

## Reconstruction of trophic pathways between plankton and the North Iberian sardine (*Sardina pilchardus*) using stable isotopes\*

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**SUMMARY:** Feeding on phyto- and zooplankton by juvenile (< 1 year old) and adult sardines (*Sardina pilchardus*) was inferred from analyses of natural abundance of stable carbon and nitrogen isotopes in samples from the northwestern Iberian Peninsula (Spain) collected at the beginning of the upwelling season and peak spawning period of sardine. Plankton samples were fractionated through nets of 20, 200, 500, 1000 and 2000  $\mu\text{m}$  mesh-size and the muscle protein of individual sardines was isolated before isotopic determinations. Up to six planktonic components and two sardine feeding types were identified from the modes in the frequency distributions of isotope abundance values. Also, the most probable pathways for carbon and nitrogen flows between compartments were analysed. The resulting food web revealed a relatively large degree of omnivory, both in plankton and sardine components, which confirms that complex trophic interactions could also occur in pelagic upwelling ecosystems. Young sardines had isotope abundance values clustered around a single mode in the frequency distribution, while adult sardines displayed two main modes. These modes are interpreted as representative of two extreme feeding types: one related to the individual capture of zooplankton prey and the other to unselective filter-feeding. Although both types of feeding could include micro- (20-200  $\mu\text{m}$ ) and mesozooplankton (200-2000  $\mu\text{m}$ ) prey, phytoplankton appears to be ingested mainly by filter-feeding. However, even adult sardines must be mainly zoophagous to achieve the observed isotopic abundance values, taking into account current assumptions on stable isotope enrichment through trophic levels. From the differences in the resulting pathways using either carbon or nitrogen isotopes, we interpreted that sardines acquire most of the protein nitrogen from zooplankton while a substantial fraction of their carbon would derive from phytoplankton. These interpretations agree with the information available for this species on the gut contents and the anatomy of the filtering apparatus.

**Key words:** phytoplankton, zooplankton, clupeids, stable isotopes, food web, upwelling, NE Atlantic.

**RESUMEN:** RECONSTRUCCIÓN DE LAS CONEXIONES TRÓFICAS ENTRE EL PLANCTON Y LA SARDINA DEL NORTE DE LA PENÍNSULA IBÉRICA (*SARDINA PILCHARDUS*) A TRAVÉS DEL ANÁLISIS DE LAS DISTRIBUCIONES DE FRECUENCIA DE LA ABUNDANCIA NATURAL DE ISÓTOPOS ESTABLES. – A partir de las medidas de la abundancia natural de isótopos estables de carbono y nitrógeno se reconstruyó la alimentación de juveniles (< 1 año de edad) y adultos de sardina (*Sardina pilchardus*) a partir de fito- y zooplankton en el noroeste de la península Ibérica. Las muestras de plancton y sardinas fueron tomadas al comienzo de la época de afloramiento y durante el máximo de freza de la sardina. Previamente a las determinaciones isotópicas, el plancton fue fraccionado por tamaños a través de tamices de 20, 200, 500, 1000  $\mu\text{m}$  de luz de malla y, en el caso de las sardinas, se tomaron muestras de la proteína muscular. A partir de las modas en las distribuciones de frecuencias de la abundancia isotópica se identificaron hasta 6 componentes tróficos del plancton y dos tipos de alimentación en las sardinas. Además, se analizaron las conexiones más probables para los flujos de carbono y nitrógeno entre los compartimentos considerados. La red trófica resultante mostró que tanto los compartimentos planctónicos como las sardinas tenían un elevado grado de omnivoría. Esto confirma que en los ecosistemas pelágicos de afloramiento también ocurren interacciones tróficas complejas. Las sardinas jóvenes presentaron una distribución unimodal de frecuencias de los valores de abundancia isotópica, mientras que las sardinas adultas tenían una distribución bimodal. Estas modas se interpretaron como la expresión de dos tipos de alimentación:

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por una parte la captura individual de presas de zooplancton y por otra la filtración no selectiva de plancton. Mediante ambos tipos de alimentación las sardinas pueden capturar micro- (20-200  $\mu\text{m}$ ) y mesozooplancton (200-2000  $\mu\text{m}$ ) pero la captura de fitoplancton parece hacerse principalmente a través de la filtración. En cualquier caso, las sardinas estudiadas deben ser predominantemente zoófagas para explicar el enriquecimiento isotópico observado teniendo en cuenta los conocimientos actuales sobre la acumulación de isótopos pesados en distintos niveles tróficos. La ingesta de fitoplancton parece contribuir más a la incorporación de carbono por las sardinas que la de zooplancton, que proporcionaría principalmente nitrógeno, según las diferencias observadas en las conexiones tróficas reconstruidas a partir de uno u otro elemento. Estas interpretaciones concuerdan con la información disponible sobre los contenidos estomacales y la anatomía del aparato filtrador de la sardina.

*Palabras clave:* fitoplancton, zooplancton, clupeidos, isótopos estables, red trófica, afloramiento, Atlántico NE.

## INTRODUCTION

Marine pelagic food webs are increasingly recognised as unstructured, in the sense that most consumer species feed on a large variety of preys during their lifetime and therefore are difficult to assign to discrete trophic levels (Isaacs, 1973). The realisation of the importance of omnivory and detritivory in these systems came from studies considering either microbial (Pomeroy, 1974; Azam *et al.*, 1983; Fenchel, 1988), plankton (Fry, 1988; Rau *et al.*, 1989; Fry and Quiñones, 1994) or large consumers like planktivorous fishes (James, 1988; Monteiro *et al.*, 1991; Lindsay *et al.*, 1998). The new results challenged classical assumptions about the organisation and structure of marine food webs, such as the predicted simplicity of trophic interactions in upwelling areas because of the direct feeding of planktivorous fishes on phytoplankton (Ryther, 1969), instead revealing relatively complex trophic relationships even in these systems (Cushing, 1978; James, 1988; Monteiro *et al.*, 1991; Moloney 1992).

However, the reconstruction of marine food webs was largely constrained by methodological difficulties in obtaining the appropriate data, often derived from time-consuming analyses of gut contents of consumer species. Among the limitations of gut content studies are the large number of observations required to obtain the appropriate information over the time and space scales of interest, and the fact that it is often not possible to identify all prey items (e.g. James, 1988; Varela *et al.*, 1988; 1990). The application of stable isotope techniques offers the possibility of qualitative and quantitative tests of hypotheses about trophic organisation because differences in the natural abundance of the isotopes of key elements (like carbon and nitrogen) between consumers and their diet are a consequence of nutrient and energy sources and exchanges, and reflect trophic relationships (Owens, 1987; Wada and Hattori, 1991). Typically, heavy isotopes accumulate in

large organisms and in tissues with low turnover rates (Tieszen *et al.*, 1983; Minagawa and Wada, 1984; Hesslein *et al.*, 1993; Fry and Quiñones, 1994). The enrichment in heavy isotopes between predator and prey is termed trophic fractionation and its value is consistent across aquatic ecosystems when averaged over a large number of trophic pathways (Minagawa and Wada, 1984; Vander Zanden and Rasmussen, 2001; Post 2002). This constancy of trophic fractionation can be used as a tool for computing the trophic position (i.e. quantification of the relative use of different trophic resources) of each species or ecosystem compartment analysed (Monteiro *et al.*, 1991; Yoshii *et al.*, 1999; Vander Zanden and Rasmussen 2001; O'Reilly *et al.*, 2002; Post 2002). As trophic positions can reach any value in between discrete trophic levels, their consideration allows for the study of the generalised omnivory and trophic complexity which characterises many ecosystems (Paine, 1988; Persson, 1999) and is particularly useful when one is studying species with diets that are difficult to quantify (e.g. Monteiro *et al.*, 1991; Sholto-Douglas *et al.*, 1991).

The determination of trophic positions from natural abundance measurements in the field requires basically the use of an appropriate isotope reference value (isotopic baseline) at or near the base of the food web and the assumption of a constant trophic fractionation between trophic levels. Recent studies of carbon and nitrogen isotopes in lake ecosystems reviewed these assumptions along with the consideration of the variability of stable isotope content within each compartment (Vander Zanden and Rasmussen, 2001; O'Reilly *et al.*, 2002; Post, 2002). These studies showed that while planktonic organisms may display considerable variability in their isotopic content because their life span is shorter than, for instance, seasonal variations in nutrient sources (O'Reilly *et al.*, 2002), long living consumers accumulate in their tissues isotopic signatures reflecting time integrated diets. Furthermore, the isotopic signature of primary consumers can be

used as reference baseline for computing trophic positions in the food web with less uncertainty than estimates based on the signature of primary producers (Vander Zanden and Rasmussen, 2001; Post 2002). Also, these studies confirmed the relatively constant fractionation between trophic levels found in previous studies (e.g. Minagawa and Wada, 1984; Peterson and Fry, 1987) and the relatively low errors produced in the estimations of trophic position when the variability in isotope fractionation values is taken into account (Vander Zanden and Rasmussen, 2001).

However, while the choice of a primary consumer for the determination of the isotopic baseline in lakes may be restricted to a few key species (e.g. Post, 2002), the diversity of species and feeding modes of planktivorous animals in marine pelagic ecosystems complicates the selection of a strictly herbivore species (James, 1988; Varela *et al.*, 1988; Rau *et al.*, 1989; Varela *et al.*, 1990; Monteiro *et al.*, 1991; Lindsay *et al.*, 1998). Several studies overcome this problem by using the isotopic signatures of several plankton size-classes to infer the organisation and changes in structure of marine pelagic food webs. Some studies employed regressions of natural abundance values versus the size of organisms when grouped by size-classes (Fry and Quiñones, 1994; Rolff, 2000). An alternative approach was the analysis of the frequency distribution of isotopic values within each size-class (Monteiro *et al.*, 1991). The latter method consists of the graphical representation of the frequency of the natural abundance values of the isotope of interest within a given compartment of the ecosystem (a plankton size-class or selected species), the identification of the modes in the distributions with trophic levels, and the reconstruction of the probable trophic links by connecting modes of different compartments. A similar procedure was previously used to illustrate trophic fractionation in diverse ecosystems (Owens, 1987). However, the reconstructed food web will be representative of those occurring in the studied ecosystem only if sampling of all compartments takes into account all the relevant sources of variability for the ecosystem and target consumer species. For instance, sampling must cover all possible habitats within the distribution range of the consumer (Monteiro *et al.*, 1991) and also consider the main variations in the source of nutrients at the base of the food web (O'Reilly *et al.*, 2002).

In this study, we apply the analysis of frequency distributions of isotope abundance to the determination of the food web structure and most probable

pathways of trophic exploitation of plankton by the sardine (*Sardina pilchardus*, Walbaum) in the upwelling ecosystem of the northern Iberian Peninsula. The target sardine species is a typical representative of the planktivorous fishes that reach large populations in upwelling areas (ICES, 2000) and displays large trophic versatility (Cépède, 1907; Oliver, 1951; Oliver and Navarro, 1952; Varela *et al.*, 1988; 1990; Conway *et al.*, 1994). This species is widely distributed along and across the northern Iberian continental shelf, extending from near-shore areas to the shelf-break (Porteiro *et al.*, 1996). Complementary information on the structure of the pelagic food web and on the variability of isotopic composition with the size of the organisms in part of the study area can be found in Bode *et al.* (2003).

## MATERIALS AND METHODS

Samples of plankton and sardine were collected off Galicia (NW Spain) during cruises PELACUS-0398 (17 March to 7 April 1998), PELACUS-0399 (14 to 23 March 1999), PELACUS-0300 (27 March to 10 April 2000) and PELACUS-0401 (2 to 18 April 2001) on RV 'Thalassa' (Fig. 1). In these cruises, made during the peak spawning of sardine and the beginning of the upwelling season, plankton was collected at stations distributed over the continental shelf and nearby ocean (with bottom depths between 50 and > 1000 m) by vertical hauls of a WP<sub>2</sub>-type net of 20 µm mesh-size from 100 m depth to the surface. Samples were fractionated through sieves of 200, 500, 1000 and 2000 µm mesh-size, which produced 4 size-classes: 20-200, 200-500, 500-1000 and 1000-2000 µm (the fraction > 2000 µm was discarded). Each fraction was carefully washed with filtered seawater, transferred to glass fibre filters, and stored frozen until further processing in the laboratory. All plankton samples were collected during the night. In addition, plankton samples (200-2000 µm) were collected at monthly intervals at one station off A Coruña (43°25.3'N, 8°26.2'W, 80 m depth) between January 2000 and December 2001 to account for seasonal variations in plankton. Although plankton species were not identified in this study, the species and size composition of phyto- and zooplankton were well described in this area. Phytoplankton was generally dominated by chain-forming diatoms, especially during blooms, with most of the biomass in the > 12 µm size-class (e.g. Bode *et al.*, 1998). In turn, most of

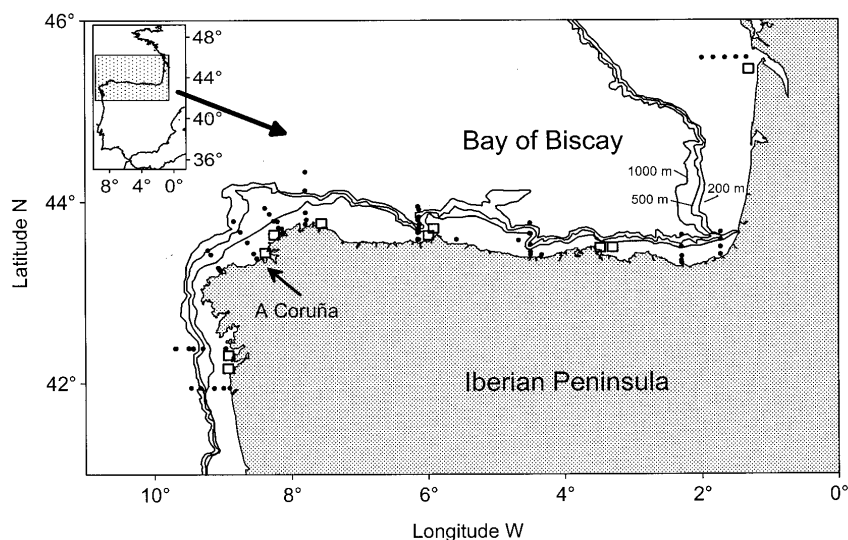


FIG. 1. – Map of sampling stations of plankton (dots) and sardine (open squares).

the mesozooplankton (200–2000  $\mu\text{m}$ ) abundance and biomass was generally due to omnivorous copepods (Valdés *et al.*, 1991). Sardines (11 to 24 cm total length) were collected during the PELACUS cruises using a pelagic trawl. Due to the spawning behaviour, sardines were concentrated near the coast during the spring, but the species is reported to be distributed over the whole continental shelf (Porteiro *et al.*, 1996; ICES, 2000). The length of each specimen was measured ( $\pm 5$  mm) and it was dissected to obtain portions of dorsal white muscle that were stored frozen for isotopic analysis.

Plankton samples were dried (50°C, 24 h), finely ground and packed in tin capsules. Plankton samples were not acidified to remove carbonates because the acidification of some spare replicates showed no significant changes in the relative abundance of  $^{13}\text{C}$  or  $^{15}\text{N}$  (Kruskal-Wallis test,  $p > 0.05$ ,  $n = 21$ ). Sardine samples were freeze-dried in the laboratory, and then treated with a mixture of chloroform, methanol and water to remove lipids (Bligh and Dyer, 1959) and minimise the differences in  $^{13}\text{C}$  caused by the variable fatty tissue content of individual fish (Sholto-Douglas *et al.*, 1991). After lipid removal, sardine samples were dried (50°C, 24 h), ground and packed in tin capsules as with the plankton samples. The natural abundance of  $^{13}\text{C}$  and  $^{15}\text{N}$  in plankton size-classes and individual sardine was determined after conversion of dried samples to  $\text{CO}_2$  and  $\text{N}_2$  in an elemental analyser (Carlo Erba CHNSO 1108) coupled to an isotope-ratio mass spectrometer (Thermo Finnigan Mat Delta Plus). Natural abundance values of  $^{13}\text{C}$  and  $^{15}\text{N}$  were expressed as isotope ratios (‰) according to the formula:

$$\delta X = [(R_{\text{sample}} - R_{\text{std}}) / R_{\text{std}}] 1000 \quad (1)$$

where  $X = ^{13}\text{C}$  or  $^{15}\text{N}$ ,  $R = ^{13}\text{C}/^{12}\text{C}$  or  $^{15}\text{N}/^{14}\text{N}$ , and std = Peedee Belemnite carbonate for  $\delta^{13}\text{C}$ , and atmospheric nitrogen for  $\delta^{15}\text{N}$ . All isotope determinations were run in triplicate. Precision of either C or N isotopic determinations was better than 0.03‰.

The trophic positions of plankton and sardine were estimated from the frequency distributions of  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  grouped in abundance classes of 0.25‰ width, as described by Monteiro *et al.* (1991). For this analysis, distributions of plankton samples were made for two size-classes of 20–200  $\mu\text{m}$  and 200–2000  $\mu\text{m}$ , and those of sardine samples were made for individuals  $< 18$  cm and  $\geq 18$  cm respectively. The sardines were separated with the purpose of separating juvenile ( $< 1$  year old) from adult individuals according to the available information on size-age relationships for the studied population (ICES, 2000). The main planktonic components were determined from the modes in isotopic frequency distributions, which also allowed for the separation of sardines with a different feeding history (Owens, 1987; Monteiro *et al.*, 1991). The determination of the most probable trophic pathways between plankton and sardines was made by linking the closest modes in the frequency distributions of each component, taking into account the average values for isotopic fractionation between trophic levels (Vander Zanden and Rasmussen, 2001). In this study we extended the original method described for  $^{13}\text{C}$  (Monteiro *et al.*, 1991) to two isotopes. In this way, separate analysis of food web pathways were made for  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$ .

The reliability of the reconstructed pathways between plankton and sardine was checked by estimating the contribution of the different plankton components to the composition of sardine muscle from the mean values of  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  of each component using the equation:

$$\delta X_s = \Delta\delta X + \Sigma (f_i \delta X_{pi}) \quad (2)$$

where  $\Delta\delta X$  is the value of the isotopic enrichment between food and tissues of the consumer (trophic fractionation),  $f_i$  are the frequencies of different plankton components with mean isotopic abundances  $\delta X_{pi}$ , and  $\delta X_s$  is the mean isotopic abundance of sardine. Initially, we used the average values of trophic fractionation derived from the literature (0.8‰ for  $\delta^{13}\text{C}$  and 3.4‰ for  $\delta^{15}\text{N}$ , Vander Zanden and Rasmussen, 2001). The values of  $f_i$  in Equation (2) were estimated by an iterative linear procedure (Fylstra *et al.*, 1998) and were compared with published reports of gut contents of *S. pilchardus* (Cépède, 1907; Oliver, 1951; Oliver and Navarro, 1952; Varela *et al.*, 1988; 1990).

## RESULTS

### Identification of food web components

Mean values of both  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  for plankton and sardine were significantly different (Kruskal-Wallis test,  $p < 0.01$ ,  $n = 250$ ), with plankton having

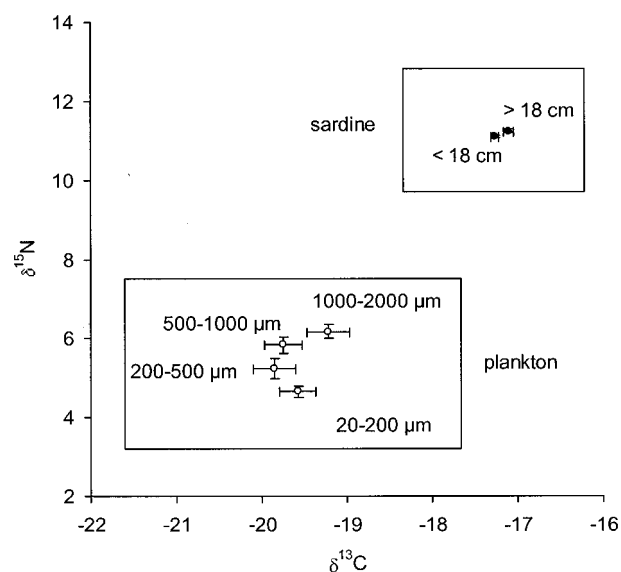


FIG. 2. – Plot of mean ( $\pm 1$  se) isotope abundance values of  $\delta^{13}\text{C}$  versus  $\delta^{15}\text{N}$  in plankton and sardine size-classes.

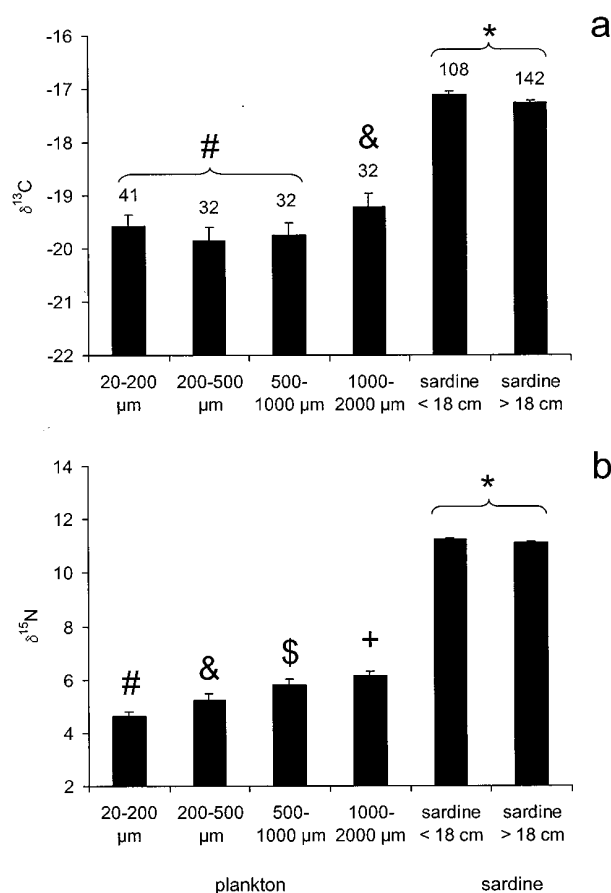


FIG. 3. – Differences between mean ( $\pm 1$  se)  $\delta^{13}\text{C}$  (a) and  $\delta^{15}\text{N}$  values (b) of the considered size-classes of plankton and sardine. Non-significant differences are indicated by linking the corresponding bars and text symbols (ANOVA and Student-Neuman-Keuls *a posteriori* test,  $p > 0.05$ ). Number of observations pooled for each size-class is indicated by numbers above bars in panel a.

the lowest values (Fig. 2). In addition, the isotopic composition of sardine samples was relatively homogeneous when compared to plankton size-classes. The separation of plankton classes was more evident when nitrogen isotopes were taken into account, which resulted in significant differences in mean values of  $\delta^{15}\text{N}$  between all size-classes (ANOVA and Student-Neuman-Keuls *a posteriori* test,  $p < 0.05$ ), while no significant differences were found between mean values of the 20-200, 200-500 and 500-1000  $\mu\text{m}$  size-classes when considering  $\delta^{13}\text{C}$  (Fig. 3). No significant differences resulted between mean values of both  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  for young and adult sardines (Fig. 3) but we maintained the separation between these classes, as there is some evidence of a gradual change in diet during growth (Bode *et al.*, 2003).

The isotopic composition of size-fractionated plankton can be considered as representative of the shelf area studied, even when most of the samples

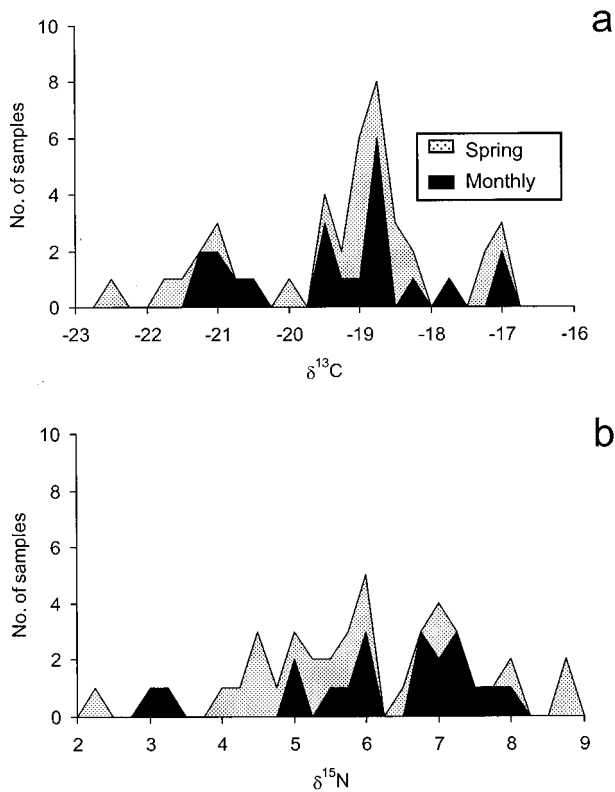


FIG. 4. – Frequency distributions of  $\delta^{13}\text{C}$  (a) and  $\delta^{15}\text{N}$  values (b) for plankton 200-2000  $\mu\text{m}$  from samples from the spring and from the monthly sampling off A Coruña.

were from the spring. To demonstrate this we tested for the differences in the frequency distribution of samples of plankton  $> 200 \mu\text{m}$  collected off A Coruña during the monthly study and those from spring cruises (Fig. 4). The results were non-significantly different considering either  $\delta^{13}\text{C}$  or  $\delta^{15}\text{N}$  (Kolmogorov-Smirnov goodness of fit test,  $p > 0.05$ ,  $n = 21$ ). Even the range of values found during the spring exceeded those found in the monthly study, indicating that the plankton sampled during spring was representative of the main nutrient sources for the pelagic shelf ecosystem. Therefore, we combined all samples from the monthly and spring cruises in subsequent analyses of frequency distribution of natural abundance values.

Despite their relatively large variability, planktonic  $\delta^{13}\text{C}$  values were distributed around several modes (Fig. 5). Values lower than  $-20\text{‰}$  can be attributed to phytoplankton, especially in high production areas (Fry and Wainright, 1991). In our study such values were found in both 20-200  $\mu\text{m}$  and 200-2000  $\mu\text{m}$  size-classes, which is consistent with the reported dominance of chain-forming diatoms in upwelling induced blooms (Varela *et al.*, 1991). The modes near  $-19.50$ ,  $-18.75$ ,  $-17.75\text{‰}$  for

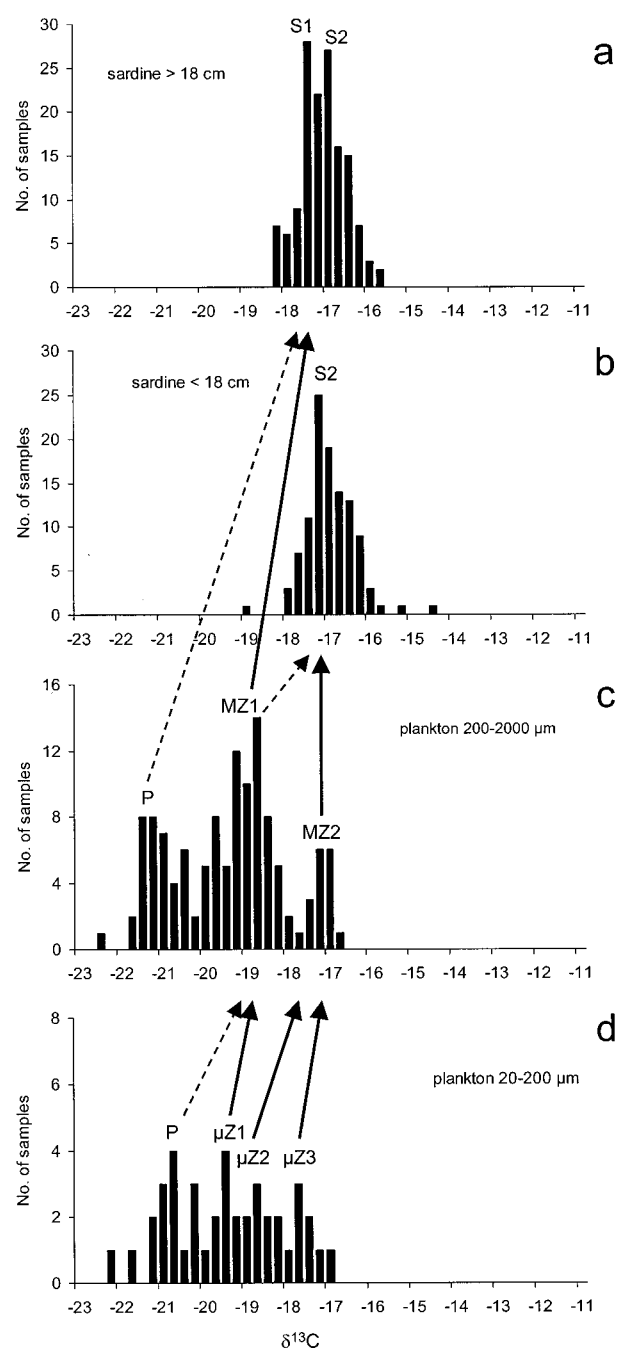


FIG. 5. – Frequency distributions of  $\delta^{13}\text{C}$  values in sardine (a, b) and plankton size-classes (c, d). The arrows indicate likely (continuous lines) and less likely trophic pathways (dashed lines) determined from comparison between the resulting isotope fractionation values and those from the literature (see text for details). P: phytoplankton;  $\mu\text{Z1}$ ,  $\mu\text{Z2}$  and  $\mu\text{Z3}$ : microzooplankton; MZ1 and MZ2: mesozooplankton; S1 and S2: sardine.

the 20-200  $\mu\text{m}$  plankton can be attributed to different microzooplankton components, while the modes near  $-18.75$  and  $-17.00\text{‰}$  of plankton  $> 200 \mu\text{m}$  would correspond to mesozooplanktonic components. Most juvenile sardines had  $\delta^{13}\text{C}$  values clustering around  $-17.00\text{‰}$ , although there was consid-

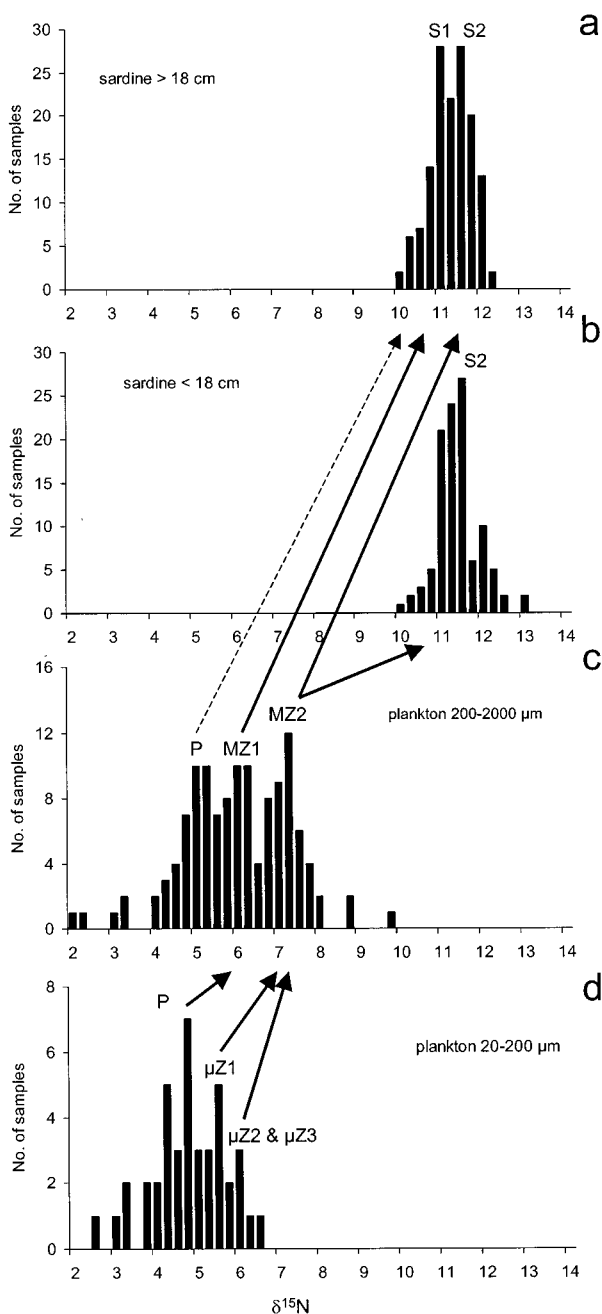


Fig. 6. – Frequency distributions of  $\delta^{15}\text{N}$  values in sardine (a, b) and plankton and size-classes (c, d). The arrows indicate likely (continuous line) and less likely trophic pathways (dashed lines). Labels for plankton and sardine components as in Fig. 5.

erable variability taking into account that these values were from a single species. Furthermore, two individuals displayed high enrichment values of  $-15.25$  and  $-14.50\text{‰}$  and another showed an unusually low  $\delta^{13}\text{C}$  value of  $-19\text{‰}$ . Sardines  $\geq 18$  cm also showed a mode in  $\delta^{13}\text{C}$  values near  $-17.00\text{‰}$  but in this case a secondary mode at  $-17.50\text{‰}$  was observed. It must also be noted that none of the adult sardines analysed had  $\delta^{13}\text{C}$  values higher than

$-15.50\text{‰}$  but seven individuals had a value of  $-18.25\text{‰}$ . This distribution of modes allowed us to infer that phytoplankton was mainly consumed by the less  $^{13}\text{C}$  enriched mesozooplankton and microzooplankton components, but these components must also consume animal prey, since the difference in  $\delta^{13}\text{C}$  values between their modes and that of phytoplankton exceeded the mean value of trophic fractionation typical of herbivores (Vander Zanden and Rasmussen, 2001). Also, the component of plankton  $> 200 \mu\text{m}$  with high values of  $\delta^{13}\text{C}$  would be predatory, consuming a microzooplankton component that would also feed on other microzooplankters, according to its  $\delta^{13}\text{C}$  value. Finally, young sardines would consume mainly predatory mesozooplankton, while the diet of adult sardines would include a higher fraction of herbivorous and omnivorous zooplankton than the diet of juveniles. However, the high  $\delta^{13}\text{C}$  enrichment of young sardines suggests that they consume low amounts of phytoplankton. The mean ( $\pm 1$  se) isotopic enrichment between adjacent modes was  $1.3 \pm 0.3\text{‰}$  ( $n = 12$ ), which is within the range of values reported for enrichment between trophic levels (Minagawa and Wada, 1984; Vander Zanden and Rasmussen, 2001; Post, 2002).

The pattern of modes in the  $\delta^{15}\text{N}$  frequency distributions was similar to that of  $\delta^{13}\text{C}$ , but in this case, the displacement of the  $\delta^{15}\text{N}$  values of modes in different size-classes and organisms allowed for a better estimation of trophic pathways (Fig. 6). According to these patterns, phytoplankton would have  $\delta^{15}\text{N}$  values of about  $5\text{‰}$ , while two microzooplanktonic components would have mean values of  $5.5\text{‰}$  and  $6.0\text{‰}$  respectively (in this case no differences were found to include a third microzooplankton component based on  $\delta^{15}\text{N}$ ). In turn, the most herbivorous of mesozooplanktonic components displayed a mode at  $6\text{‰}$ , while the mode for predatory mesozooplankton was at  $7.25\text{‰}$  and some samples reached  $\delta^{15}\text{N}$  values up to  $9.75\text{‰}$ . As with  $\delta^{13}\text{C}$ , young sardines displayed a single mode at  $\delta^{15}\text{N}$  value of  $11.5\text{‰}$ , although there were some individuals with  $\delta^{15}\text{N} > 12\text{‰}$  and the distribution for adult sardines had two modes at  $\delta^{15}\text{N}$  values of  $11.0\text{‰}$  and  $11.5\text{‰}$  respectively (Fig. 6). The probable trophic pathways inferred from such patterns are consistent with those derived from carbon isotopes (Fig. 5). Furthermore, the mean  $\delta^{15}\text{N}$  value of the mode attributed to phytoplankton was within the range of values reported for this component in other areas (Owens, 1987; Goering *et al.*, 1990; McClelland and Valiela, 1998) and the high isotopic enrichment



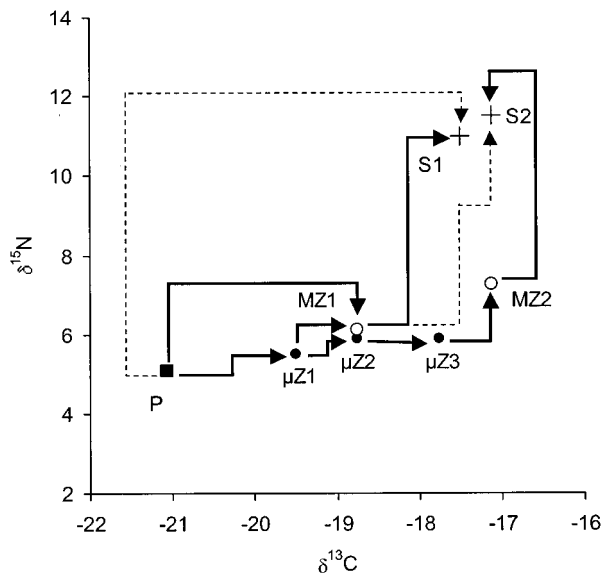


FIG. 7. – Interpretation of the reconstructed pelagic food web from the analysis of frequency distributions of  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  in plankton and sardine. Plankton and sardine components are marked with different symbols (square: phytoplankton, filled circles: microzooplankton, open circles: mesozooplankton, crosses: sardine) and labelled as in Fig. 5. The position of each component in the diagram was according to the  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values of their modes in the frequency distributions (Figs. 5 and 6). Continuous lines indicate the most probable pathways and dashed lines indicate less probable pathways. Some pathways were omitted for clarity (e.g. from MZ1 to MZ2).

between the modes of phytoplankton and sardines indicates that little nitrogen derived from phytoplankton was contained in sardine muscle. In addition, the mean ( $\pm$  se) enrichment value between modes linked by the inferred pathways was  $2.4 \pm 2.1\text{‰}$  ( $n = 12$ ), which is within the range found between adjacent trophic levels in other studies (Minagawa and Wada, 1984; Vander Zanden and Rasmussen, 2001; Post, 2002).

### Identification of trophic pathways

The trophic structure of the studied pelagic ecosystem can be represented by a relatively simple diagram in which phytoplankton is located at the base of the food web and directly feeds micro- and mesozooplankton herbivores that would be the primary consumers (Fig. 7). Secondary consumers would be microzooplankton, while large planktonic predators would feed on a variety of prey that would include both micro- and mesozooplankton. Sardines would have two main feeding modes. On the one hand, they would consume mostly zooplanktonic prey, probably predatory mesozooplankton but also microzooplankton. Such feeding mode is dominant among young sardines. On the other hand, sardines

would prey on predatory mesozooplankton but also include herbivores and even phytoplankton in their diet. The latter feeding mode seems to be only displayed by adult sardines.

### Reconstruction of sardine feeding habits

From the mean isotopic value of each identified plankton and sardine component we estimated that a simple three-step food web including phytoplankton, herbivorous and predatory mesozooplankton, and sardines would explain the direct incorporation of up to 17% of phytoplankton in adult sardine muscle taking into account  $\delta^{13}\text{C}$  values and the mean value of trophic fractionation of  $0.8\text{‰}$  (Fig. 8a). However, when considering  $\delta^{15}\text{N}$  values and a mean value of trophic fractionation of  $3.4\text{‰}$  we were not able to explain the isotopic composition of sardine muscle, which suggests that the mean value of isotopic fractionation from other studies is not applicable in this case. Therefore, we made the estimations of sardine diet by using the mode value of  $\delta^{15}\text{N}$  enrichment between the most enriched zooplankton component and the mode of the most enriched sardines (i.e.  $4.25\text{‰}$ ), which meant that the young sardines would feed exclusively on predatory mesozooplankton. In this extreme case, the fraction of phytoplankton-nitrogen incorporated by adult sardines would not exceed 5% (Fig. 8b). Given the relatively high heavy isotope content of predatory mesozooplankton, the sardines would attain their mean isotope abundance values through the incorporation of  $> 50\%$  of carbon and  $> 60\%$  of nitrogen from this type of prey. It must be noted that in this simple food web the contribution of herbivorous mesozooplankton (MZ1) to the diet of sardine is similar to that of herbivorous microzooplankton ( $\mu\text{Z1}$ ), as their modal isotopic values are equivalent.

Considering a more complex food web, with the inclusion of one additional prey type from the microzooplankton and similar assumptions about the mean isotopic fractionation per trophic link as in the preceding case, would further reduce the consumption of phytoplankton and mesozooplankton herbivores. In this case, only 15% of carbon and 1% of nitrogen from phytoplankton would be enough to produce the  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values of S1 sardines respectively, while the more enriched microzooplankton component  $\mu\text{Z3}$  would contribute up to 22% (Fig. 8c, d). Finally, the inclusion of all the identified plankton components in the estimations (i.e. a 6-step food web) increased the contribution of



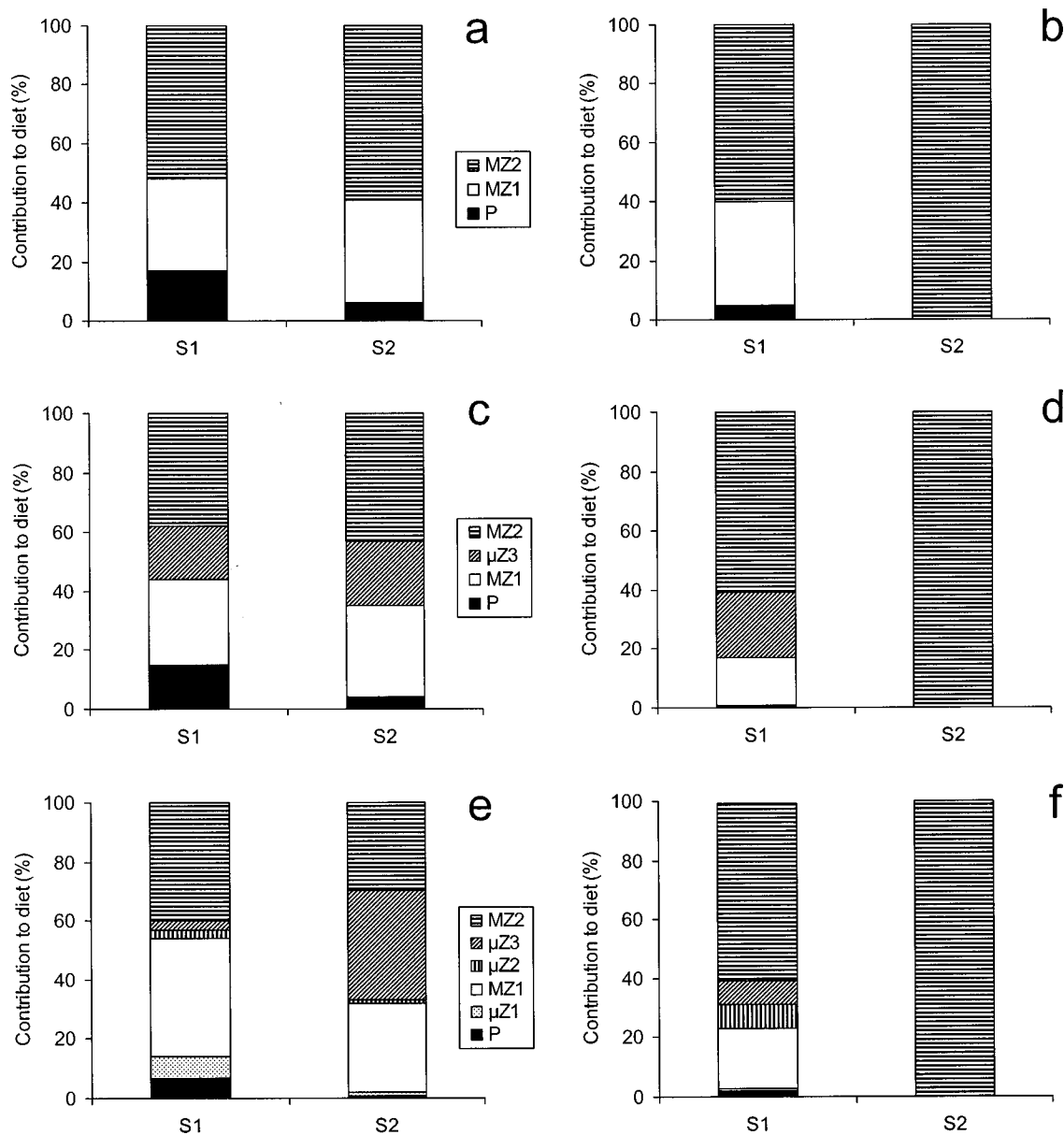


FIG. 8. – Estimations of mean contributions of different plankton components to the carbon and nitrogen composition of sardine muscle according to the measured values of stable isotope abundance (see Methods). The estimations were made with  $\delta^{13}\text{C}$  (a,c,e) and  $\delta^{15}\text{N}$  (b,d,f) and for a two-step (a,b), three-step (c,d), and six-step pathway food web. Plankton and sardine components are marked as in Fig. 3.

mesozooplankton up to 80% in the case of carbon isotopes and to 60% in the case of nitrogen (Fig. 8e, f). These increases were made at the expenses of reductions in the use of phytoplankton, which was estimated to represent < 10% of the diet of sardines in this complex food web.

## DISCUSSION

The reconstructed food web confirms that trophic complexity in upwelling ecosystems is higher than previously thought (Ryther, 1969) because the

direct trophic link between phytoplankton and pelagic fishes is not a dominant pathway, at least for the most abundant clupeids (Cushing, 1978; Monteiro *et al.*, 1991). However, despite the generalised omnivory found in plankton and clupeids, the maximum number of trophic levels would probably not increase very far from 4 (Ryther, 1969), as energy losses after several trophic steps would place an upper limit. Thus, Moloney (1992) showed that food web models of upwelling ecosystems predict a moderate increase in the number of trophic steps from primary producers to planktivorous fishes when omnivory and microbial consumers are taken into

TABLE 1. – Mean dominance values (% of total number or biomass) of planktonic prey types found in gut-contents of the north Iberian sardine (larvae or adults) as reported in the literature. All unidentified organic items were grouped as detritus. Quantification of relative dominance was made by measuring prey abundance (number) or biomass in the gut-content.

Sardine	Phytoplankton	Zooplankton	Detritus	References
larvae (by number)	0	88	12	Conway et al., 1992
adults (by number)	36	59	5	Oliver, 1952; Oliver and Navarro, 1952; Varela et al., 1988
adults (by biomass)	42	57	1	Varela et al., 1990

account, compared with models considering only the traditional food chain phytoplankton - zooplankton - fish. The existence of omnivory would facilitate the necessary adaptation of consumers to the large spatial and temporal variability of plankton in these systems (e.g. Margalef and Estrada, 1981; Varela et al., 1991; Tenore et al., 1995).

The omnivory of *Sardina pilchardus* was well documented in gut-content studies (Table 1), although early studies tended to support the predominance of phytoplankton in its diet (Cépède, 1907; Oliver, 1951; Oliver and Navarro, 1952), while recent studies showed a variable consumption of zooplankton prey (Varela et al., 1988; 1990). On average, > 50 % of the preys (either measured by number or by biomass) found in the guts of adult (i.e. non larval) sardines were zooplankton, mostly adult and juvenile copepods. At the same time, all studies report a significant fraction of phytoplankton ingested by adults (> 30 %). In contrast, larval sardines were nearly exclusively zoophagous (Conway et al., 1994). As in other clupeids (James, 1988), the number of gill rakers and denticles composing the filtering apparatus of sardines increase with fish growth (Andreu, 1953; 1960), favouring the retention of phytoplankton. According to the results of this study and gut-content data, *Sardina pilchardus* has a variable diet as the consequence of being able to perform both particle-feeding (in which each prey is individually captured) and filter-feeding (in which the fish strains all particles retained by its filtering apparatus). The former feeding mode allows for the capture of zooplankton and is represented in our results by S2-type sardines, while the latter is more suitable for the capture of phytoplankton, particularly diatom chains, and corresponds to S1-type sardines. A recent study showed that the values of both  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  in the muscle of *S. pilchardus* from the study area decreased linearly with size when the fish reached 18 cm (Bode et al., 2003), which can be interpreted as the gradual increase in filter-feeding with fish growth.

Besides the increasing predominance of filter-feeding with age, the switching between the selective particle-feeding and non-selective filter-feeding can be triggered by factors like the decrease below a certain threshold of large zooplankton prey and display diel periodicity, as shown in other clupeids (Blaxter and Hunter, 1982; Van der Lingen, 1998b). James (1988) reviewed the information available on the diets of microphagist clupeids and showed that in most species there were conflicting reports about the predominant feeding mode, both because of methodological inadequacies of gut-content studies and because these species have the ability to forage over several trophic levels. Furthermore, there is evidence that zooplanktonic prey are assimilated more easily and provide food of higher quality than phytoplankton, though phytoplankton and microzooplankton, particularly the numerically abundant nauplii and juvenile stages of copepods, can provide a significant sustaining staple food when large prey are scarce (Cushing, 1978; Blaxter and Hunter, 1982; Van der Lingen, 1998a, b). Thus, filter-feeding allows for the ingestion of additional food particles (such as phytoplankton cells) when the fish try to consume small zooplankton (such as nauplii). All these studies support our interpretation of the foraging behaviour of the northern Iberian sardine estimated from the differences between the isotopic abundance in plankton and in the muscle of sardine.

The pelagic food web considered in this study is relatively simple but represents the fundamental trophic steps from the primary producers (i.e. phytoplankton) to a key consumer (i.e. the sardine) in this offshore, upwelling ecosystem (Tenore et al., 1995). The structure is similar to the food web inferred for the Benguela upwelling and its main clupeid species using a similar methodology (Monteiro et al., 1991). A more complex food web would be necessary to adequately explain trophic interactions in the case of a littoral ecosystem in which benthic-pelagic exchanges were important (e.g. Post, 2002). However, some assumptions need to be considered in order to ascertain whether isotopic values in the samples

are valid representatives of the different compartments due to the different turnover of the involved organisms and tissues. On the one hand, while the samples of the smallest size class of plankton seem to contain a significant fraction of phytoplankton according to the values of  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  (Owens, 1987; Goering *et al.*, 1990; McClelland and Valiela, 1998), these samples are representative only of the time of sampling, since phytoplankton shows an isotopic composition close to the source nutrients. As the spring cruises were made at the beginning of the upwelling season, with prevailing upwelling-favourable winds (Lavin *et al.*, 2000), our samples can be considered as representative of the high production periods typical of this ecosystem (e.g. Varela *et al.*, 1991; Tenore *et al.*, 1995). For this reason they may not take into account periods in which the productivity of the system is based on regenerated nutrients (Bode and Varela, 1994). However, we have shown that plankton samples from the 200-2000  $\mu\text{m}$  fraction from the spring had a similar isotopic composition to those collected over two annual cycles off A Coruña. In addition, other studies reported a general coupling between phytoplankton and zooplankton at a scale of weeks (Varela *et al.*, 1991; Tenore *et al.*, 1995). Therefore, we can safely assume that the plankton samples included in this study were representative of the main trophic conditions in the study area, as they included upwelling and non-upwelling conditions. In contrast, the isotopic composition of samples of sardine muscle can integrate trophic episodes for weeks or months, as shown in other fish species (Tieszen *et al.*, 1983, Hesslein *et al.*, 1993), taking into account recent feeding on plankton derived from upwelling but also feeding on plankton during non-upwelling periods in winter. However, the ultimate source of inorganic nutrients for sardine during both periods can be considered to be the same because upwelling introduces new nutrients in the surface layer that are the same as the nutrients appearing in the well mixed water-column during winter (e.g. nitrate). Furthermore, given the approximately weekly periodicity of upwelling episodes in the study area during most of the year (Nogueira *et al.*, 1997) it can be expected that the sardine muscle adequately integrates the variability in the isotopic composition of the different nutrient sources as the sardines travel along and across the shelf, while gut contents would display a larger variability due to the necessary adaptations to local food. It is therefore possible that local food webs may depend on specific sources of nutrients, as

demonstrated in other shelves (e.g. Perry *et al.*, 1999), and their consideration may allow for a quantification of their contribution to the feeding of a particular sardine population.

Although the model used in Equation (2) to infer the relative contributions of the different plankton components to the composition of sardine muscle is widely employed in the determination of diets in other studies (Owens, 1987; Yoshii *et al.*, 1999; Post 2002), it has a limited predictive capacity because it has 3 to 6 unknowns (i.e. the frequency values  $f_i$ ) that need to be estimated. This causes the equation to have multiple solutions for a given set of isotopic values. In addition, it requires an isotopic baseline and assumes that the enrichment between trophic levels is constant. However, recent studies have demonstrated that while both  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  enrichment are quite variable when a particular trophic step is considered, mean values of enrichment like those employed in this study are robust estimates of trophic fractionation since they were derived from a large number of trophic interactions and systems (Minagawa and Wada, 1984; Vander Zanden and Rasmussen, 2001; Post 2002). As noted in the results section, the mean enrichment values resulting from the pathway linking modes of Figs. 5 and 6 are within the range of values cited in the literature.

An isotopic baseline computed from a long-living consumer is generally preferred because of the low variability in its isotopic composition (Vander Zanden and Rasmussen, 2001; Post, 2002). However, we used the isotopic signatures of phytoplankton to estimate the trophic positions, as in another similar study (Monteiro *et al.*, 1991), because of the difficulty in selecting a single consumer species that would be representative of the pelagic ecosystem in the whole upwelling area where the sardine is distributed (Porteiro *et al.*, 1996; ICES, 2000). In this study, relative, rather than absolute, trophic positions were enough for a preliminary description of the food web. Furthermore, the percent composition of the sardine diets obtained by assuming three to six trophic steps may be considered as indicative of extreme types of feeding, from nearly exclusive zoophagy to a significant use of filter-feeding. Such feeding types are supported by gut content observations (Cépède, 1907; Oliver, 1951; Oliver and Navarro, 1952; Varela *et al.*, 1988, 1990) and the structure of the filtering apparatus of the studied sardine species (Andreu, 1953, 1960).

The food web structure and sardine diet types obtained with both isotopes are remarkably similar,

which suggests that flows of carbon and nitrogen run well coupled in this ecosystem. It must be noted that neither carbon nor nitrogen isotopic enrichment of the muscle of sardines could be reached with a predominantly phytophagous diet, unless unrealistically high trophic fractionation values were assumed. Similarly, a preference for zooplankton prey of relatively high trophic position (i.e. predators) would increase the amount of high quality nutrients per weight of prey, as shown in studies of other clupeids (e.g. Van der Lingen, 1998a), and would produce the observed  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values of sardine muscle with relatively low trophic fractionation values. Nevertheless, some differences between the fraction of carbon and nitrogen from zooplankton or phytoplankton which is accounted for by the isotopic composition of the sardine muscle proteins analysed (Fig. 8) suggests the existence of different metabolic pathways for both elements. Thus, enrichment in  $^{15}\text{N}$  between ingested food and proteins is expected because of amino acid deamination and transamination during assimilation because of the preferential mobilisation of  $^{14}\text{N}$  (Gannes *et al.*, 1997). Also, starving animals may become enriched in  $^{15}\text{N}$  as a consequence of the catabolism of proteins (Doucett *et al.*, 1999), as may be the case of the studied sardines since the cruise was made at the beginning of the spawning season, when the fish show minimum storage of energetic compounds such as lipids (Oliver, 1951; Blaxter and Hunter, 1982). Sholto-Douglas *et al.* (1991) interpreted the negative correlation found between  $\delta^{15}\text{N}$  and fish length in two coexisting clupeids as the consequence of protein and amino acid metabolism. In our study, the high trophic fractionation of nitrogen isotopes required to explain the direct consumption of phytoplankton by sardines could be interpreted as the consequence of the necessary conservation of nitrogen along the food web, which is consistent with the low fractionation values found between plankton size-classes (Bode *et al.*, 2003).

As  $\delta^{15}\text{N}$  enrichment in the pathway linking modes of zooplankton and sardines in Figure 6 vary between 3.8 and 5.9‰, which includes the mean trophic fractionation value of 3.4‰ for consumers (Vander Zanden and Rasmussen, 2001), we can conclude that metabolic fractionation during assimilation of ingested food is the main process influencing nitrogen fractionation in sardines feeding mostly on zooplankton. The possible occurrence of starvation would be minimal because high rates of consumption of muscle proteins would have produced higher

fractionation values. Similarly, the higher nitrogen fractionation required for the assimilation of phytoplankton (5.9‰) suggests that most phytoplankton nitrogen is not assimilated. The latter hypothesis is supported by the observation of undigested phytoplankton cells in most microphagous clupeids while zooplankton is readily digested (James, 1988; Van der Lingen, 1998a), although the lower  $\delta^{13}\text{C}$  fractionation required for the direct pathway from phytoplankton to S1 sardines (Fig. 5) compared to that of  $\delta^{15}\text{N}$  (Fig. 6) suggests that some phytoplankton-derived carbon may be assimilated. Thus, the muscle protein of the northern Iberian sardine would have derived most of its nitrogen from zooplanktonic prey while a substantial fraction of its carbon would come from phytoplankton.

The predominantly zoophagous feeding of *S. pilchardus*, particularly in young individuals, opens the possibility of competition for food resources with other coexisting microphagist clupeids in the region. For instance, gut-content studies in the anchovy *Engraulis encrasicolus* of the Bay of Biscay during spring indicate that this species feeds exclusively on zooplankton, particularly on copepods (Plounevez and Champalbert, 1999), which are also found in sardine guts (Varela *et al.*, 1988; 1990). However, studies on similar coexisting species of sardines and anchovies in other areas have found diverse adaptations of feeding behaviour by which these species are able to avoid food competition (Louw *et al.*, 1998). The results of our study showed that, despite the known plasticity of sardine feeding, there are two predominant feeding modes in adult sardines: one related to the individual capture of zooplankton prey and the other related to filter-feeding. Monteiro *et al.*, (1991) reached similar conclusions when studying  $\delta^{13}\text{C}$  values in anchovies and plankton of the Benguela upwelling. According with the different enrichment in carbon and nitrogen isotopes between plankton resources and sardine muscle observed, these two extreme feeding modes would involve the differential incorporation of carbon and nitrogen in structural tissues according to their source. The reconstructed food web, although simple and general, would allow specific hypotheses on sardine feeding and habitat exploitation to be tested. For instance, future studies may investigate the existence of local food webs based on particular nutrient sources, such as those in coastal areas receiving the influence of large rivers (e.g. in the south-eastern Bay of Biscay), and quantify their utilisation by sardines. Also, the determination of the

natural abundance of stable isotopes in various biochemical compounds (e.g. lipids) and prey species of recently incorporated food, along with isotopic determinations in various tissues of sardines, may help to clarify the differential exploitation of phytoplankton versus zooplankton by sardines at various time scales.

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