

## Freshwater food web studies: a plea for multiple tracer approach

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### ABSTRACT

#### Freshwater food web studies: a plea for multiple tracer approach

Food webs are complex systems of interactions between ecosystem species. Beyond the direct analysis of stomach contents, stable isotopes of carbon ( $\delta^{13}\text{C}$ ) and nitrogen ( $\delta^{15}\text{N}$ ) have been used widely to evaluate these trophic relationships and calculate the relative contribution of food sources to a consumer's diet using mixing models. However, there are still some constraints on the use of these traditional tracers that limit their output. Here, we briefly comment on the potential of using multiple tracers (i.e., stable isotopes of C, N and H; trace metals), and applying recent numerical approaches (i.e. Bayesian mixing models) to advance the understanding of complex aquatic food webs. Stable isotopes of hydrogen ( $\delta^2\text{H}$ ), normally used to examine large-scale migration patterns of terrestrial animals, have been recently proposed as a complementary trophic tracer in aquatic ecosystems. The principle for this application is the large isotopic difference in  $\delta^2\text{H}$  among food items that can be found in some aquatic systems. Other potential trophic indicators are such substances that accumulate through diet (e.g., trace metals). These substances are traditionally studied from bioaccumulation or toxicological perspectives, but there are indications that encourage their use for tracing food web interactions. Bayesian mixing models, which are able to incorporate several sources of variability and multiple food sources in the model, can help to solve puzzling results. In summary, we suggest that the simultaneous use of multiple tracers will provide more reliable results than any of them in isolation. The challenge is to develop methods to combine them enhancing their strengths and minimizing uncertainty.

**Key words:** Stable isotopes, food webs, deuterium, trace metals, Bayesian mixing models.

### RESUMEN

#### Estudio de redes tróficas acuáticas: una llamada al uso de múltiples marcadores

Las redes tróficas son sistemas complejos de interacciones entre las especies del ecosistema. Más allá del análisis directo de los contenidos estomacales, los isótopos estables del carbono ( $\delta^{13}\text{C}$ ) y nitrógeno ( $\delta^{15}\text{N}$ ) se han utilizado con frecuencia para evaluar las conexiones tróficas y calcular la contribución relativa de cada alimento en la dieta de los consumidores mediante modelos de mezcla. Sin embargo, todavía hay algunas restricciones en el uso de estos marcadores tradicionales que limitan su rendimiento. En este artículo comentamos brevemente la posibilidad de utilizar varios marcadores (i.e., los isótopos estables del C, N y H; metales traza), y aplicar nuevos métodos numéricos (i.e., modelos de mezcla bayesianos) para avanzar en la comprensión de las complejas redes tróficas acuáticas. Los isótopos estables del hidrógeno ( $\delta^2\text{H}$ ), que normalmente se usan para examinar patrones de migración de los animales terrestres a grandes escalas, se han propuesto recientemente como un trazador trófico complementario en los ecosistemas acuáticos. Su aplicación se basa en la gran variabilidad de  $\delta^2\text{H}$  entre las fuentes de alimento que se puede encontrar en algunos sistemas acuáticos. Otros posibles indicadores tróficos son tales sustancias que se acumulan a través de la dieta (por ejemplo, metales traza). Estas sustancias han sido tradicionalmente estudiadas desde perspectivas de bioacumulación o toxicológicas, pero hay indicios que estimulan su aplicación en el estudio de las interacciones tróficas. Los modelos bayesianos de mezcla, que son capaces de incorporar varias fuentes de variabilidad y múltiples fuentes de alimentos, pueden ayudar a resolver casos ambiguos. En resumen, sugerimos que el uso simultáneo de varios marcadores proporcionará resultados más fiables que cualquiera de ellos de forma individual. El reto está en desarrollar métodos para combinarlos aprovechando sus fortalezas y minimizando las incertidumbres.

**Palabras clave:** Isótopos estables, redes tróficas, deuterio, metales traza, modelos bayesianos de mezcla.

## INTRODUCTION

Ecosystems are organized communities of producers, consumers, and decomposers along with an abiotic environment that influences species growth, reproduction, and dispersal (Covich, 2001). The early approach to trophic structure was a simple linear food chain where species aggregate into discrete trophic levels (plants-herbivores-carnivores) with declining numbers of individuals at higher levels (Elton, 1927). Soon, this simplistic view of ecosystem trophic structure was revised. Lindeman (1942) introduced the tropho-dynamics viewpoint in an article of exceptional significance for ecology. This paradigm included some basic principles, such as that energy transfer efficiency between trophic levels should limit the size and length of the food chain; and that increase in biomass at higher trophic levels can only be sustained if biomass turnover at lower levels is higher. The latter, for instance, occurs in marine pelagic food webs, in which short-living algal cells (i.e., phytoplankton) support a large biomass of longer-living zooplankton, and the biomass of heterotrophs exceeds that of autotrophs (Odum, 1971; Gasol *et al.*, 1997). Currently, the food chain view (Fig. 1a) has been replaced by the more realistic

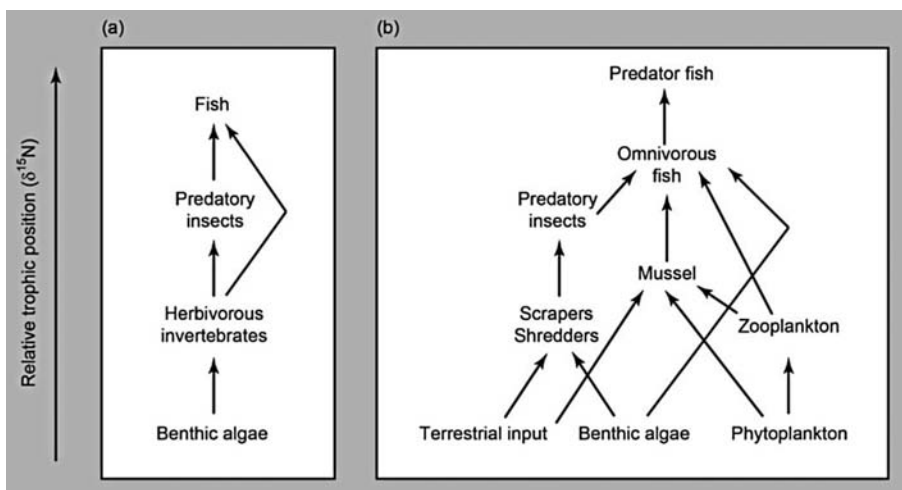
food web concept (Fig. 1b), which due to its complexity is a challenging research topic.

Food webs are networks of trophic interactions within an ecosystem, characterized by the number of species involved and the nature, number and intensity of their connections. The static view of trophic structure of ecosystems has been substituted by a concept that sees complexity and dynamism as an intrinsic property of food webs (Pimm, 1984; Polis & Strong, 1996). However, how variable trophic links are over space and time, how we can measure these complex interactions are questions difficult to approach.

Progress in the understanding of this complexity and dynamism requires development of new theoretical and operative frameworks, and the eventual merging of them. Here, we discuss pros and cons of the use of some new tracers (i.e.,  $\delta^2\text{H}$ , trace metals) and numerical approaches (i.e., Bayesian mixing models) that might be helpful to advance the understanding of the food web structure in aquatic ecosystems.

## STABLE ISOTOPE ECOLOGY

Stable isotopes have been used widely in ecological research during the last decades (West *et al.*,



**Figure 1.** Rather than ordinary food chains (a), trophic relationships between aquatic organisms constitute complex food webs (b), which are difficult to disentangle without a set of different tracers. *Más allá de simples cadenas tróficas (a), las relaciones tróficas entre los organismos acuáticos forman complejas redes tróficas (b), las cuales son difíciles de desenmarañar sin un conjunto diverso de trazadores.*

2006). Particularly, after the development of continuous flow methods (CF-IRMS) of isotopic analyses, which are faster and cheaper techniques, stable isotope analyses have expanded to a widespread use in biology. Stable isotopes are forms of a given element that differ in atomic mass since they have the same number of protons, but different number of neutrons. This mass difference generates a variation in abundance of the heavier to the lighter isotope in organism tissues due to the different reaction and transport rates for molecules. Stable isotope measurements are generally expressed as the relative isotope-ratio difference or isotope delta ( $\delta$ ) values. They are usually reported in parts per thousand (‰) deviations from an international standard, as follows:

$$\delta X = [(R_A/R_{\text{std}}) - 1] \quad (1)$$

where  $R_A$  and  $R_{\text{std}}$  are the isotope ratio of the heavier and lighter isotope of the element  $X$  (e.g.,  $^{13}\text{C}/^{12}\text{C}$ ,  $^{15}\text{N}/^{14}\text{N}$ , and  $^2\text{H}/^1\text{H}$ ) in the sample and the international standard (e.g., PDB, AIR, and VSMOW, respectively).

In ecological studies, stable isotopes of carbon ( $\delta^{13}\text{C}$ ) and nitrogen ( $\delta^{15}\text{N}$ ) have been applied to gain insight into food web structures (e.g., Peterson & Fry, 1987; Cabana & Rasmussen, 1994). Basically, the isotope ratio of a consumer reflects those of its diet with some trophic isotopic discrimination. The discrimination factor can vary according to species and tissues but, usually, average values from meta-analysis studies are applied in the calculations. Isotopic differences provide insight for investigating the relative use of food sources, contributions of different habitats to the entire food web, general degree of omnivory and other relevant aspects of the food web structure (France, 1995; Vander Zanden *et al.*, 1999). Stable isotopes provide some advantages to assess feeding relationships compared to traditional approaches (e.g., stomach content studies). For instance, they can trace the animal diet over different time periods, compared to the snapshot that using stomach content represents. Isotope turnover rates of each tissue vary and may provide in-

formation of animal diet over different time intervals (Karasov & Martínez del Rio, 2007). Tissues with fast turnover rates will achieve earlier the isotopic equilibrium and will reflect diet changes at shorter temporal scales.

## BAYESIAN MIXING MODELS

Mixing models evaluate the relative contribution of different food items to consumer's diet (Phillips & Gregg, 2003), and their estimation can be refined incorporating differences in food stoichiometry (Phillips & Koch, 2002). Among several existing models, the one-isotope, two-source model (Boecklen *et al.*, 2011) is extensively used. It is based on the following assumptions:

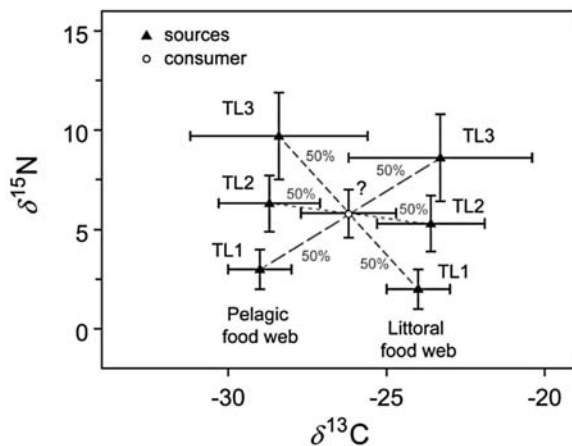
$$\delta_T = f_A (\delta_A + \Delta_A) + f_B (\delta_B + \Delta_B) \quad (2)$$

$$1 = f_A + f_B \quad (3)$$

where  $\delta_T$ ,  $\delta_A$ , and  $\delta_B$  are the isotope value in the consumer's tissue, source A and source B, respectively;  $f_A$  and  $f_B$  are the fractional contribution for each source; and  $\Delta$  is the diet-tissue trophic discrimination.

Trophic discrimination factors (e.g.,  $\Delta^{15}\text{N}$  and  $\Delta^{13}\text{C}$ ) must be assigned a priori to each dietary food component to build mixing models. There are some aspects that require attention before applying these models. For example, consumers may lie outside the mixing polygon delimited by all the potential sources because some key end-member source is lacking or there are large differences in stoichiometry among food sources.

Recent stable-isotope mixing models (e.g., SIAR and MixSIR; Moore & Semmens, 2008; Parnell *et al.*, 2010) implement the Bayesian approach that may enable more accurate estimates to track trophic links in complex food webs than traditional approaches. The advantages of these models to previous approaches are the possibility to incorporate the variation in diet-tissue trophic discriminations (McCutchan *et al.*, 2003; Caut *et al.*, 2009) and prior information. Diet-tissue trophic discrimination can vary among consumers depending on sampled tissue (Pinnegar



**Figure 2.** Hypothetical case illustrating the limitation of using two isotopic tracers in complex food webs. Three equally feasible solutions for the diet of a consumer can be estimated based on the  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values of its potential food. There are three combinations of two 50 % food items (illustrated by different grey broken lines) from distinct trophic levels (TL): (i) TL1 (pelagic) and TL3 (littoral); (ii) TL3 (pelagic) and TL1 (littoral); and (iii) TL2 (pelagic) and TL2 (littoral). Uncertainties of the isotopic values were estimated as standard deviations (SDs) from 1,000 bootstrap iterations of the mixing model. New values of model parameters were drawn from normal distributions described by the estimated means and SDs for each iteration. No trophic discrimination was assumed. *Caso hipotético que ilustra la limitación del uso de dos trazadores en redes tróficas complejas. Se dan tres soluciones igualmente plausibles para la dieta de un consumidor estimadas a partir de los valores de  $\delta^{13}\text{C}$  y  $\delta^{15}\text{N}$  de su comida potencial. Hay tres combinaciones de dos fuentes de comida con una contribución del 50 % (ilustradas por distintas líneas segmentadas grises) procedentes de distintos niveles tróficos (TL): (i) TL1 (pelágica) y TL3 (litoral); (ii) TL3 (pelágica) y TL1 (litoral); y (iii) TL2 (pelágica) y TL2 (litoral). La variabilidad de los valores isotópicos se estimó como desviaciones estándar (DS) de 1000 iteraciones de remuestreo del modelo de mezcla. Nuevos valores de los parámetros del modelo se extrajeron de distribuciones normales determinadas por las medias y DS para cada iteración. Se asumió que no hay discriminación trófica.*

& Polunin, 1999), protein composition and proportion in the diet (Kelly & Martínez del Río, 2010; Robbins *et al.*, 2010), food nitrogen content (Adams & Sterner, 2000), an animal growth and ingestion rates (Gaye-Siessegger *et al.*, 2004; Martínez del Río *et al.*, 2009). The uncertainty of model estimates increases when consumers potentially feed on many sources (Phillips & Gregg, 2003). This uncertainty may be reduced using stomach content analysis to disregard some food sources (Catalan *et al.*, 2004), or introducing it in the Bayesian mixing models as prior informa-

tion. In any case, stomach content is always useful to examine unexpected associations between food and consumers. However, it is necessary to keep in mind that stable isotopes and stomach contents are techniques that can refer to different time scales.

When using only two isotope tracers (e.g.,  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ ), consumers that potentially feed on totally different food sources can lie in the same isotopic niche (Fig. 2). Despite the advances of Bayesian mixing models, there is no way to disentangle the correct food composition. Figure 2 illustrates such case with a hypothetical example: the isotope values of a unique consumer in the  $\delta^{13}\text{C}$ - $\delta^{15}\text{N}$  space could be feasibly interpreted by three completely different diet compositions within a complex food web. In addition to this complex case, limited power for discerning source contributions also occurs when there is little isotopic differentiation among sources or high variation in the diet-tissue trophic discrimination (Phillips & Gregg, 2003; Moore & Semmens, 2008; Bond & Diamond, 2011). In complex food webs, additional constraints might be useful to simplify the mixing models; for instance, gut contents could be used to discard certain sources and, in cases where the isotopic values of some sources are not statistically different, these sources can be pooled to reduce their number. However, increasing the number of tracers, which increase the degrees of freedom for the estimation of trophic links, may be more powerful for solving misleading cases.

## STABLE ISOTOPES OF HYDROGEN

Stable hydrogen isotope ratios ( $\delta^2\text{H}$ ) have been used for tracking large-scale terrestrial migration movements and wildlife provenance. This approach is based on the well-known spatial isotope landscapes (or isoscapes) and the strong correlation between  $\delta^2\text{H}$  values in precipitation and those in tissues from a given location (Hobson *et al.*, 2012). Hydrogen isotopes have also been used to distinguish between allochthonous and autochthonous sources (leaf litter versus primary producers) in a consumer diet (Doucett *et al.*, 2007). Aside from these applications, the

large  $\delta^2\text{H}$  differences among the basal carbon sources of the food web in aquatic systems indicate that there is potential for distinguishing food web pathways. This means high  $\delta^2\text{H}$  variability among food web components and, thus, increased interaction resolution if used in combination with the traditional stable isotopes.

The use of  $\delta^2\text{H}$  as a dietary tracer in aquatic ecosystems is promising. However, previously, the mechanisms and processes that determine the variation of H isotopes in aquatic systems must be understood sufficiently in order to build a theoretical  $\delta^2\text{H}$  framework for food web studies. Recent applications of  $\delta^2\text{H}$  as an aquatic food web tracer assume a trophic compounding effect of water rather than trophic isotope discrimination (Solomon *et al.*, 2009; Soto *et al.*, 2011b), as an explanation to the trophic  $\delta^2\text{H}$  patterns found by Birchall *et al.* (2005). This apparent trophic discrimination is caused by the H isotopic exchange with water *in vivo* during protein synthesis (Soto *et al.*, 2013). Controlled experiments have shown that the water contribution to tissue H varies with the type of organism. Therefore, in contrast with C and N, H in the consumer's tissues is derived both from diet and environmental water. The contribution of ambient water to tissue H at each trophic step through the food web determines the  $^2\text{H}$  enrichment in consumers compared to their diet. Furthermore, there are other mechanisms of variation for H isotopes in organisms; for instance, the effect of the metabolic water derived from the metabolism of lipids. Ideally, in a given location, researchers should be able to determine diet  $\delta^2\text{H}$  values for a consumer knowing the stable isotopic composition of the environmental water and that of metabolic water from ingested food components using mass-balance models.

Analytically, there are other factors that do not make trivial the  $\delta^2\text{H}$  application for trophic purposes. (i) The isotopic exchangeability of H in organic samples with ambient vapour adds uncontrolled uncertainty to  $\delta^2\text{H}$  measurements. This can make  $\delta^2\text{H}$  results not comparable among laboratories unless they use some method to correct for the exchangeable H, such as the Comparative Equilibration method (Wassenaar & Hobson, 2000, 2003). (ii) Variation in the lipid content

of tissues induces uncertainty because lipids are highly depleted in  $^2\text{H}$  compared to the protein of the same animal tissue (Hobson *et al.*, 1999; Soto *et al.*, 2013). In addition, lipids do not have exchangeable H with ambient water vapour in the laboratory (Wassenaar & Hobson, 2000), in contrast with the calibrated standards used with the Comparative Equilibration method. Thus, lipids should be removed from samples before  $\delta^2\text{H}$  measurements to avoid uncertainties in the evaluation of trophic relationships (Jardine *et al.*, 2009).

### TRACE METAL BIOACCUMULATION

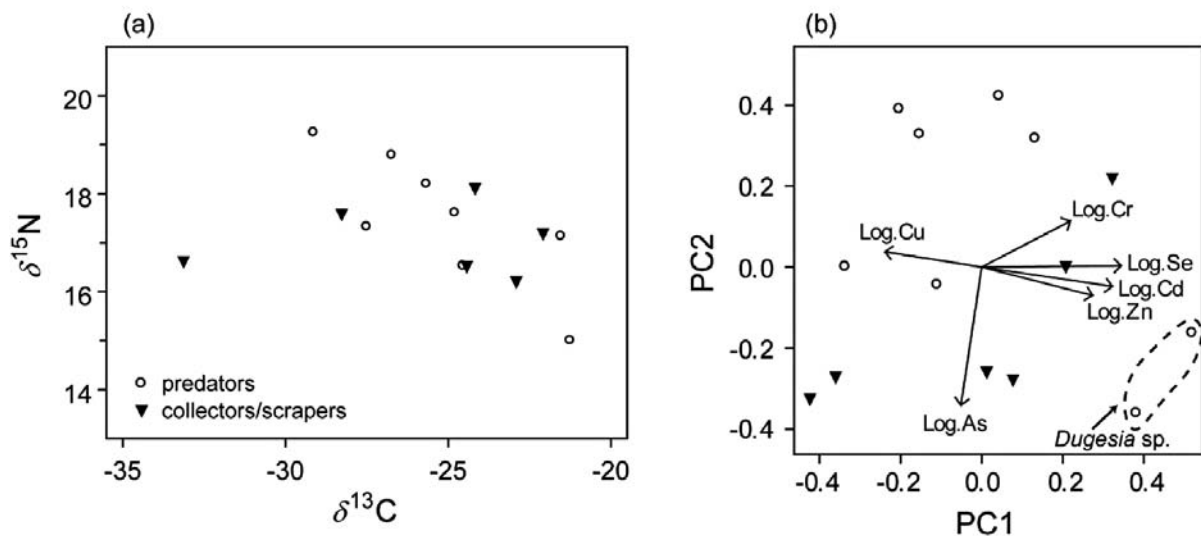
Trace metal bioaccumulation in aquatic organisms has been studied widely during last decades, being a major concern due to the impacts in human health (Luoma & Rainbow, 2008). However, trace metals have been seldom used to trace dietary sources (Stewart *et al.*, 2004) compared with their potential. Trace metals are ubiquitous elements whose environment concentrations depend on the natural background and contamination spills due to industrial production and agricultural treatments. Trace metal bioaccumulation in aquatic organisms results from exposure to medium and diet and involves complex mechanisms such as assimilation, storage, metabolism, and elimination of contaminants. Comparative studies considering several food web components for distinct contaminants are rare but hold a great potential for environmental biomonitoring and food web considerations (Soto *et al.*, 2011a). Food web components showed differences in trace metal concentrations among aquatic species with the same apparent trace metal exposure (Soto *et al.*, 2011a) and the difference in biomonitoring capacity is also the basis for the proposal that trace metals can complement stable isotopes in unveiling aquatic food web structure.

Concentrations of chemical elements that bioaccumulate in an organism should correlate to some degree to concentrations in its diet. Similarly to stable isotopes and according to trace metal bioaccumulation models, the trace metal concentrations in aquatic species are

affected by physiological characteristics of the species (e.g., growth, elimination rates, ingestion rates), which usually are related to animal size (Trudel & Rasmussen, 1997; Trudel *et al.*, 2000). Therefore, the relative differences of trace metal bioaccumulation among organisms can provide insights into species trophic relationships, and also the species metabolic requirements. Trace metals may become particularly useful when i) studying species with similar rates of key physiological processes (e.g., ingestion, trace metal elimination), and ii) the variation of trace metal concentration in food sources is high.

Combining trace metals and stable isotopes for evaluating trophic links appears promising. Cabana & Rasmussen (1994) showed that Hg

bioaccumulation was related to the trophic position ( $\delta^{15}\text{N}$ ) and that the knowledge of the degree of omnivory could predict realistically the Hg biomagnification. Biomagnification of trace metals along food chains occurs because uptake from the diet is higher than elimination. On the contrary, As concentrations are biodiminished along food chains (Chen & Folt, 2000). The same explanation applies to  $\delta^{15}\text{N}$  in which organisms retain  $^{15}\text{N}$  preferentially over  $^{14}\text{N}$ . There are cases in which trace metal discriminate better among trophic levels than stable isotopes. An example is shown in figure 3, corresponding to the macroinvertebrate food web of Flix reservoir. Predators and consumers (collector/scrapers) can scarcely be differentiated using C and N stable isotopes.



**Figure 3.** A case study example showing a situation in which trace metals discriminate better among trophic groups than stable isotopes. Data correspond to macroinvertebrates from the Flix reservoir (Ebro River, Spain) sampled in 2006. (a) The  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values (Soto *et al.*, 2011b). (b) Principal component biplot of trace metal concentrations (trace metal data from Soto *et al.*, 2011a). The principal components (PC1 and PC2, respectively) explained 50 % and 17 % of the variation, respectively. Predators are clearly discriminated from consumers by the biodiminished arsenic (As) concentrations. Only the peculiar case of Turbellaria (*Dugesia* sp.) does not follow the pattern. Predator taxa include: *Dugesia* sp. (Turbellaria), Hydrophilidae adults (Coleoptera, Insecta), Coenagrionidae (Odonata, Insecta), and *Naucoris* sp. (Naucoridae, Heteroptera, Insecta); and collectors/scrapers include: *Physa* sp. (Gastropoda, Mollusca), *Cloëon* sp. (Baetidae, Ephemeroptera, Insecta), and Hydrophilidae larvae (Coleoptera, Insecta). *Un caso de estudio que ilustra una situación en que los metales traza discriminan mejor entre grupos tróficos que los isótopos estables. Los datos corresponden a macroinvertebrados del embalse de Flix (río Ebro, España) muestreados durante el 2006. (a) Valores de  $\delta^{13}\text{C}$  y  $\delta^{15}\text{N}$  (Soto *et al.*, 2011b). (b) Biplot de un análisis de componentes principales de las concentraciones de metales traza (datos de metales traza de Soto *et al.*, 2011a). Las componentes principales (PC1 y PC2) explican un 50 % y un 17 % de la variación, respectivamente. Los depredadores se discriminan claramente de los consumidores por el efecto de biodiminución del arsénico (As). Sólo el caso peculiar de los Turbelarios (i.e., *Dugesia*, sp.) no sigue la pauta. Los depredadores incluyen: *Dugesia* sp. (Turbellaria), adultos de Hydrophilidae (Coleoptera, Insecta), Coenagrionidae (Odonata, Insecta), y *Naucoris* sp. (Naucoridae, Heteroptera, Insecta); y los consumidores: *Physa* sp. (Gastropoda, Mollusca), *Cloëon* sp. (Baetidae, Ephemeroptera, Insecta), y larvas de Hydrophilidae (Coleoptera, Insecta).*

However, the trace metal signatures clearly differentiate between the two trophic groups. More research regarding the consistency of the multivariate trace metal patterns should be undertaken.

There are already some studies that point towards a use of multiple tracers. Soto *et al.*, (2011b) found that Hg and As concentrations in fish from a reservoir were positively and negatively correlated, respectively, with trophic indicators based on C and N stable isotopes. The combination of trace metals and stable isotopes ( $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ ) were also useful to show a potential trophic meaning of  $\delta^2\text{H}$  values which was unclear only using  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  (Soto *et al.*, 2011b). A case study from San Francisco Bay, organisms feeding on bivalves had much higher selenium concentrations than those species that fed on crustaceans because of the lower loss rate constant of selenium in bivalves (Stewart *et al.*, 2004). Organochlorine contaminants have also been occasionally used in feeding ecology, in cases where the interpretation of trophic position with only stable isotopes as indicators could be imprecise (Fisk *et al.*, 2002).

In summary, we suggest that increasing the number of tracers by combining trace metals (or other bioaccumulated contaminants) and stable isotopes can be a powerful technique in systems where the isotope values of consumers result insufficient to separate feeding modes.

## FINAL REMARKS

The use of  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  is helpful for revealing the food web structure of aquatic ecosystems, but usually insufficient for disentangling complex food webs. It cannot be expected that only two tracers solve the myriad of possible interactions that occur in food webs. Complementary trophic tracers of considerable potential are other stable isotopes (i.e.,  $\delta^2\text{H}$ ) and substances that bioaccumulate through food webs (i.e., trace metals, organic pollutants).

For a confident use of  $\delta^2\text{H}$  in aquatic food web studies, the potential confounding effect of seasonal and spatial hydrogen isotopic variation of environmental water should be taken into ac-

count along with other mechanisms that drive the hydrogen isotopic variability.

Differential trace metal accumulation patterns occur among food web organisms. This diversity is the basis to suggest trace metal use for studying food webs. Any increase in understanding the bioaccumulation process will serve also the application of trace metals as food web tracers. The combined use of both techniques (stable isotopes and trace metals) can be highly complementary, particularly, when values in the potential food sources show little variability for one of them.

In addition to using more tracers, the numerical estimations can also be improved. Bayesian mixing models are a powerful tool to obtain reliable results because can take into account sources of variability in the data interpretation. These models can be performed at both population and individual levels to trace the links of complex food webs. Quantitative estimation of trophic food webs may have applications that range from theoretical (e.g., food web stability) to applied investigations (e.g., invasive species diet).

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