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Life after death: shallow-water Mediterranean invertebrate communities associated with mammal bones

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Abstract

Invertebrate and microbial marine communities associated with mammal bones are interesting and poorly understood habitats, mainly known from studies on deep-water whale remains. In order to characterize these communities in the shallow-water Mediterranean, we present here the results of a pioneering experiment using mammal bones. Minke whale, pig and cow bones were experimentally deployed on three different background communities: rocky substrate, soft-bottom and a *Posidonia oceanica* meadow. Bones were deployed for a year at about 20 m depth and collected every 3 months, and the invertebrate fauna colonizing the bones was identified to the lowest possible taxonomic level. As expected, mammal bones showed remarkable differences when compared with background communities. Within bones, four different clusters could be identified, primarily on the basis of the polychaete fauna, the most abundant and diverse group in the survey. Clusters A1–A3 corresponded to high to moderately altered successional stages composed by a fauna closer to that of anthropogenically enriched shallow-water environments. These clusters were characterized by the occurrence of the opportunist polychaetes *Ophryotrocha puerilis*, *Neanthes caudata* (Cluster A1), *Protodorvillea kefersteini* (Cluster A2) and *Ophryotrocha alborana* (Cluster A3). Cluster B was characterized by the presence of the polychaete *Oxydromus pallidus* together with typical invertebrate background fauna, which suggests that this community, after a year of deployment, was closer to that found in natural conditions. As opposed to similar shallow-water studies in other geographic areas, no occurrence of the polychaete *Osedax* (commonly known as bone-eating worms) was reported from our experiments. Apart from the study on the invertebrate communities, insights about the population dynamics of three of the most abundant species (*O. puerilis*, *O. alborana*, *N. caudata*) are given as well as remarks on a hypothetical trophic network based on fecal pellet analysis.

Introduction

Whale-falls and the invertebrate and microbial marine communities they host are one of the most extraordinary and poorly understood habitats in the world. Interest in these particular habitats grew after the accidental discovery of these communities on a whale carcass found in the eastern Pacific (Smith *et al.* 1989), and a series of studies has investigated different aspects of the biology, ecology, taxonomy and phylogeny of the invertebrate fauna associated with natural and experimentally implanted whale carcasses (*e.g.* Baco & Smith 2003; Smith & Baco 2003; Smith 2006; Braby *et al.* 2007; Lundsten *et al.* 2010; Glover *et al.* 2013). To a lesser extent, other mammal

bones such as cow and pig bones have also been studied in relation to the invertebrate fauna that they host (Jones *et al.* 2008; Anderson 2010). Thus, marine invertebrate communities associated with mammal bones have been studied almost in every ocean in the world at depths ranging from a few meters to about 3000 m depth.

Sunken whale-falls constitute massive inputs of long-lasting organic matter in the context of an otherwise food-limited benthic environment. At least for whale remains deposited in the deep-sea, whale carcasses are expected to go through four stages of succession (Bennett *et al.* 1994; Smith & Baco 2003), including a mobile-scavenger stage (soft tissue is removed by dense aggregations of necrophages), an enrichment-opportunistic stage (dense assemblages of opportunistic invertebrates, mainly polychaetes and crustaceans, colonize bones and adjacent organically enriched sediments), a sulfophilic stage (chemoautotrophic sulfur-based assemblages play a dominant role) and a reef stage (after the depletion of organic matter retained in the bones). Once scavengers and opportunistic species have consumed the flesh from carcasses, whale bones sustain invertebrate communities for years thanks to the slow degradation rate of the two main organic components that fill the bones, primarily lipids and proteins (Smith & Baco 2003). In these substrates, chemoautotrophic communities develop due to the presence of sulfide-oxidizing bacteria, including chemoautotrophic bacterial mats and endosymbionts, which dominate these particular ecosystems making the organic matter retained in the bones available for different macrofaunal invertebrates (Treude *et al.* 2009). Previous studies have concluded that this bonanza of nutrients linked with the subsequent enhanced bacterial activity promotes the occurrence of different nutritional modes among the organisms inhabiting the bones (Bennett *et al.* 1994): organisms directly feeding on primary whale organic matter; organisms grazing on free-living bacteria proliferating on the bone surface and within the bone trabecular matrix; chemoautotrophs using inorganic compounds (*e.g.* sulfides) produced by anaerobic bacteria; metazoan predators; and suspension feeders, these organisms being more common after the depletion of the organic matter.

Among the invertebrates living in association with mammal bones, the annelid polychaetes of the genus *Osedax* are perhaps one of the most remarkable examples. These bone specialists, occurring at a wide range of depths, are sessile polychaete siboglinids that live anchored to the bone surface thanks to a ramified root system in which they host endosymbiotic bacteria that degrade the organic matter in the bone (Rouse *et al.* 2004). However, the greatest diversity of organisms appears to live free on the bone surface and/or inside the bone in the galleries that appear after the natural degradation of the organic matter retained in the trabecular matrix. Polychaete dorvilleids of the genus *Ophryotrocha* are one of the most represented taxa, with congeneric species described from whale-fall experiments in different geographic areas and from a wide bathymetric range (Wiklund *et al.* 2009, 2012; Taboada *et al.* 2013). These opportunistic and sometimes extremely abundant polychaetes have been suggested to feed mainly on filamentous *Beggiatoa*-like bacteria that proliferate in the bone surface and within bone cracks and trabeculae; however, these organisms can not be considered as bone specialists as some of them are also present in anthropogenically enriched habitats (Wiklund *et al.* 2009).

To date, few studies have comprehensively described the invertebrate communities living in association with mammal bones and how these communities fluctuate through time (Bennett *et al.* 1994; Baco & Smith 2003; Smith & Baco 2003; Fujiwara *et al.* 2007; Lundsten *et al.* 2010). This is especially true for shallow-water communities, comparatively less studied than those from deep-water, which have only been investigated from modern whale remains from the North Sea and the Sea of Japan, and from fossil remains in the Mediterranean (Glover *et al.* 2005; Dahlgren *et al.* 2006; Fujiwara *et al.* 2007; Dominici *et al.* 2009; Danise & Dominici 2014; Danise *et al.* 2014). These shallow-water reduced habitats have recently been suggested to be more similar to other shallow-water organically enriched substrates, such as benthic assemblages affected by fish farms or sewage discharges (Danise *et al.* 2014). By contrast, deep-water whale-falls, far from the influence of the organic matter derived from photosynthesis, have strong affinities with other deep-sea chemoautotrophic habitats such as hydrothermal vents and cold seeps, where sulfide has an inorganic origin (Smith *et al.* 1989; Bennett *et al.* 1994; Smith 2006).

Aiming to increase the knowledge of shallow-water communities associated with mammal bones, we conducted a pioneering experiment in the shallow-water Mediterranean. Bones used in the experiments included not only defleshed vertebrae from a minke whale (*Balaenoptera acutorostrata* Lacépède, 1804), in order to obtain comparable results with experiments conducted in other geographic regions, but also bones from cows (*Bos primigenius taurus* Linnaeus, 1758) and pigs (*Sus domesticus* Erxleben, 1777), commonly used by local fishermen in the Mediterranean as bait to fish octopuses. The experiments were conducted to test three hypotheses in relation to the invertebrate communities that mammal bones host: (i) the bone-associated fauna will be different from the background fauna associated with the control areas and will be closer to anthropogenically enriched shallow-water areas, as suggested by Danise *et al.* (2014); (ii) differences in organic content and porosity of the bones will lead to differences in the

invertebrate communities in the different bones; and (iii) the species composition of bone communities will change through time and will be progressively replaced by organisms from the background fauna.

Material and Methods

Sample collection and preservation

Bones

Bones used in our experiments were obtained from a caudal fin of a common minke whale (*B. acutorostrata*) stranded in Asturias in 2008, and vertebrae from cows (*B. primigenius taurus*) and pigs (*S. domesticus*) from a local market. Bones were defleshed and drilled in order to ease their further attachment to experimental moorings. Cow and pig bones were cut into halves; whale bones were cut into four pieces to make them more comparable in size with the other two types of bones. After this, bones were immediately frozen at −20 °C until deployment on the sea bed.

Experiments were deployed at about 20 m depth on the sea bed of the Mediterranean coast of Blanes (NW Mediterranean, 41°40.536′ N, 2°48.839′ E; Fig. 1) in March 2011; bones were attached to pieces of ballast to avoid displacement from their original position. This locality was chosen because of the presence of three different habitats: rocky bottoms, sandy bottoms and a *Posidonia oceanica* (Linnaeus) Delile, 1813, meadow. This allowed the deployment and later collection of bones within single SCUBA dives. During the study, seawater temperature ranged from 12.8 to 20.7 °C (data from OOCS-CEAB-CSIC; Table 1), within the range of the temperatures recorded in recent years. The study area is relatively close to the Blanes Canyon, influenced by the Northern Current, a permanent southwestwards along-slope flow (Ahumada-Sempoal *et al.* 2013).

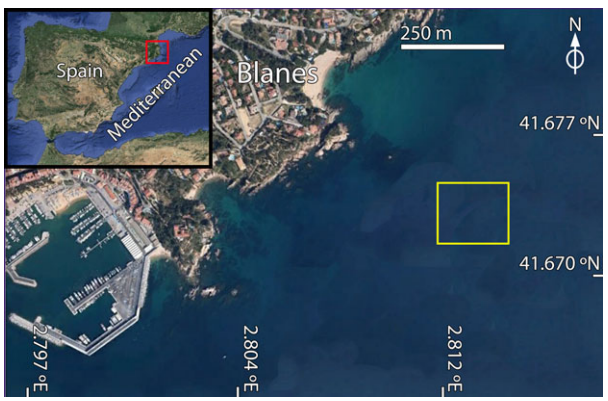

Fig. 1. Map showing the approximate area (yellow rectangle) where the experiments took place.

Table 1. Summary of the experimental bones and controls analysed during the study.

	Trimester 0		Trimester 1			Trimester 2			Trimester 3			Trimester 4		
	Sand	Rock	*Posidonia oceanica*	Sand	Rock	*P. oceanica*	Sand	Rock	*P. oceanica*	Sand	Rock	*P. oceanica*	Sand	Rock	*P. oceanica*
Bone experiment															
whale	×18*	×18*	×12*	×3	×3	×3	×3	×3	×3	×3	×3	–	–	×1	–
pig	×12*	×12*	×12*	×3	×3	×3	×3	×3	×3	×1	×3	×3	–	×1	×1
cow	×12*	×12*	×12*	×3	×3	×3	×3	×3	×3	×3	×3	×3	×3	×3	×3
Sampling date	29/03/11	29/03/11	29/03/11	29/07/11	29/07/11	29/07/11	11/10/11	11/10/11	11/10/11	01/02/12	01/02/12	01/02/12	11/05/12	11/05/12	11/05/12
Control	×3	×3	×3	×3	×3	×3	×3	×3	×3	×3	×3	×3	×3	×3	×3
Sampling date	18/05/11	29/03/11	29/03/11	02/08/11	29/07/11	29/07/11	21/10/11	11/10/11	11/10/11	01/03/12	01/02/12	01/02/12	23/05/12	11/05/12	11/05/12
Temp. (°C)	14.4	14.4	14.4	20.7	20.7	20.7	18.6	18.6	18.6	12.8	12.8	12.8	14.1	14.1	14.1

*Bone deployment.

Three replicates per type of bone [whale (Wh), cow (Cw) and pig (Pg)] were removed every three months [trimester 1–4 (T1–4)] at each of the substrates [rock (Ro), sand (Sa) and *Posidonia oceanica* (Ps)] (Table 1). Samples were then placed in separate plastic bags with seawater and immediately taken to the laboratory at the Department of Animal Biology, Faculty of Biology, University of Barcelona (Spain). Once there, bones were placed into individual containers with filtered seawater (0.22 μm) without supplementary oxygen, forcing the system to become anoxic. Daily or every 2 days, organisms inhabiting the bones were collected from the bone surface or the bottom of the containers using Pasteur pipettes and forceps, and transferred to plates with filtered seawater. This process was repeated until no invertebrate was noticed in the bone/containers, and took from a few days to about 3 weeks, depending on the degree of colonization of the bone.

Organisms were left overnight to eliminate gut content. Fecal pellets were preserved in 10% formalin buffered in seawater and photographed with an Invenio 5S 5MPixel CMOS camera attached to a light microscope and a stereo-microscope to further analyse the diet of the organisms. Prior to preservation, organisms were anesthetized in a 7% solution of $MgCl_2$ in fresh water, observed *in vivo* and photographed under a stereo-microscope, and finally fixed with 10% formalin buffered in seawater (taxonomic identification) or absolute ethanol and stored at −20 °C (molecular studies). All the invertebrates were identified to the lowest possible taxonomic level, counted and weighed. We used methyl green to distinguish ciliary patterns among *Ophryotrocha* species. The live photographs and light micrographs obtained were edited using Adobe PHOTOSHOP CS5, making the background black and enhancing contrast. Most of the light microscope images were obtained after 'Z-stacking' images at different focal distances using the program DELTAPIX-PRO.

Size frequency histograms were drawn for three of the most common species found in the bones [*Ophryotrocha puerilis* Claparède & Metschnikow, 1869, *Ophryotrocha alborana* Paxton & Åkesson, 2011; and *Neanthes caudata* (Delle Chiaje, 1827)] by measuring their maximum body length using the program DELTAPIX-PRO. Size frequencies were used to infer polymodal frequency distribution histograms using Bhattacharya's method by a routine of the FiSAT (FAO-ICLARM Stock Assessment Tools) computer program (Gayanilo *et al.* 1996).

Control samples

Controls were collected from areas adjacent to where bones were deployed to assess possible migration and the contribution of organisms from different habitats to the bones. Every 3 months, samples from algae attached to rock, sand and *Posidonia oceanica* were collected, coinciding with the period of bone collection (Table 1). Rock samples were obtained by scratching quadrats of 20 × 20 cm area (Bellan-Santini 1969) and consisted of assemblages of the red alga *Sphaerococcus coronopifolius* Stackhouse, 1797, and unidentified green, brown and calcareous algae. *Posidonia oceanica* samples consisted of three shoots of the plant per trimester (to infer density of these communities we considered the density of the *P. oceanica* meadow as 240 shoots · m^{-2}; M. Terradas J. Mora, & S. Taboada, unpublished results). Sand samples were obtained using a 600-cm^2 van Veen grab from a boat and consisted of medium to coarse sediments similar to those present in nearby beaches. A portion of the sediment was kept for granulometric (data not displayed in this study) and organic content analyses, the rest being sieved through a 500-μm mesh. Controls of the three substrates were fixed in 10% formalin buffered in seawater and later sorted at the laboratory at the Department of Animal Biology, Faculty of Biology, University of Barcelona.

Laboratory methods

Organic matter content

Organic matter content was calculated for bones and control sand substrates. Bone organic content was calculated for each of the bones used in the experiments; initial organic content of bones was calculated from several pieces of fresh bones not used during the experiments. For the sand samples, a subset of the sample collected with the van Veen grab was used to calculate the organic content. Both bone and sand samples were homogenized, dried for 72 h at 60 °C, and calcined with a muffle furnace at 550 °C for 10 h. The percentage of organic matter was obtained using the difference in weight of samples before and after calcination.

Bone quantitative and qualitative features

Bone surface areas were measured following the procedure proposed by Bennett *et al.* (1994): bones were covered by cut-outs of a single smooth layer of aluminum foil that was subsequently weighed.

In order to qualitatively characterize the features of every bone, once in the laboratory all bones were described. A particular emphasis was placed on characters related to bacterial activity. The qualitative features included: smell of SH_2, presence of bacteria, presence of encrusting algae and blackening of bones. Blackening is an indication of reducing processes while the presence of filamentous, sulfur bacterial mats is indicative of high fluxes of free hydrogen sulfide at the water–bone interface (Schippers & Jorgensen 2002; Jorgensen & Nelson 2004).

Barcoding of selected species

DNA extractions from a selection of individuals of the species *Ophryotrocha puerilis*, *Ophryotrocha alborana* and *Ophryotrocha robusta* Paxton & Åkesson, 2010 were sequenced to corroborate their taxonomic identification. Cytochrome *c* oxidase subunit I (COI) fragments were amplified, sequenced and compared with the existing *Ophryotrocha* sequences available in the NCBI database (www.ncbi.nlm.nih.gov). Details about the molecular procedures used can be found elsewhere (Taboada *et al.* 2013).

Data analysis

All of the invertebrates obtained from the bones and control samples were sorted, identified to the lowest possible taxonomic level, counted, and weighed, to generate an abundance matrix (individuals · taxon^{-1}) and a biomass matrix (mg wet weight · taxon^{-1}) (Supporting Information Appendices S1 and S2).

The abundance data matrix was analysed using the PRIMER® v. 6 statistical package (Clarke & Gorley 2006). As no apparent differences were observed in the results after removing rare species (contributing <1% to the total), we decided to keep the whole set of taxa in the different statistical analyses. Distances were calculated on pooled replicates based on Bray–Curtis similarity distances after dispersion-weighting (Clarke *et al.* 2006) and standardizing data to reduce the influence of the most abundant (opportunistic) taxa and to eliminate the effects of the different sizes of the samples. Multivariate patterns of variation among distances between controls and bones were visualized by non-metric multidimensional scaling (MDS) based on the whole invertebrate data set. Differences between controls and bones were investigated using an analysis of similarity (ANOSIM) test (Clarke 1990) (n = 9999 permutations). This test was also used to investigate the differences observed between controls and bones with only annelid data grouped by genus and family considered. Clarke & Warwick (2001) stated that reducing community redundancy data by using only family-level analysis is perfectly adequate for monitoring organic enrichment situations. An MDS plot was also calculated considering only control data to explore differences among the different substrates and differences among controls were also calculated using an ANOSIM test.

The whole set of bone samples was graphically represented using an MDS plot and a hierarchical agglomerative clustering analysis using the group average linkage method, which allowed different clusters within our survey to be inferred. To complement the information obtained with the MDS plot (based on ranks), we performed a principal co-ordinates (PCO) analysis, based on actual dissimilarities and super-imposed vectors of selected taxa using the Pearson correlation. A similarity percentage analysis (SIMPER; Clarke & Warwick 2001) was used to explore which taxa were principally responsible for the clusters identified in the dendrogram. The effect of reducing the accuracy of taxonomic identification, using higher taxonomic levels such as genus and family, was also tested just for the annelid data set.

The Simpson index (D) was calculated for all of the clusters and controls (grouped by the type of substrate). This index is commonly used to measure diversity when sample size is not homogeneous (Clarke & Warwick 2001) and ranges from 0 (one taxon dominates the community) to 1 (taxa equally present).

To provide a general overview of the contribution of the different phyla to the controls and clusters we used the matrix of abundance · m^{-2} (individuals · m^{-2}). Trophic structure of controls and clusters was also determined using the information on biomass. Trophic relationships are particularly influenced by organic matter input; thus, changes in the trophic structure may be interpreted as changes in the inputs of organic matter (Pearson & Rosenberg 1978). Every taxon was assigned to one of the following five trophic groups (see Fauchald & Jumars 1979): C, carnivores/omnivores; F, filter feeders; S, surface-deposit feeders; SS, subsurface-deposit feeders; M, mixed (filter and surface-deposit feeders). The wet weight obtained for every organism was transformed to dry weight using the conversion factors proposed by Ricciardi & Bourget (1998), thus obtaining a biomass matrix (mg of dry weight · m^{-2}).

Results

Controls *versus* bones

A total of 128 samples (controls and bones) were analysed during this study, comprising 287 taxa (137 of them identified to species level). Controls accounted for 40 samples (three replicates per trimester for each of the control sites except for the rock substrate with two replicates per trimester) with a total abundance of 5483 invertebrates, while the 88 bones (see Table 1 for details) yielded 6708 organisms. For both controls and bones, annelids were the most biodiverse phylum, accounting for ~ 67% of the total diversity; the total number of annelid taxa was 118 and 115 in controls and bones, respectively, with polychaetes being the predominant group (see Appendix S1). Annelids were followed by molluscs and crustaceans for the controls (15.7% and 9.6%, respectively) and by crustaceans and molluscs for the bones (12.3% and 10.5%, respectively). As for abundance

values, annelids also represented the most dominant group, with ~ 65% of the individuals for the controls and ~ 95% for the bones. The next most abundant groups for the controls were mollusks and crustaceans (14.6% and 8.7%, respectively).

The MDS in Fig. 2a shows the clear differences observed between controls and experimental bones (in both cases the replicates were pooled within samples), also supported by the ANOSIM analysis ($R = 0.933$; $P < 0.0001$). Identical results were also obtained after removing species that contributed <1%. Significant differences were also found among the different controls, indicating that different invertebrate communities inhabit these substrates ($R = 0.81$; $P < 0.0001$; Fig. 2b). Following the recommendations by Clarke & Warwick (2001), we analysed our data at a high taxonomic level (genus and family) focusing just upon annelids, the most abundant and diverse group within the survey and one of the groups most commonly used in similar studies. Differences between controls and bones were observed even at the family level, although R values for the ANOSIM comparisons were lower ($R = 0.441$ for genus; $R = 0.496$ for family). However, for the controls, no significant pairwise differences were observed between the rock and *Posidonia oceanica* sampling sites when using the annelid genus and family data subsets ($R = 0.199$, $P = 0.011$ using genus subset; $R = 0.077$, $P = 0.134$ using family subset).

Bones

Some of the bones collected during the last two trimesters (after 9–12 months of deployment) appeared to be heavily damaged, most likely due to erosive processes. These included pig and whale bones, which appeared to degrade faster than cow bones. For cases in which replicates were almost entirely degraded, they were not considered in the analysis (see Table 1). For this reason, multivariate statistical analyses such as PERMANOVA, which allows the partitioning of variance according to several factors and measures possible interactions among them, could not be performed with our bone data set.

The replicates of the whole bone data set were pooled to build a MDS plot and a hierarchical agglomerative cluster that showed four differentiated groups named Clusters A1, A2, A3 and B (Figs 3 and 4). PCO analysis confirmed the results obtained from the cluster analysis, with the first two axes of the ordination explaining 27.7% and 19.9% of the full distribution of samples (Fig. 5). Organic matter content did not appear to decrease over time (Table S1) and thus could not be correlated with the biological data. By contrast, qualitative observations such as the smell of SH_2 and bacterial activity appeared to be well correlated with the clusters inferred from the hierarchical ordination (Fig. 4); in general, bacterial activity and smell of SH_2 were absent in the samples making up Cluster B but were mostly present in the samples in Clusters A1–A3.

Cluster A1 was mainly composed of samples from the first and second trimesters regardless of the type of bone and substrate. Cluster A2 contained cow bones deployed in the sand substrate from the third and fourth trimesters, while cow and pig bones from trimesters 2–4 and from the three substrates characterized Cluster A3. Finally, Cluster B was composed of pig and whale bones predominately from the third and fourth trimesters from the three substrates. Samples within each of the clusters were grouped and analysed with the SIMPER routine to determine the contribution of each taxa to the observed similarity between groups (Table 2). Average similarity among clusters ranged from 13.5% in Cluster A3 to 23.0% in Cluster B. Few taxa were responsible for most of the abundance (up to 90% contribution) in Cluster A1, while for Clusters A2, A3 and B the percentage contribution was shared between a higher number of taxa. In Clusters A1–A3, dorvilleid annelids of the species *Ophryotrocha puerilis*, *Ophryotrocha alborana* and *Protodorvillea kefersteini* (McIntosh, 1869) were the most abundant organisms, although the nereidids *Neanthes caudata* and *Nereis perivisceralis* Claparède, 1868, also

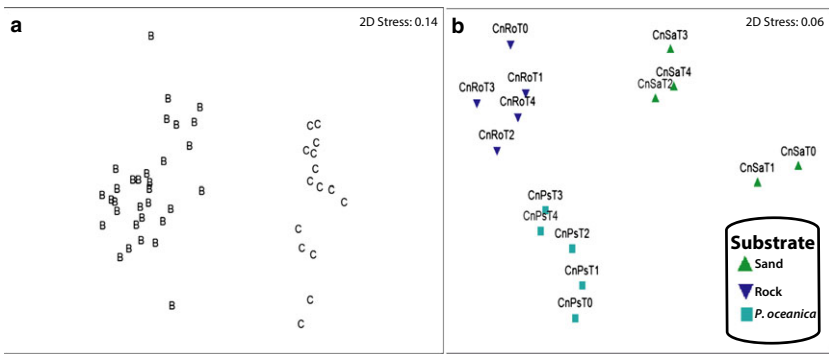

Fig. 2. Non-metric multidimensional scaling ordination of Bray–Curtis similarities from dispersion-weighting and standardized abundance matrix (a): comparing bones (B) with controls (C); and (b): only using controls. Sample abbreviations: Cn, control; Ps, *Posidonia oceanica*; Ro, rock; Sa, sand; T0, deployment of experiments; T1, trimester 1; T2, trimester 2; T3, trimester 3; T4, trimester 4.

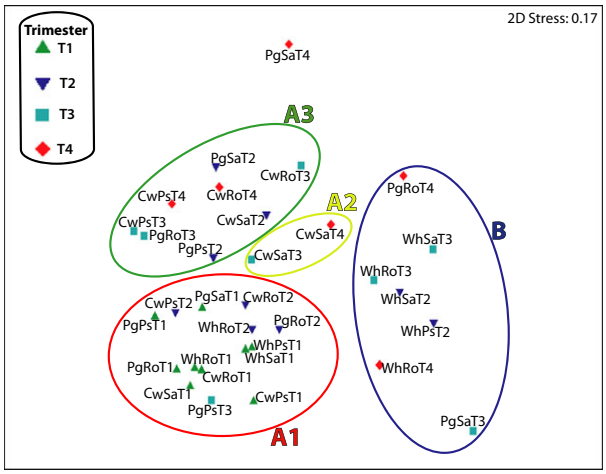

Fig. 3. Non-metric multidimensional scaling ordination of Bray–Curtis similarities from dispersion-weighting and the standardized abundance matrix using bones. A1, A2, A3 and B refer to the clusters defined in Fig. 4. Sample abbreviations: Cw, cow; Pg, pig; Wh, whale; Ps, *Posidonia oceanica*; Ro, rock; Sa, sand; T1, trimester 1; T2, trimester 2; T3, trimester 3; T4, trimester 4. See Table S1 for details.

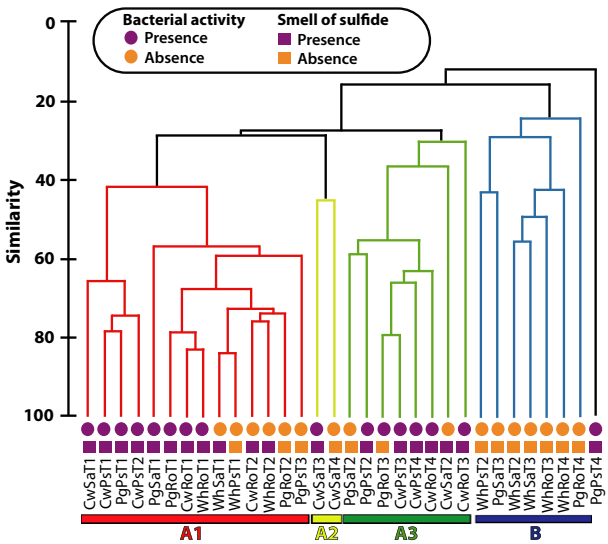

Fig. 4. Hierarchical agglomerative cluster (group average method) based on the Bray–Curtis similarities from dispersion-weighting and the standardized abundance matrix using bones. A1, A2, A3 and B correspond to the four different clusters used to group samples. Presence (purple) and absence (orange) of bacterial activity (circles) and smell of SH_2 (squares) associated with every set of samples.

played an important role. Cluster B was highly dominated by *Oxydromus pallidus* Claparède, 1864, an hesionid also present in the previous clusters, followed by other polychaetes such as *N. perivisceralis*, *Chrysopetalum debile* (Grube, 1855) and *Glycera tesselata* Grube, 1840, amongst other organisms. Abundance by phylum was also mea-

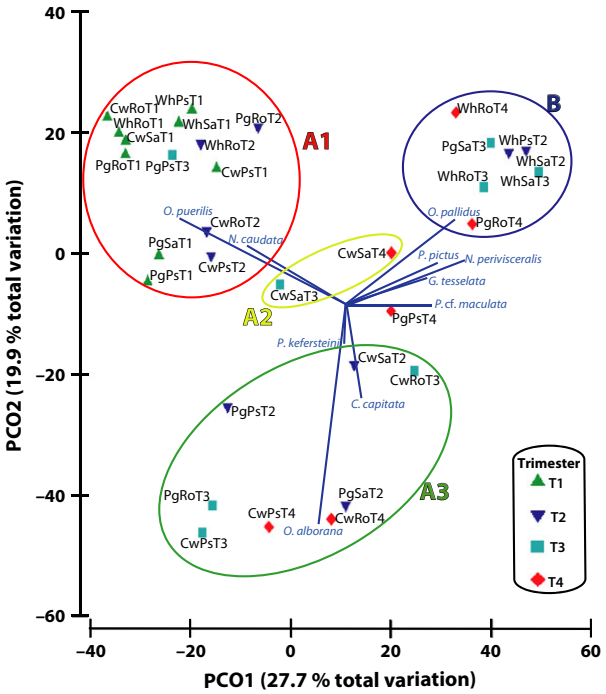

Fig. 5. Principal co-ordinates (PCO) analysis of Bray–Curtis similarities from dispersion-weighting and the standardized abundance matrix using bones. A1, A2, A3 and B refer to the clusters defined in Fig. 4. Super-imposed vectors using the Pearson correlation of a selection of taxa. See Fig. 3 for abbreviations.

sured for the different clusters, with the highest values seen for annelids (>80%). Annelids also played a predominant role for the controls but their contribution was less important in sand and *Posidonia oceanica*, where molluscs, arthropods and nematodes were found to make up remarkably high proportions (Fig. 6). Mean Simpson diversity indexes were lowest for Cluster A1 followed by Cluster A3, while values close to 0.9 were recovered for the rest of the clusters (A2 and B) as well as for the controls (Fig. 7).

Similarly to the analysis conducted with the controls, we also analysed our bone data set to higher taxonomic levels (genus and family), again focusing just on annelids. Cluster analysis showed an identical dendrogram as the one for the whole invertebrate data set. This picture was compared with the clusters obtained pooling annelids by genus and family (Figs S1 and S2), where a clear difference between Clusters A and B was still recovered but accuracy to determine Clusters A1–A3 was lost.

Population dynamics and colonization mechanisms

Frequency sizes were measured to infer population dynamics for three of the most abundant species collected

Table 2. Characteristic taxa of each of the clusters identified in the experimental bones using a SIMPER (SIMilarity PERcentage) analysis.

Taxon	Average abundance	Average similarity	Similarity/SD	Contribution (%)	Cumulative (%)
Cluster A1 (average similarity = 20.54)					
Ophryotrocha puerilis (ANN)	52.19	11.37	0.72	55.36	55.36
Neanthes caudata (ANN)	14.79	5.36	0.58	26.12	81.48
Oxydromus pallidus (ANN)	7.74	2.13	0.53	10.37	91.85
Ophryotrocha alborana (ANN)	2.57	0.33	0.2	1.59	93.44
Capitella capitata (ANN)	0.69	0.25	0.2	1.2	94.64
Glycera tesselata (ANN)	1.02	0.18	0.2	0.88	95.52
Cluster A2 (average similarity = 13.69)					
Protodorvillea kefersteini (ANN)	3.5	6.52	0.86	47.58	47.58
Ophryotrocha puerilis (ANN)	1.67	1.76	0.38	12.87	60.45
Oxydromus pallidus (ANN)	1	1.15	0.47	8.4	68.85
Neanthes caudata (ANN)	0.83	1.03	0.46	7.54	76.39
Nereis perivisceralis (ANN)	0.83	0.9	0.48	6.6	82.99
Syllis sp. (ANN)	0.83	0.7	0.48	5.12	88.1
Sphaerosyllis pirifera (ANN)	0.5	0.36	0.26	2.63	90.74
Polycirrus aurantiacus (ANN)	0.33	0.29	0.26	2.12	92.85
Cluster A3 (average similarity = 16.69)					
Ophryotrocha alborana (ANN)	34.21	5.64	0.5	33.79	33.79
Ophryotrocha puerilis (ANN)	14.79	3.98	0.74	23.85	57.64
Oxydromus pallidus (ANN)	1.42	1.65	0.52	9.9	67.54
Nereis perivisceralis (ANN)	1.17	0.88	0.31	5.29	72.83
Neanthes caudata (ANN)	1.21	0.72	0.36	4.29	77.12
Capitella capitata (ANN)	3.63	0.55	0.24	3.3	80.42
Sphaerosyllis pirifera (ANN)	0.63	0.51	0.28	3.07	83.49
Phyllodoce cf. *maculata* (ANN)	0.88	0.5	0.22	2.98	86.47
Ophryotrocha robusta (ANN)	2.5	0.49	0.27	2.92	89.39
Protodorvillea kefersteini (ANN)	1	0.34	0.21	2.03	91.43
Pomatoceros triqueter (ANN)	0.83	0.33	0.2	1.98	93.41
Ophryotrocha sp. (ANN)	0.33	0.27	0.24	1.63	95.03
Cluster B (average similarity = 23.04)					
Oxydromus pallidus (ANN)	15.6	13.07	1.26	56.73	56.73
Nereis perivisceralis (ANN)	10.4	2.16	0.56	9.39	66.12
Chrysopetalum debile (ANN)	3.27	1.45	0.7	6.29	72.42
Glycera tesselata (ANN)	1.73	1.42	0.56	6.15	78.57
Leptochelia savignyi (ART)	3.33	1.29	0.38	5.61	84.18
Phyllodoce cf. *maculata* (ANN)	3.13	0.61	0.46	2.64	86.82
Polyophthalmus pictus (ANN)	1.93	0.36	0.34	1.54	88.36
Neanthes caudata (ANN)	1.07	0.26	0.32	1.13	89.49
Sphaerosyllis pirifera (ANN)	1.33	0.25	0.21	1.08	90.57
Caulleriella cf. *bioculata* (ANN)	1.67	0.22	0.27	0.96	91.53
Prosthiostomum siphunculus (PLA)	0.73	0.21	0.28	0.9	92.43
Polycirrus aurantiacus (ANN)	1.53	0.19	0.29	0.83	93.26
Prosobranchia sp. (MOL)	0.33	0.16	0.15	0.7	93.96
Ophryotrocha puerilis (ANN)	2.27	0.15	0.21	0.65	94.61

ANN, Annelida; ART, Arthropoda; MOL, Mollusca; PLA, Platyhelminthes.

from the bones (*Ophryotrocha puerilis*, *Ophryotrocha alborana* and *Neanthes caudata*). *In vivo* observations confirmed different life history stages (egg masses, larvae, juveniles, adults and brooding adults) in *O. puerilis*. The frequency-size plots for this species showed that individuals from a wide range of sizes were present in different bones from Trimester 1 as well as in the only bone analysed from Trimester 2 (Fig. 8a). Recruitment appeared to be continuous in most of the bones analysed during Trimester 1, with the presence of three (in sample WhRoT1.1) and two (in samples WhRoT1.1, WhRoT1.3, WhSaT1.1, WhPsT1.1) cohorts of individuals.

As in *O. puerilis*, *O. alborana in vivo* observations confirmed the occurrence of juvenile, adults and gravid females; also similarly to *O. puerilis*, a wide range of frequency sizes with a predominance of medium-sized indi-

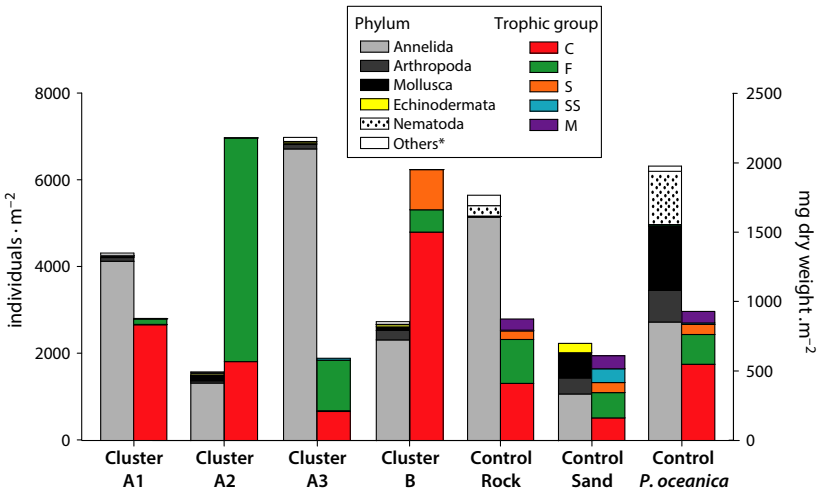

Fig. 6. Abundance and biomass contribution of the different phyla and trophic groups to controls and bone clusters. *, includes Chordata, Cnidaria, Nemertea, Platyhelminthes, Porifera, Sipunculida and Xenacoelomorpha. Abbreviations: C, carnivores/omnivores; F, filter feeders; S, surface-deposit feeders; SS, subsurface-deposit feeders; M, mixed group (filter and surface-deposit feeders).

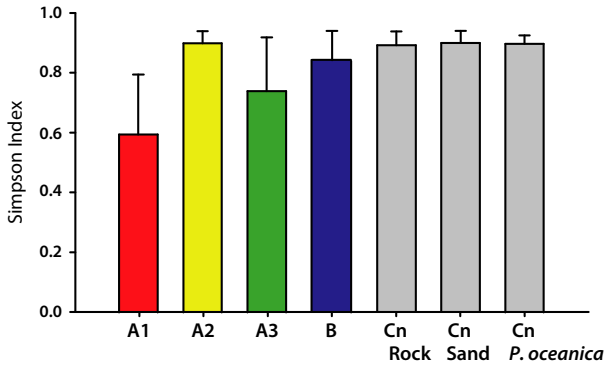

Fig. 7. Means and SDs of Simpson diversity index for controls and bone clusters. Abbreviations: Cn, Control.

viduals (1453–1820 μm) was recorded for *O. alborana* in the two trimesters analysed (Fig. 8b). Unimodal distribution sizes in the four bones analysed seem to support a unique event of colonization.

Finally, the analysis of frequency sizes for all the bones colonized by *Neanthes caudata*, except for CwPsT2.3, confirmed a unimodal and symmetric distribution of sizes (and therefore only one cohort). Bone CwPsT2.3 clearly showed a predominance of small-sized organisms, suggesting early recruitment, perhaps by the adults present in the bone (Fig. 8c).

Trophic relationships

Prior to calculating the trophic strategies present in the different bone clusters and controls, accidental organisms and juveniles of megafaunal invertebrates [*e.g.* the echinoderms *Paracentrotus lividus* (Lamarck, 1816) and *Ophiothrix fragilis* (Abildgaard, in O.F. Müller, 1789)] were removed from the analysis. The contributions of the five different trophic strategies considered here are shown in Fig. 6. Carnivores/omnivores followed by filter-feeders were the most represented trophic guilds in rock and *Posidonia oceanica* controls, accounting for ~ 80% of the total biomass; sand communities were characterized by a predominance of filter-feeders and carnivores/omnivores (~ 60%); and the subsurface-feeders, surface-feeders and mixed group accounted for ~ 10–15% each. As for bone clusters, carnivores/omnivores clearly dominated Cluster A1, mainly due to the contribution of *Neanthes caudata* and *Ophryotrocha puerilis*. Clusters A2 and A3 shared a similar picture: surface-feeders represented ~ 2/3 of the community biomass with a predominant influence of occasional large-sized terebellids and cirratulids in both clusters, and carnivores/omnivores accounted for ~ 1/3 of the community biomass, mainly due to the presence of medium-sized nereidids (*N. caudata*, *Nereis perivisceralis*). Finally, Cluster B was characterized by a predominance of carnivores/omnivores followed by surface- and filter-feeders. The contribution of carnivores/omnivores in this case was primarily due to the occurrence of occasional large-sized annelids (*e.g. Glycera tesselata*, *Hesione pantherina* Risso, 1826, *Phyllodoce* cf. *maculata*).

Fecal pellet analysis was mainly conducted for two of the most common species in the survey, *O. puerilis* and *N. caudata*, although information about feeding habits was also gathered for other organisms present in the bones (Table S2, Figs S3 and S4). The aim of this analysis was to preliminarily characterize the trophic network of the organisms present in the most altered bones (Clusters A1–A3). In most cases, the bones in these clusters were characterized by the presence of bacteria, either in the form of *Beggiatoa*-like filamentous bacterial mats (still pending identification) or in the form of bacteria embedded within a mucous layer; these bacteria were often correlated with the presence of ciliate protozoans. Fecal pellet

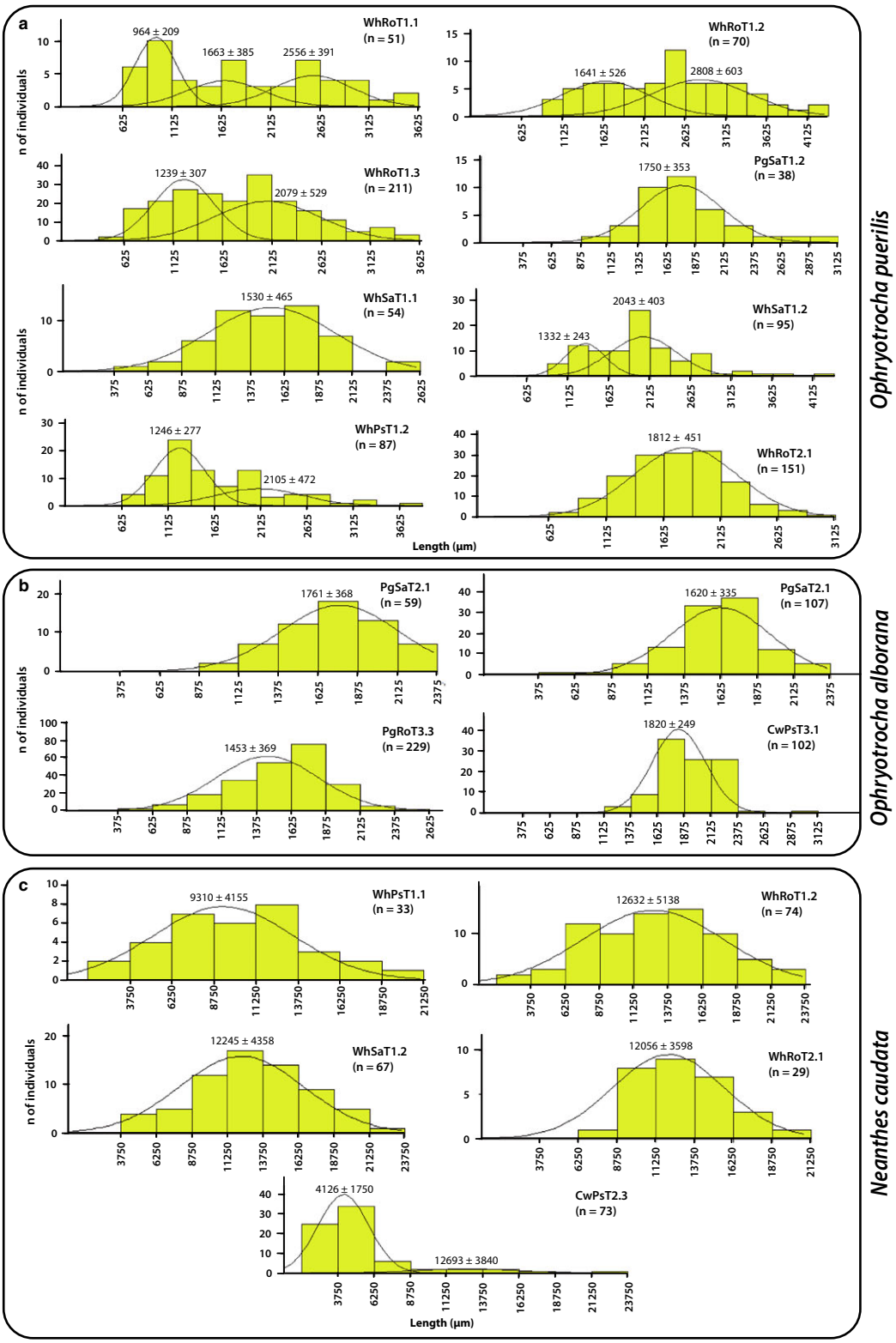

Fig. 8. Size-frequency histograms (based on total length) of the populations of (a): *Ophryotrocha puerilis*; (b): *Ophryotrocha alborana*; and (c): *Neanthes caudata*. Curved lines represent normalized expected frequencies. Average length (±SD) of the size groups determined by Bhattacharya's method. n, number of individuals per sample. See Table S1 for details of sample abbreviations.

analysis on samples containing individuals of *N. caudata* showed that the majority of samples contained packages of compound chaetae of conspecific individuals, and occasionally, mandibles of *N. caudata*, which may indicate cannibalistic behavior. The carnivorous diet of *N. caudata* also encompassed *Ophryotrocha* spp., crustaceans and *N. perivisceralis*. Other than these, remains of bacteria and algae were also common in *N. caudata* pellets. Tubular structures were observed in some parts of the bone; these empty structures, attached to the bone surface as an extension of the galleries present in the bone, were built by *N. caudata* from the inside by depositing fecal pellets and other inorganic elements (*e.g.* pieces of bone) at the end of the tube.

As for *O. puerilis*, fecal pellet analysis indicated that bacteria was the most common dietary resource, although algae were also observed when the bones had encrusting algae. Interestingly, some of the fecal pellets analysed had packages of compound chaetae resembling that of *Ophryotrocha* spp. and in two cases they were accompanied by *Ophryotrocha* spp. jaws, which may suggest, as for *N. caudata*, cannibalistic behavior. Fecal pellets of other species were also analysed but with lower replicative efforts: *Glycera tesselata* had remains of conspecific organisms, the nemertean *Cephalothrix rufifrons* (Johnston, 1837) had *Ophryotrocha* spp. jaws, Nereididae sp. 3 had remains of Nereididae compound chaetae (probably *N. caudata*) and the crustacean *Nebalia kocatasi* Moreira, Kocak & Katagan, 2007, had remains of algae.

Besides the information obtained from the fecal pellet analysis, the species *O. puerilis*, *O. alborana* and *Capitella capitata* (Fabricius, 1780) were occasionally seen feeding on bacterial aggregations.

Discussion

Our work is the first to describe shallow-water invertebrate communities associated with modern mammal bones in the Mediterranean Sea. We demonstrated that at least some of the bones in the present study (Clusters A1–A3) became islands hosting macrofaunal and microbial communities noticeably different from those of the background. So far, the only similar studies conducted in the Mediterranean (Dominici *et al.* 2009; Danise *et al.* 2010; Danise & Dominici 2014) come from whale fossil skeletons in shelf sediments of the Tyrrhenian Sea, which makes comparisons with our results very difficult. In these previous studies, malacofauna did not differ from the fauna present at the site before whale sinking except for the occurrence of two chemosymbiotic species. In contrast, the remarkable differences between the controls and bones in our study were evident even when using the annelid data set at family level, according to what has been reported in other studies comparing organically enriched environments with non-altered environments (Clarke & Warwick 2001).

Similarly to the findings of Bennett *et al.* (1994) , some of the bones in our experiments showed heterogeneous abundance and species compositions that could not be correlated with the type of bone, the time since their deployment or the substrate where the bones were deployed. Smith & Baco (2003) also pointed out that successional stages in a whale carcass often overlap and that sometimes there is no clear distinction between different stages. In our case, this heterogeneity may indicate that bones were subjected to different abiotic factors (*e.g.* erosion by sediment) or else it might be the result of asynchronic population founder events. At least four different successional stages, corresponding to Clusters A1–A3 and B, could be distinguished within our experimental bones using both diversity and trophic parameters. Clusters A1–A3 contained communities primarily sustained by chemoautotrophic bacteria fueled by sulfides. The first successional stage, Cluster A1, was mainly composed of samples with the opportunistic polychaetes *Ophryotrocha puerilis*, *Neanthes caudata* and *Oxydromus pallidus*, although other species with a lower contribution present in this cluster such as the polychaete *Capitella capitata* and the leptostracan *Nebalia kocatasi* are widely distributed in altered environments (Pearson & Rosenberg 1978; Moreira *et al.* 2007, 2009; Simonini *et al.* 2010). Cluster A3 was characterized by the replacement of *O. puerilis* by its congener *Ophryotrocha alborana* in terms of relative abundance, which may indicate that *O. alborana*, to the best of our knowledge only known from a couple of harbors in the SW Mediterranean Sea (Paxton & Åkesson 2011), would be a late opportunistic species. Also within Cluster A3 there seemed to be a replacement of the nereidid *N. caudata* (dominant nereidid in Cluster A1) by *Nereis perivisceralis*. As for Cluster A2, *Protodorvillea kefersteini* and *O. puerilis* accounted for the major proportion of the group's abundance, the former commonly appearing in association with high inputs of organic matter and low oxygen concentrations (see Pearson & Rosenberg 1978). Finally, Cluster B was mainly characterized by the presence of *Oxydromus pallidus*, a hesionid polychaete common in polluted environments (see Pearson & Rosenberg 1978), and by the presence of species frequently observed in the background fauna such as the polychaetes *Nereis perivisceralis*, *Chrysopetalum debile*, *Glycera tesselata* and *Polyophthalmus pictus* (Dujardin, 1839), or the crustacean *Leptochelia savignyi* (Krøyer, 1842) (Viéitez *et al.* 2004; R. Sardá, personal observation). Thus, we suggest that Cluster B could be regarded as a community close to returning to background conditions, as opposed to Clusters A1–A3, which are representatives of high to

moderately polluted communities. It should also be noted that differences among the identified clusters were smaller when the annelid data set was analysed at higher taxonomic levels (genus, family), emphasizing the need to use species-level identification in order to monitor shifts in successional stages.

The communities regarded as altered in our experiments (Clusters A1–A3) appeared to be closer to other shallow-water organically enriched marine environments than to deep-water reducing environments. This is in agreement with a recent study by Danise *et al.* (2014) on the malacofauna associated with a shallow-water whale carcass near the Swedish coast that concluded that shelf-depth whale-falls are natural analogues of organic polluted areas. Organic matter inputs are considered one of the principal causes for faunal changes in nearshore benthic marine environments. These polluted environments are generally characterized by the ubiquitous presence of a few opportunistic species belonging to the polychaete families Capitellidae, Spionidae and Dorvilleidae (Pearson & Rosenberg 1978). In the Mediterranean, different studies on the benthic community composition of anthropogenically enriched areas have corroborated these generalities. Karakassis *et al.* (2000) concluded that the capitellid *Capitella capitata* and the dorvilleid *Protodorvillea kefersteini* were the characteristic species of the most impacted sediments beneath fish farms. More recently, in their study after the cessation of sewage discharges in the Barcelona metropolitan area, Serrano *et al.* (2011) concluded that the initial invertebrate community, clearly dominated by *C. capitata* and the spionid *Malacoceros fuliginosus* (Claperède, 1870), was replaced 20 years later by a more diverse community dominated by *Ophryotrocha hartmanni* Huth, 1933, and the capitellids *Mediomastus fragilis* Rasmussen, 1973, and *C. capitata*. Besides fish farms and sewage discharges, *Ophryotrocha* is known to be widely distributed in Mediterranean harbors (Simonini *et al.* 2010). Interestingly, the abundance of *O. puerilis* was reported to be low with respect to that of other congeneric species co-existing in the same harbor (Prevedelli *et al.* 2005), in contrast to the high abundances that we reported for this species in our experimental bones. Other studies on whale- and wood-falls in different geographic areas (Wiklund *et al.* 2009, 2012; Taboada *et al.* 2013) agree that *Ophryotrocha* representatives play a central role in shallow-water communities inhabiting naturally organically enriched substrates such as mammal bones.

A common feature shared by the most abundant species found in the altered assemblages in the present study (Clusters A1–A3) is their ability to reproduce rapidly and efficiently. This is a characteristic shared by opportunists, which tend to be small, short-lived species with rapid attainment of sexual maturity, reproducing several times a year through direct development, thus reducing dispersal (Pearson & Rosenberg 1978; Levin 1984). Most of these species are hermaphroditic, ensuring more efficient mating, as is the case for *Ophryotrocha puerilis* and *O. alborana* (Åkesson 1967; Paxton & Åkesson 2007, 2011). Our data on the size-frequency distribution of *O. puerilis* indicated possible multiple recruitments events during the first trimester (summer season), with remarkably high abundances (Fig. 8a). This contrasts with what has been reported for this species in previous studies that recorded maximum abundances during winter and spring months (Simonini 2002; Prevedelli *et al.* 2005). The nereidid *Neanthes caudata* is a monotelic (*i.e.* females reproduce only once in their lifetime) brooding species with direct development (Reish 1957), this last feature being corroborated here after the remarkable recruitment episode observed in one of the bones examined (Fig. 8c). Direct development, known to occur in *O. puerilis*, *O. alborana* and *N. caudata*, ensures that, once colonized, ephemeral habitats such as mammal bones will be efficiently exploited by the founder colonizers; in other words, initial colonization of bones may be followed by reseeding of established populations, something already noticed in similar experiments in other areas (Bennett *et al.* 1994). However, whether colonization of bones by external populations occurs once followed by reseeding or *via* multiple events is a question that still remains unsolved. In this context, we are currently undertaking molecular investigations using the *COI* molecular marker that will attempt to verify the origin of the populations of two of the most common species in the survey, *O. puerilis* and *O. alborana*.

Following the categorization proposed by Bennett *et al.* (1994), a range of different nutritional modes could be distinguished in our assemblages. We reported no organisms feeding directly on the organic matter retained in the bones (*e.g.* endosymbiosis with bacteria in *Osedax* spp.), in contrast to the findings of previous studies of deep-water communities and of other shallow-water whale carcasses (Bennett *et al.* 1994; Glover *et al.* 2005, 2013; Braby *et al.* 2007; Fujiwara *et al.* 2007). Focusing again on the most altered bones (Clusters A1–A3), and based on the fecal pellet content of some of the organisms and on *in vivo* observations, we may hypothesize about a possible trophic network. Chemoautotrophic bacteria (*Beggiatoa*-like filamentous and bacteria embedded within a mucous layer) would be at the base of the trophic web as primary producers (Treude *et al.* 2009). These bacteria would be a potential food resource and physical niche to hide from predation to different ciliate protozoans, as suggested in a recent study (Buck *et al.* 2013). The mixture of bacteria and ciliates could in turn become the food resource for some of the most common grazers in our survey such as

Ophryotrocha puerilis, *O. alborana*, *Protodorvillea kefersteini* and *Capitella capitata*. The annelid *Oxydromus pallidus* is an example of predator although other occasional organisms could be identified as potential predators (*e.g.* the annelid *Glycera tesselata*, the nemertean *Cephalothrix rufifrons*). Finally, suspension feeders would be represented by a range of species in the families Serpulidae, Cirratulidae and Terebellidae [*e.g. Spirobranchus triqueter* (Linnaeus, 1758), *Caulleriella* cf. *bioculata*, *Polycirrus* spp.]. Interestingly, the fecal pellet analysis suggested that *N. caudata* is a very versatile omnivore with a tendency to prey on conspecific organisms. Although cannibalism was primarily attributed to *N. caudata* males preying on spent females after hatching (Reish 1957), we can not dismiss the possibility that it may also be a strategy to control overpopulation. All things considered, it appears that the trophic network involving the organisms inhabiting the bones (a habitat presumably closed to the exterior) in the most altered assemblages examined here was more complex than expected from the relatively low diversity that they hosted, and certainly requires further study with complementary and more accurate approaches (*e.g.* stable isotope analysis).

Our study confirms that, at least for pig and cow bones, invertebrate communities of opportunistic species associated with shallow-water mammal bones in the Mediterranean are capable of developing for at least a 1-year period. Indeed, bones of these species can host such communities for several years: observations on cattle bones regularly used to fish octopuses in a nearby area confirmed that similar communities (polychaete fauna and microbial community) developed in the bones for more than 2 years (S. Taboada, unpublished data). On the contrary, whale bones seemed to degrade faster, perhaps because these bones are more porous and hence expose a higher percentage of bone surface for degradation, and/or because the whale bones in our experiments came from a juvenile, whose skeletons are known to decompose much more rapidly due to their vertebrae being poorly calcified compared with adults (Smith & Baco 2003). Other than that, the relatively high water temperatures of the Mediterranean could intensify the remineralization induced by bacteria of the organic matter retained in the bones, something that has already been suggested in similar studies with whale-falls in the Sea of Japan (Fujiwara *et al.* 2007).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Hierarchical agglomerative cluster (group average method) based on the Bray-Curtis similarities from dispersion-weighting and standardized abundance matrix using Bones pooled by genus.

Figure S2. Hierarchical agglomerative cluster (group average method) based on the Bray-Curtis similarities from dispersion-weighting and standardized abundance matrix using Bones pooled by family.

Figure S3. (A) Living *Beggiatoa*-like bacterial mats; (B) Light micrograph of formalin-preserved *Beggiatoa*-like bacterial mats; (C) Light micrograph of formalin-preserved of bacteria embedded within a mucous layer; (D) Living ciliate protozoans.

Figure S4. (A) Light micrograph of formalin-preserved fecal pellets of *Neanthes caudata* composed by rests of bacteria; (B) Light micrograph of *N. caudata* formalin-preserved fecal pellet with the jaw apparatus of *Ophryotrocha* sp.; (C) Light micrograph of *N. caudata* formalin-preserved fecal pellet with a mandible and chaetae of *N. caudata*; (D) Light micrograph taken *in vivo* of a tube (arrow) built by *N. caudata* attached to the bone surface; (E) Light micrograph of *Glycera tesselata* formalin-preserved fecal pellet with four mandibles and chaetae of *G. tesselata*; (F) Living nemertean *Cephalotrix rufifrons*. Inset with detail of *Ophryotrocha* sp. mandibles in the distal part of the nemertean's gut.

Appendix S1. Total abundance of individuals per sample (individuals. taxon^{-1}). See Fig. 2 for sample abbreviations.

Appendix S2. Total biomass per sample (mg fresh weight. taxon^{-1}). See Fig. 2 for sample abbreviations.

Table S1. Quantitative and qualitative data from the experimental bones and controls.

Table S2. Fecal pellets analysis of different species collected from the experimental bones.