verts* (Sainte-Edwidge, QC, Canada) and were acclimated to laboratory conditions for at least 3 weeks prior to their use. Exposure solutions were spiked using dilutions of a standard Y solution (10,000 µg/ml Y in 3% HNO<sub>3</sub> by weight, TraceCERT<sup>®</sup>, FLUKA).

## **Accumulation of Yttrium from water**

Fish ( $1.6 \pm 0.2$  g) were exposed to Y-enriched dechlorinated tap water for 5 d in groups of seven individuals in 20 L aerated aquaria lined with polypropylene (PP) bags (one aquarium for each concentration level; pH 7.8,  $63 \text{ mg CaCO}_3 \text{ L}^{-1}$ ,  $15 \pm 0.2$  °C). Chemical characteristics of the tap water are given in Supporting Information (SI; Table S1). Fish were not fed 24 h before and during the exposure period. Five Y nominal exposure levels plus the control ( $0 \text{ } \mu\text{g Y L}^{-1}$ ) were tested: 52, 86, 144, 240 and  $400 \text{ } \mu\text{g Y L}^{-1}$ . In addition, dissolved Y concentrations were measured every day during the exposure period (SI, Table S2). Yttrium exposure conditions over the bioconcentration assay are given in SI, Table S2. We observed a decrease over time of Y dissolved concentrations, likely due in part to Y adsorption to exposure vessels and to Y precipitation (subsequent analysis of tap water used revealed the presence of sufficiently high concentrations of phosphate to suggest precipitation).

## **Trophic transfer experiment**

### ***Exposure of prey to Y***

*D. magna* neonates (< 24 h) were reared for 7 d in 15 L aquaria lined with a PP bag filled with dechlorinated tap water (300 individuals per aquarium). They were then transferred for 24 h to another set of aquaria also lined with a PP bag but filled this time with 15 L of reconstituted water (pH  $7.8 \pm 0.1$ ,  $89 \pm 2 \text{ mg CaCO}_3 \text{ L}^{-1}$ ,  $21 \pm 1$  °C) spiked with Y. After this exposure period, Y-spiked *D. magna* were transferred for 5 min in a 1 mM EDTA

solution to remove Y adsorbed to their body surface, rinsed with Milli-Q water, before being dried on Kimwipes® and stored in a 50-mL tube at -80 °C.

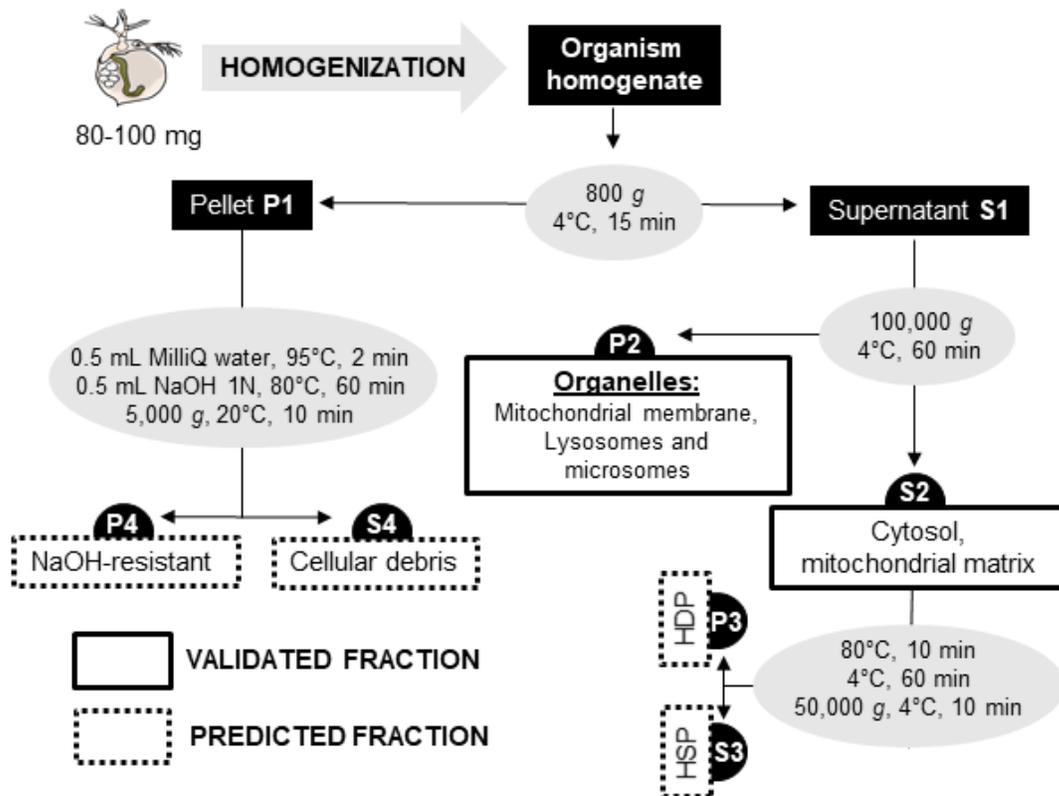
To obtain a range of Y-laden *D. magna*, we prepared five batches of *D. magna* exposed to one of the five following nominal concentrations all of which were subsequently analytically determined: 0 (control), 86, 144, 240 and 400  $\mu\text{g Y L}^{-1}$ . When the mass needed for each level of the feeding experiment was reached, Y-laden daphnids were pooled into groups of  $20 \pm 4$  mg of wet weight (ww), corresponding to the daily meal for one fish, and stored at -80 °C into 96-wells microplate (Greiner Bio-one) pre-soaked in  $\text{HNO}_3$  (15%, v/v, Optima grade, Fisher Scientific) and rinsed in Milli-Q water, each well containing one group.

#### ***Subcellular fractionation of D. magna***

Five samples of about 80-100 mg of *D. magna* of each Y exposure concentration were subjected to the subcellular fractionation procedure, based on Wallace et al. (2003) and customized for *D. magna* (Cardon et al., 2018) (Fig.1). Each *D. magna* sample was suspended in Tris-HCl (25 mM; OmniPur) sucrose buffer (250 mM; pH 7.4; Sigma Aldrich) at a ratio of 1:4 (weight [mg]: buffer volume [ $\mu\text{L}$ ]). Then, the suspended sample was ground with a Potter-Elvehjem pestle at 570 rpm 2 s repeated 10 times, with a 30-s interval of rest between each homogenization period. The resulting homogenate was centrifuged at 800 g for 15 min. The supernatant was collected and the pellet was resuspended in Tris-sucrose buffer at a ratio of 1:4 before disruption with an ultrasonic probe (Branson 250, with a 4.8 mm diameter microtip probe), at a power of 22 W, with pulses at  $0.2 \text{ s}^{-1}$  (20%) for 1 min. The resulting homogenate was pooled and mixed using a vortex with the first supernatant for a final ratio between sample and buffer of

1:8. Subsequently homogenates were separated into five partially validated fractions by differential centrifugation as described in Figure 1: P2) organelles (mitochondrial membranes, lysosomes and microsomes); P3) Heat-Denatured Proteins (HDP) including enzymes; S3) Heat-Stable Proteins (HSP) including metallothioneins; S4) Debris (nuclei, cellular membrane and debris); and P4) NaOH-resistant fraction (granules and potentially the daphnid chitinous exoskeleton).

Centrifugations (< 25,000 g) were performed using an IEC Micromax centrifuge (Thermo IEC) whereas a WX ULTRA 100 centrifuge (Sorval, Ultra Thermo Scientific) equipped with a F50L-24 X1.5 rotor (Fisher Scientific) was used for ultracentrifugation ( $\geq 25,000 g$ ).



**Figure 1** – Subcellular fractionation protocol customized for *D. magna* as developed in Cardon et al. (2018). P: Pellet; S: Surpernatant; HDP: Heat Denatured Proteins; HSP:

Heat Stable Proteins. Validated fractions are those that are derived from our enzymatic validation protocol (see text); predicted fractions are those that cannot be validated enzymatically and are therefore predicted from the literature.

### ***Exposure of *O. mykiss* to Y-laden daphnids***

Fish ( $0.93 \pm 0.14$  g) were exposed individually in 1-L aerated glass beakers filled with dechlorinated tap water (pH 7.8,  $63 \text{ mg CaCO}_3 \text{ L}^{-1}$ ,  $15 \pm 0.2$  °C). Seven individuals were used for each Y exposure levels of daphnids. Prior to the experiment, fish were fed with control daphnids for 10 d. Then, for 5 d, fish were fed 2% of their body weight (wet weight of prey to wet weight of fish) daily with their corresponding Y-laden daphnids. All daphnid pellets were consumed within seconds of being added. Every day, faeces were removed with a 50-mL glass pipette after a 1-h feeding period to avoid subsequent Y release to the medium. In addition, to monitor potential Y efflux from fish (from urine and feces), the water of each experimental chamber was sampled using a 20-mL PP syringe, then filtered through a  $0.45 \mu\text{m}$  polyethersulfone syringe filter to measure dissolved Y concentrations daily. Chemical characteristics of the water medium at the beginning of the trophic transfer experiment and Y exposure condition over the trophic transfer assay are given respectively in SI, Table S1 and Table S3.

### **Fish treatment and storage at the end of the experiments**

Coefficients of variation of fish average wet weight between each exposure level did not exceed 5%. At the end of the experiment, fish were depurated for 24 h. They were then euthanized with clove extract, rinsed with EDTA (1 mM), then with Milli-Q water, dried with Kimwipes®, and finally weighed and measured for their fork length. Their gills,

intestine, liver, muscle tissues and remaining body parts were collected with a surgical razor blade. Tissues were put in 1.5 mL pre-weighed and acid-washed PP microcentrifuge tube, and remaining body parts were collected in 50 mL tube. They were then weighed, freeze dried, weighed again and stored at -80 °C until Y analyses.

### **Yttrium measurements and quality control**

All labware was soaked in HNO<sub>3</sub> (15%, v/v, Optima grade, Fisher Scientific) and rinsed seven times in Milli-Q water before use, to minimize Y accidental contamination. Centrifuged pellet fractions resulting from the fractionation of *D. magna* (NaOH-resistant fraction, organelles and HDP), aliquots sampled as homogenate and *O. mykiss* tissues were freeze-dried for 24 h, weighed and stored at -80 °C. Every subcellular fraction and *O. mykiss* tissues were digested at 65 °C in 500 µL of HNO<sub>3</sub> (70%, v/v) whereas *O. mykiss* remaining parts were subjected to the same procedure but in 4 mL of HNO<sub>3</sub>. Then, 9.5 mL and 45 mL of Milli-Q water were added in the digestates of *D. magna* fractions and *O. mykiss* tissues, respectively.

Concentrations of Y in water and in organism fractions and tissues were measured with an inductively coupled plasma-mass spectrometer (ICP-MS; Thermo Elemental X Series). To ensure quality of these measurements, samples of similar weight of a certified standard reference material, BCR 668 (mussel tissue, Institute for Reference Materials and Measurements) underwent the same digestion procedure and analysis. Mean ( $\pm$  SD) recoveries of BCR 668 reference samples (n = 10) were within the range of certified values for Y ( $90.0 \pm 0.1\%$ ). To verify metal recovery following subcellular fractionation, 40-µL subsamples of tissue homogenate were analyzed. Recovery was expressed as the ratio of the sum of the Y burden in the five fractions divided by the total homogenate Y

burden in *D. magna*, multiplied by 100. Mean ( $\pm$  SD) recovery values of Y was  $99 \pm 4\%$  (n = 25).

### Calculation and statistical analyses

Yttrium concentrations in *O. mykiss* tissues and *D. magna* homogenates were expressed by wet weight ( $\text{mg kg}^{-1}$  ww). Yttrium burden in a given subcellular fraction of *D. magna* was divided by the sum of Y burden in all fractions and multiplied by 100 to assess the relative contribution of each subcellular fraction to the total Y burden in terms of percentages (%).

Assimilation efficiency (AE) was calculated according to Lapointe et al. (2009) as follows:

$$AE = \left[ \frac{M_{O. mykiss} - M_{\text{control}}}{M_{D. magna}} \right] \times 100$$

where  $M_{O. mykiss}$  is the Y burden in fish fed Y-laden daphnids,  $M_{\text{control}}$  is the average Y burden in fish fed uncontaminated daphnids and  $M_{D. magna}$  represents the total amount of Y provided to fish from Y-laden *D. magna*. This equation for AE defines the bioaccumulation factor. It roughly estimates AE if it is assumed that the prey is consumed and that physiological losses of Y and growth of the test subject during the exposure and depuration phases are inconsequential. In our study, all food pellets given to the fish were eaten within minutes. Further, we assumed that fish growth during the experiment was minimal. Also, efflux rates of Y from fish to water were also considered low, since aqueous Y levels remained relatively low during the experiment (Table S3).

Data are expressed as mean  $\pm$  standard deviation (SD). Significant differences were

tested with a one-way analysis of variance (ANOVA), followed by Tukey pairwise comparison test ( $p < 0.05$ ). Linear regression analyses were performed to study the relationship between Y accumulation according to Y exposure level. The assumptions of normality and homoscedasticity were verified by Shapiro-Wilk's and Levene's tests, respectively. When these assumptions were not met, a natural log transformation was successfully applied on the data. Statistical analyses were performed using R software version 3.4.4.

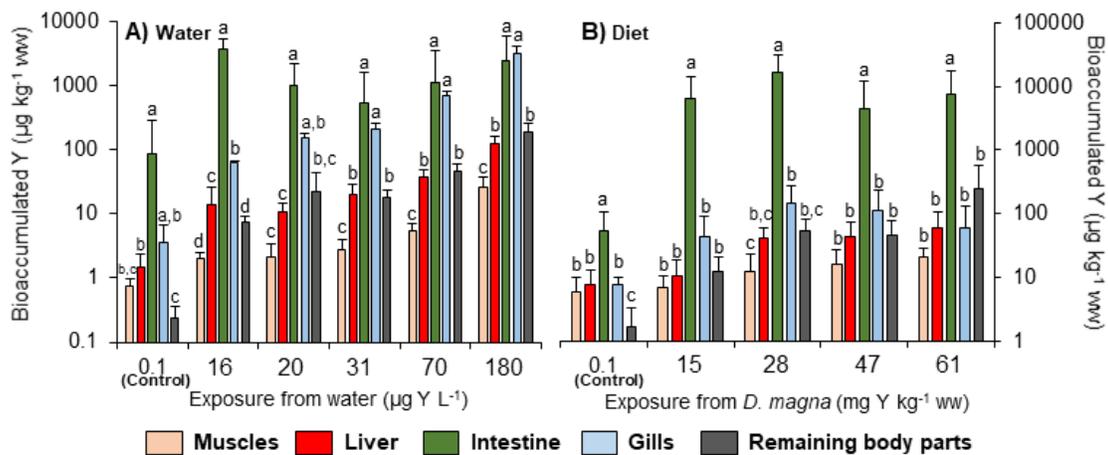
## Results and Discussion

There are few studies on fish biodistribution and subcellular fractionation of group A metals (Kinraide, 2009; Nieboer and Richardson, 1980), including REE. Therefore, in the discussion that follows, our results on Y in *O. mykiss* will be essentially compared to metals belonging to the intermediate group such as Cd, or group B such as Cu, for which more data on accumulation, biodistribution and subcellular fractionation in fish are available.

### **Bioaccumulation and distribution of Y in *O. mykiss* tissue depending on the exposure source**

We compared the bioaccumulation of Y from water and diet in different trout organs. In the water-only exposures, when excluding the maximal exposure level, the intestine presented the top values of bioaccumulated Y (Fig. 2A). However, unlike other tissues, no relationship was found between the Y content in the intestine and the exposure concentration (Fig. 2A). In contrast, a strong positive correlation ( $R^2 = 0.7$ ,  $p = 0.003$ )

was observed between Y concentration in gills and water exposure level (SI, Fig. S1A). At the maximal exposure of 180  $\mu\text{g Y L}^{-1}$ , the Y concentrations in the gills and the intestine were statistically similar. Hence, the Y concentration of the gills increased with aqueous Y and at the highest exposure attained a concentration equal to the concentration of the intestine (Fig. 2A). At this maximal exposure, concentrations of Y in the liver and in the remaining body parts were more than ten times lower than in the gills and the intestine. In general, in the water exposure test, the concentrations of Y in the liver and in the remaining parts of the fish body were similar and at an intermediate level between the values measured in the muscles and gills. For clarity, the highest concentration in the gill occurred at the highest exposure.



**Figure 2** - Bioaccumulation of Y (Mean  $\pm$  SD;  $\mu\text{g kg}^{-1}$  ww; n=5-7) in different tissues of *O. mykiss* following two types of exposures **A)** from water ( $\mu\text{g Y L}^{-1}$ ) or **B)** from diet ( $\text{mg Y kg}^{-1}$  ww of daphnid), over an exposure range. Different letters indicate a significant difference of accumulation among the different tissues of *O. mykiss* for a given exposure level (ANOVA, followed by Tukey pairwise comparison test,  $p < 0.05$ ). The lowest exposure level for both experiments represents the control. Exposure

conditions over these tests, especially Y dissolved concentrations, are given in SI, Tables S2 and S3.

When exposing fish to Y through diet, concentrations of Y in the intestine were 7 to 1370 times higher than those in all other tissues (Fig. 2B). In contrast, no significant differences were measured between Y concentrations in the liver (min-max: 11-61  $\mu\text{g Y kg}^{-1}$  ww), in the remaining parts of the fish body (12-243  $\mu\text{g Y kg}^{-1}$  ww) and in the gills (44-143  $\mu\text{g Y kg}^{-1}$  ww) (Fig. 2B), when excluding the control. Furthermore, unlike the water exposure trial, no relationship between Y exposure concentrations in gills and the level of Y in *D. magna* (SI, Fig. S1B) was observed.

Regardless of the source of exposure, the Y content determined in muscle tissues was significantly lower than in other tissues with average values between 1 and 25  $\mu\text{g Y kg}^{-1}$  ww (Fig. 2). In addition, for both exposure trials, the burden of Y in the intestine was, on average, higher than the whole fish less the intestine. Overall, a similar pattern of Y partitioning between *O. mykiss* tissues was observed for both exposure routes, with the following sequence of Y concentrations in body parts: muscles < liver < gills < intestine.

Other field studies have reported low Y concentrations in muscles compared to other tissues. In ten freshwater fish species from a reservoir in the state of Washington (USA), muscles had lower REE concentrations (on average at least three times lower for Y) than in the whole body and carcass (Mayfield and Fairbrother, 2015). Moreover, significantly larger differences were measured in three fish species from Canadian temperate lakes for which whole-body REE concentrations were on average at least 40 times higher than muscle concentrations (Amyot et al., 2017). In general, metal concentrations in fish

muscles have been reported to be lower in most cases than in other organs (Subotic et al., 2014).

Our results further suggest that gills are among the main tissues for Y accumulation in fish. Since gills were thoroughly rinsed with EDTA solution and water, it is unlikely that these high levels are caused by external particles (from food or feces) trapped in gills. This importance of gills as an REE accumulation site has previously been reported (Sun et al., 1996; Tu et al., 1994). Analysis of Y subcellular fractionation in *O. mykiss* gills could be another step to investigate REE accumulation in this organ. Preferential accumulation of Zn in cellular debris fractions (including cell membranes) of *O. mykiss* gills cells has already been reported (Sappal et al., 2009). Hence, we hypothesize that Y will be mainly accumulated in cellular debris fractions of gills cells as well.

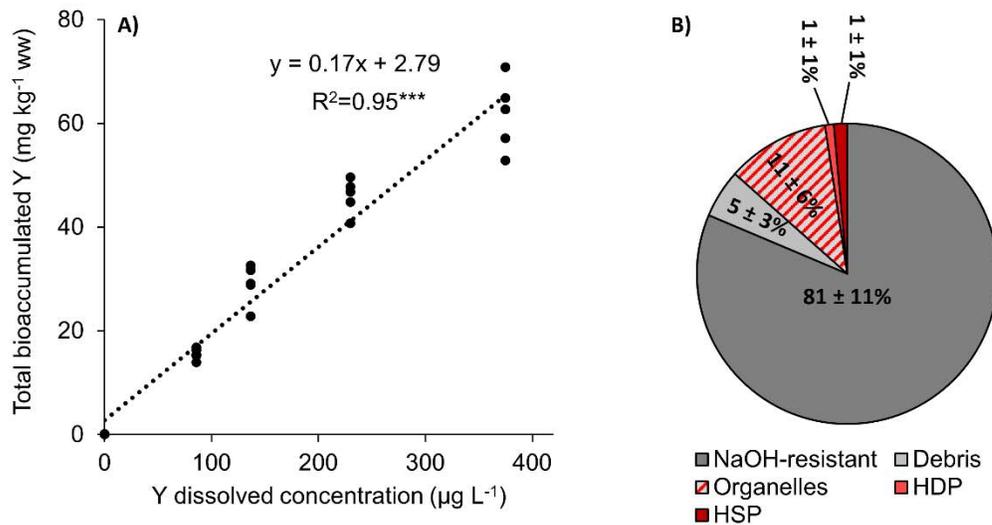
The relatively high concentrations of Y in the gills may have detrimental effects on fish health. REE are known to be antagonists of Ca uptake; lanthanum for example, is commonly used as a Ca<sup>2+</sup> channel blocker in studies on metal uptake by gills (Hogstrand et al., 1996). Indeed, gills are considered as the first route of Ca uptake by freshwater fish (Marshall, 2002), Ca uptake by the intestine being considered as secondary (Flik et al., 1996). Hence, the accumulation of Y in the gills of *O. mykiss* could lead to Ca deficiency in this organism. The assimilation of essential metals, such as Zn, which can be taken up through Ca<sup>2+</sup> channels (Hogstrand et al., 1996) could also be limited due to the presence of Y. Therefore, we suspect that Y could have adverse effects on the ionoregulation within *O. mykiss*. Like La, we suspect that Y blocks Ca and Zn internalization through the apical gill membrane but does not enter the gill cell cytosol (Perry and Flik, 1988).

The observed preferential accumulation of Y in internal organs like the intestine, seems in agreement with the literature. For instance, laboratory studies on the biodistribution of seven REEs including Y have reported the following concentration pattern in *Cyprino carpio* : muscles < skeleton < gills < internal organs (Sun et al., 1996; Tu et al., 1994). Note that organs included in internal organs in these two studies are not defined. This pattern was also reported in *O. mykiss* for other metals, including Cd, Cu and Se, following an exposure to a diet enriched with these metals (Handy, 1992; Misra et al., 2012). For Se and Cu however, liver showed higher concentration than gills. Similarly, the importance of the liver (compared to muscle) as a site of REE accumulation in vertebrates has already been reported in Arctic ecosystems (MacMillan et al., 2017). Furthermore, in 28-d exposures to similar Y concentrations, the Y concentration in the liver of *O. mykiss* was about ten times higher than the one we measured here after 5 days (Cardon et al., 2019). This suggests that Y concentration in *O. mykiss* tests may not have reached a steady state at the end of our tests. Therefore, we can assume that liver could be a more important organ of Y accumulation for fish in the field than what we evaluated. Another trial involving both water and diet as exposure routes and a longer period of exposure to Y, could be a way to test this hypothesis.

### **Range of bioaccumulation and subcellular fractionation in daphnids**

A strong linear relationship ( $R^2 = 0.95$ ,  $p = 3.0 \cdot 10^{-16}$ ) was observed between Y bioaccumulation by *D. magna* and the Y exposure level (Fig. 3A). The levels of Y accumulation by *D. magna* ranged from  $0.05 \pm 0.03$  to  $62 \pm 7$  mg Y kg<sup>-1</sup> ww. At the subcellular level, Y was mainly found in the NaOH-resistant fraction with, on average,

81 ± 11% of the total Y (Fig. 3B). Organelles contained 11 ± 6% of the total Y, while the remainder (<10%) was divided between the other fractions (debris, HDP and HSP).



**Figure 3** - Characterization of Y accumulation in daphnids. **A)** Relationship between the Y exposure level and the total of Y bioaccumulated by *D. magna* ( $\text{mg Y kg}^{-1} \text{ ww}$ ,  $n = 5$ ); **B)** Average percentage of Y for all exposure levels (mean  $\pm$  SD; %;  $n = 5$ ) in each subcellular fraction of Y-exposed *D. magna*.

Such high values of Y bioaccumulated by *D. magna* (Fig. 3A) have previously been reported in laboratory studies (Cardon et al., 2018; Yang et al., 1999), but are at least 1000 times higher than those reported in zooplankton from the field (Amyot et al., 2017; MacMillan et al., 2017). Yttrium subcellular fractionation reported here is similar to the Y subcellular fractionation in *D. magna* assessed with a longer exposure period of 7 d (Cardon, 2018).

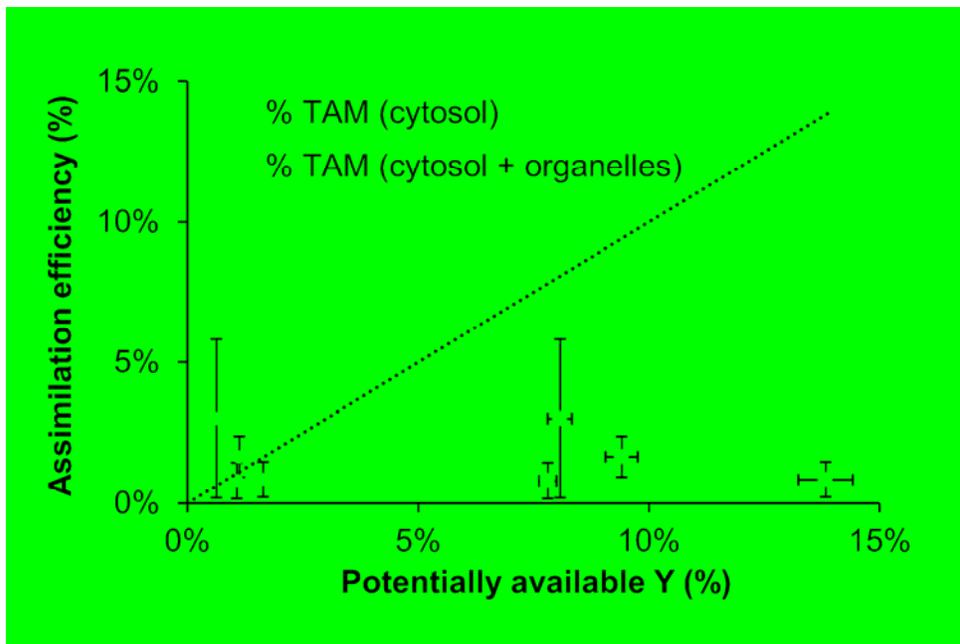
According to several authors (Dubois and Hare, 2009a; Wallace and Luoma, 2003), only the part of metal bound to the putative trophically available fraction in the prey cells, the so-called trophically available metal (TAM), will be transferred to their consumer. Therefore, the trophic transfer potential of a metal could be assessed based on its subcellular fractionation in the prey. Our results suggest a weak potential of Y trophic transfer from *D. magna* to its predator. Indeed, by considering only Y found in the cytosol (HDP and HSP) of *D. magna* as the TAM (Wallace and Lopez, 1996), less than 3% of Y in this organism would be theoretically trophically available. Even if we include Y associated to organelles in the TAM pool, as recommended by several authors (Dubois and Hare, 2009a; Wallace and Luoma, 2003), this transfer potential remains under 15%. In contrast, the proportion of TAM (including organelles), reported for Ni, Tl and Zn in *D. magna* exceeded 40% (Lapointe et al., 2009; Wang and Guan, 2010).

Note that daphnids used as prey in this experiment were contaminated through exposure to Y-enriched water and not Y-enriched diet. It is possible that subcellular fractionation in *Daphnia* and subsequent trophic transfer to trout could be different if an Y-enriched diet would have been used.

### **Relationship between assimilation efficiency and trophically available metal**

Percentages of Y found in the cytosol of *D. magna* appeared to be very close to the AE in *O. mykiss*. Over the trophic transfer trial, percentages of Y in *D. magna* cytosol ranged from 0.63 to 1.6% while AE ranged from 0.80 to 3.0% (Fig. 4). When adding organelles, the TAM fraction increased significantly (range: 7.8 to 14%) and departed from the measured AE (Fig. 4). Moreover, the spread of % of potentially available Y increased with the addition of Y recovered in *D. magna* organelles to the pool of TAM. These

results suggest that the Y in *D. magna* cytosol was trophically available while the Y associated to organelles was not. In contrast, many authors have concluded that at least a part of the metal from the organelles fraction was available: for instance, Cd transferred from *Potamocorbula amurensis* to *Palaemon macrodactylus* (Wallace and Luoma, 2003) and from *Gammarus lawrencianus* to *Palaemonetes pugio* (Seebaugh et al., 2006); Cd, Zn, Ni, Tl and Se transferred from *Chironomus riparius* to *Sialis velata* (Dubois and Hare, 2009a, 2009b; Dumas and Hare, 2008). Even NaOH-resistant fraction was considered as potentially available in some studies (Cheung and Wang, 2005; Rainbow et al., 2007).



**Figure 4** - Relationship between assimilation efficiency of dietary Y in *O. mykiss* and the proportion of Y assumed to be trophically available (TAM) in *D. magna* with or without the inclusion of organelles in TAM (means SD, n = 5-7). The dashed line illustrates a 1:1 relationship.

Different prey species can have similar proportions of a theoretically available metal in their cells but still have large differences of AE for their consumers (Pouil et al., 2016). In fact, it is usually accepted that subcellular components included in the TAM pool differs depending on the prey and the predator (Rainbow et al., 2011). For instance, for a given prey, the assimilation of metal-containing fractions by a predator will depend on the strength of its digestive processes To get insight on the relative strength of this digestive process for *O. mykiss*, we can compare metal AE of several fish with those of invertebrate predators fed with the same prey. Three marine fish, *Lutjanus argentimaculatus*, *Penophthalmus cantonensis* and *Ambassis urotaenia*, assimilated 6-33% and 5-46% of respectively Cd and Zn from copepod and clam prey (Xu and Wang, 2002) while two invertebrates, *Palaemonetes varians* and *Hinia reticulata*, assimilated on average more than 60% of the same metals from the same prey (Rainbow and Smith, 2010). Similarly, *Sialis velata*, an invertebrate predator, assimilated more than 70% of Cd, Ni and Tl from two invertebrate prey (Dubois and Hare, 2009b; Dumas and Hare, 2008), while several fish, including *O. mykiss*, fed with similar metal-laden prey, assimilated less than 10% of these metals (Bécharde et al., 2008; Lapointe et al., 2009; Ng and Wood, 2008; Steen Redeker et al., 2007a). Finally, the barnacle, *Balanus amphitrite*, assimilated more than 63% of Cr from zooplankton prey (Wang et al., 1999) versus less than 12% for fish fed with similar prey (Ni et al., 2000). All these examples could suggest a “softer” digestive process for some fish including trout.

Beyond the type of digestive processes, the similar AE and TAM values we measured for Y could be simply the result of the low AE of nonessential metal for fish. Some studies comparing trophic transfer of essential metals such as Zn or Se from a variety of prey to

fish have reported close values between AE and metal bound to prey cytosol (Zhang and Wang, 2006). Nonetheless so far, except for Hg, most studies on the relationship between nonessential metal trophic transfer to fish and the percentage of this metal theoretically available in their prey observed an AE far lower than predicted and hence departed from the expected 1:1 line (representing a perfect correlation) with TAM. Indeed, in the following examples, metals present in the cytosol of the prey represented more than 30% of the total bioaccumulated metal while AE of the fish fed with these prey did not exceed 10%. It was the case for the trophic transfer of Cd from *Tubifex tubifex* to *Cyprinus carpio* (Steen Redeker et al., 2007b), for Cd from *Lumbriculus lumbriculus* to *O. mykiss* (Ng and Wood, 2008), for As from different prey (clam, copepod and fish) to *Terapon jarbua* (Zhang et al., 2011), for Ag from (Seabream, shrimp and ragworm) to *Scophthalmus maximus* (Pouil et al., 2015) and for Ni from *D. magna* or *C. riparius* to *Pimephales promelas* (Lapointe et al., 2009). Predictions of trophic transfer of nonessential metals to fish based on TAM analysis in its prey should thus be made with caution; indeed, the composition of the TAM fraction should not be assumed but should be directly validated through feeding experiments such as the ones presented here.

Overall, our results indicating low subcellular fractionation towards trophically available metal fractions are in agreement with the low AE for Y. Hence, this work provides mechanistic evidence that could explain low REE levels reported in fish in the field studies.

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