



ORIGINAL ARTICLE

Sponge waste that fuels marine oligotrophic food webs: a re-assessment of its origin and nature

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Keywords

Benthic ecology; caves; cell renewal; choanocyte; DOM; egestion; invertebrate excretion; nutrient loop; nutrient recycling; oligotrophy; POM; Porifera; reefs.

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Abstract

It has recently been realized that sponges take up much of the dissolved organic matter (DOM) available in the water of reefs. The energy derived from this DOM is suggested to be invested in renewing the sponge filter cells (choanocytes) every few hours, generating an outflow of detrital particulate organic matter (POM) that is rapidly ingested by other invertebrates. By this DOM-to-POM recycling, sponges are proposed to fuel the food web of oligotrophic marine communities, including reefs, caves and deep-sea environments. In four species studied herein by electron microscopy, the POM found in the outgoing aquiferous canals had a complex composition, with large between-species differences. It may include choanocytes (0–52%), and also mesohyl cells, such as archeocytes (9–20%) and spherulous, and granular cells with inclusions (27–90%). Exocytosed vesicles also occurred. Surprisingly, to end up into the outgoing canals, the internal mesohyl cells are squeezed between the epithelial cells (endopinacocytes) of the canal wall. Mesohyl cells were also able to transfer their inclusions to the endopinacocytes, which in turn extruded their acquired vesicle loads into the canal lumen. The unanticipated abundant participation of mesohyl cells and endopinacocytes in the production of POM appears to be an ordinary process that occurs continuously in the sponges, mostly related to elimination of digestion leftovers and excretion by-products. Therefore, POM is generated by sponges irrespective of whether the primary food source is particulate (evidence from this study) or DOM (previous literature). Altogether, these results indicate that the cellular mechanisms behind the relevant organic-matter recycling carried out by sponges are more diverse than initially anticipated. The varying ratios of choanocytes/mesohyl cells in the POM across species suggest that different sponge species may impact differently the energetics of food webs of the respective oligotrophic habitats where they dominate.

Introduction

Sponges are prominent members of coral reefs where they mediate the transfer of energy and matter through the flux of organic carbon and dissolved inorganic nutrients (Reiswig 1974; Pile 1997; Gili & Coma 1998; Maldonado *et al.* 2012). A new perspective on their trophic role comes from the recent finding by de Goeij *et al.* (2013)

that reef sponges take up most of the dissolved organic matter (DOM) available in the water column before it is transferred away from a reef. The fate of that DOM carbon used by sponges has been a mystery, as respiration requires only about 40% of the total carbon taken up, and the remainder is not converted into detectable growth. de Goeij *et al.* (2013) proposed that DOM energy may be invested in renewing the entire cell layer of choanocytes (monociliated filtration cells) every few hours. The choanocyte renewal would produce a significant outflow of particulate organic matter (POM) rich in carbon

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and nitrogen that could be rapidly assimilated by a variety of invertebrates, thereby fueling the reef food chain. By this mechanism, sponges are proposed to play a crucial trophic role, fueling food chains of not only coral reefs but also many other oligotrophic marine communities, including caves, varied deep-sea habitats, etc.

By invoking a peculiarly rapid cell kinetics discovered in the sponge *Halisarca caerulea* after labeling mitotic cells with 5-bromo-2'-deoxyuridine (BrdU), it was initially proposed that choanocytes were entirely replaced and shed every 4 h (de Goeij *et al.* 2009) and that this extensive cell shedding might be the main source of the POM outflow in other demosponges as well (de Goeij *et al.* 2013). Subsequent work by de Goeij's team on *H. caerulea* has found choanocyte proliferation rates more than twice as slow as their own former estimates, with values similar to those also estimated for seven additional demosponges (Alexander *et al.* 2014). In this latter study, it was noticed that, unlike in the choanocyte chambers, very few cells of the mesohyl become BrdU labeled, which was interpreted as mesohyl cells having much lower proliferation rates than choanocytes. An even smaller amount of those mesohyl cells were tentatively identified in the outgoing POM of the sponges by light microscopy (Alexander *et al.* 2014). Interestingly, it was also reported that the total amount of choanocytes observed in the outgoing POM of the eight studied demosponges using light microscopy was not as much as expected from the estimated cell proliferation rates using BrdU (Alexander *et al.* 2014). Such a combination of results, even if unnoticed by the authors, opens up the idea that production of outgoing POM and cell proliferation are processes only partially associated with each other and that POM generation may derive from a more complex combination of cellular routes.

It must be acknowledged that the characterization of sponge cell dynamics pursued by de Goeij's team is a difficult challenge, the results of which are strongly determined by the intricacies of the use of BrdU, a method with some unclear aspects when applied to sponges. For instance, no control procedure has been performed so far to assess whether the DNA chemical labeling by BrdU may be responsible for either part of the cell shedding or for increasing the natural ratios of cell division. Although BrdU has often been used as a 'vital' label for tracking proliferative cancer cells, among its discovered side effects are that it may alter DNA transcription (Hill *et al.* 1974), cause mutation at high concentration (Konishi *et al.* 2011), release gene silencing caused by DNA methylation (Weiss 2013) and more. Likewise, there is no information about the rate at which this compound diffuses into the deep sponge mesohyl. However, it is obvious that while choanocytes come into contact with BrdU almost instan-

taneously upon its addition to seawater, mesohyl cell takes an undetermined longer period, a slower BrdU diffusion through the sponge tissue during which the reactivity of the compound at a given concentration may also be altered. Therefore, the level at which mesohyl cells proliferate compared with choanocytes remains unclear because the results of the two previous approaches may be biased by a slow BrdU infusion into the sponges. In addition, it is not surprising that the inferred high rates of cell proliferation have been found not to couple with either similarly high cell shedding or detectable net body growth. The sponge mesohyl is a very lax tissue, with amoeboid cells wandering at low density in an ample gel-like intercellular medium consisting of collagen fibrils, dissolved macromolecules and often bacteria and cyanobacteria. A good portion of the daily or even weekly mesohyl cell proliferation could transiently be accumulated into the mesohyl, resulting in just a bit denser tissue in terms of cell numbers, and not translating directly into cell shedding or detectable net body growth. Likewise, proliferation of choanocytes does not necessarily mean immediate cell renewal, as choanocyte chambers can modify their size and new choanocyte chambers be formed. Such a new choanoderm biomass would again protrude into an easily compressible mesohyl, causing no detectable body growth but just increasing the folding of the sponge choanoderm.

In summary, because available estimates of choanocyte proliferation using BrdU provide only a partial explanation to understand the production of outgoing POM by sponges, an alternative, complementary approach may be required to further clarify this biological process. To this aim, the ultrastructure of the POM material found '*in situ*' in the excurrent canals of four sponge species was here investigated by transmission electron microscope (TEM). This approach provides a descriptive snapshot into the cellular process of POM production and by no means is it intended to quantify POM production over time. Seeking to complement the results from previous studies based on cell proliferation by de Goeij *et al.* (2009, 2013) and Alexander *et al.* (2014), the current research aimed to document in detail the relative abundances of the cell types contributing to the outgoing POM at a given time and whether the cellular mechanisms of POM production vary substantially across species.

Material and Methods

Site characteristics and sample collection

Four demosponge species living in oligotrophic habitats at the Chafarinas Islands (35°11.00' N, 2°26.00' W; Alboran Sea, Western Mediterranean) were investigated by TEM. The growth habit of the examined species ranged

from thinly encrusting in *Protosuberites epiphytum* (Fig. 1A) to thickly encrusting in *Thymosia* sp. nov. and *Dictyonella marsilli* (Fig. 1B and C), and submassive in *Aplysina cavernicola* (Fig. 1D). The sampled individuals of the species *P. epiphytum* and *D. marsilli* inhabited a submerged cave at 15 m depth (Halcon Point Cave at El Rey Island; 35°10'54.00" N, 2°25'19.76" W). The individuals of *Thymosia* sp. and *A. cavernicola* inhabited both a semi-submerged cave (3 m deep; 35°10'40.66" N, 2°26'37.46" W) at Congreso Island and an overhanging cliff in the submerged Laja Bank (35°11'7.47" N, 2°26'12.19" W) at depths of 30–37 m.

Electron microscopy protocols

Small tissue portions ($n = 3$) were carefully excised from two individuals of each species during SCUBA dives in

October 2010. By using a surgical scalpel, minimum damage was caused to both the collected tissue samples and to the donor sponges (which were not collected and remained healthy 1 year later). Collected tissue samples remained stored in seawater to be fixed immediately upon dive completion (approximately 45–60 min after collection). Fixation took place by immersing 2×2 mm tissue pieces for 4 h in a fixative cocktail consisting of 2% glutaraldehyde, 2% osmium tetroxide, 65% sodium acetate buffer, 11% sucrose and 20% distilled water. Initial dehydration steps took place in 50% ethanol (2 h) and then 70% ethanol, in which samples were preserved for 1 month until returning to the laboratory on the mainland. Dehydration was then resumed in 70% (10 min), 80% (10 min), 90% (3×10 min), 96% (3×10 min) and 100% ethanol (3×10 min), followed by propylene oxide (2×10 min). Embedding in Spurr

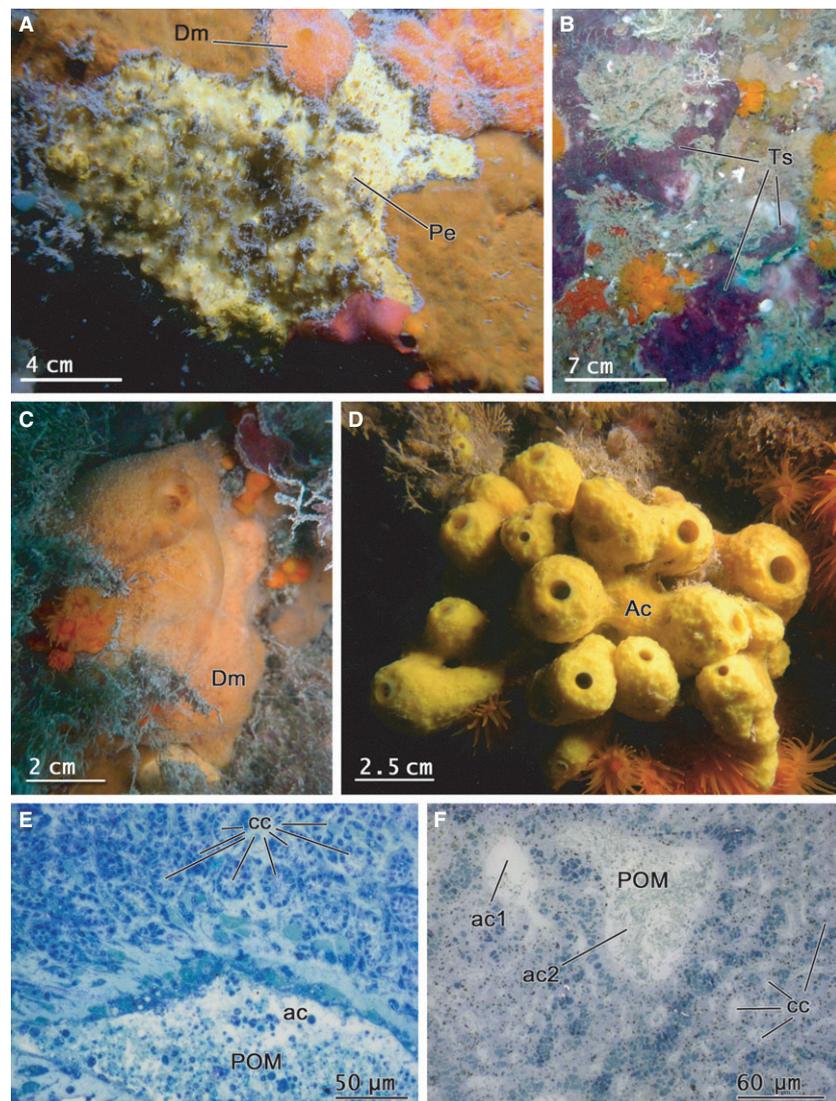


Fig. 1. *In situ* views of the sampled animals and views of the general tissue structure. (A): Individuals of *Protosuberites epiphytum* (Pe) and *Dictyonella marsilli* (Dm). (B): Individuals of *Thymosia* sp. (Ts). (C): Close-up of an individual of *D. marsilli*. (D): Individual of *Aplysina cavernicola* (Ac). (E): Semi-thin section of the mesohyl of *P. epiphytum* showing numerous choanocyte chambers (cc) and an aquiferous canal (ac) full of particulate organic matter (POM). (F): Semi-thin section of a *Thymosia* sp. individual showing a large aquiferous canal (ac2) filled with POM and a smaller aquiferous canal (ac1) by the side that is virtually clean of discarded material. Abundant choanocyte chambers (cc) can also be seen in the surrounding mesohyl.

resin required five immersion steps with gentle shaking during each one: 6 h in a 3:1 propylene-oxide/resin solution, 12 h in 2:2 propylene-oxide/resin solution, 7 h in a 1:3 propylene-oxide/resin solution and two 6-h steps in pure resin. Resin was hardened at 60 °C for

5 days. Ultrathin sections were obtained with an Ultracut Reichert-Jung ultramicrotome, mounted on gold grids and stained with 2% uranyl acetate for 30 min, then with lead citrate for 10 min. Observations were conducted with a JEOL 1010 TEM operating at 80 kV

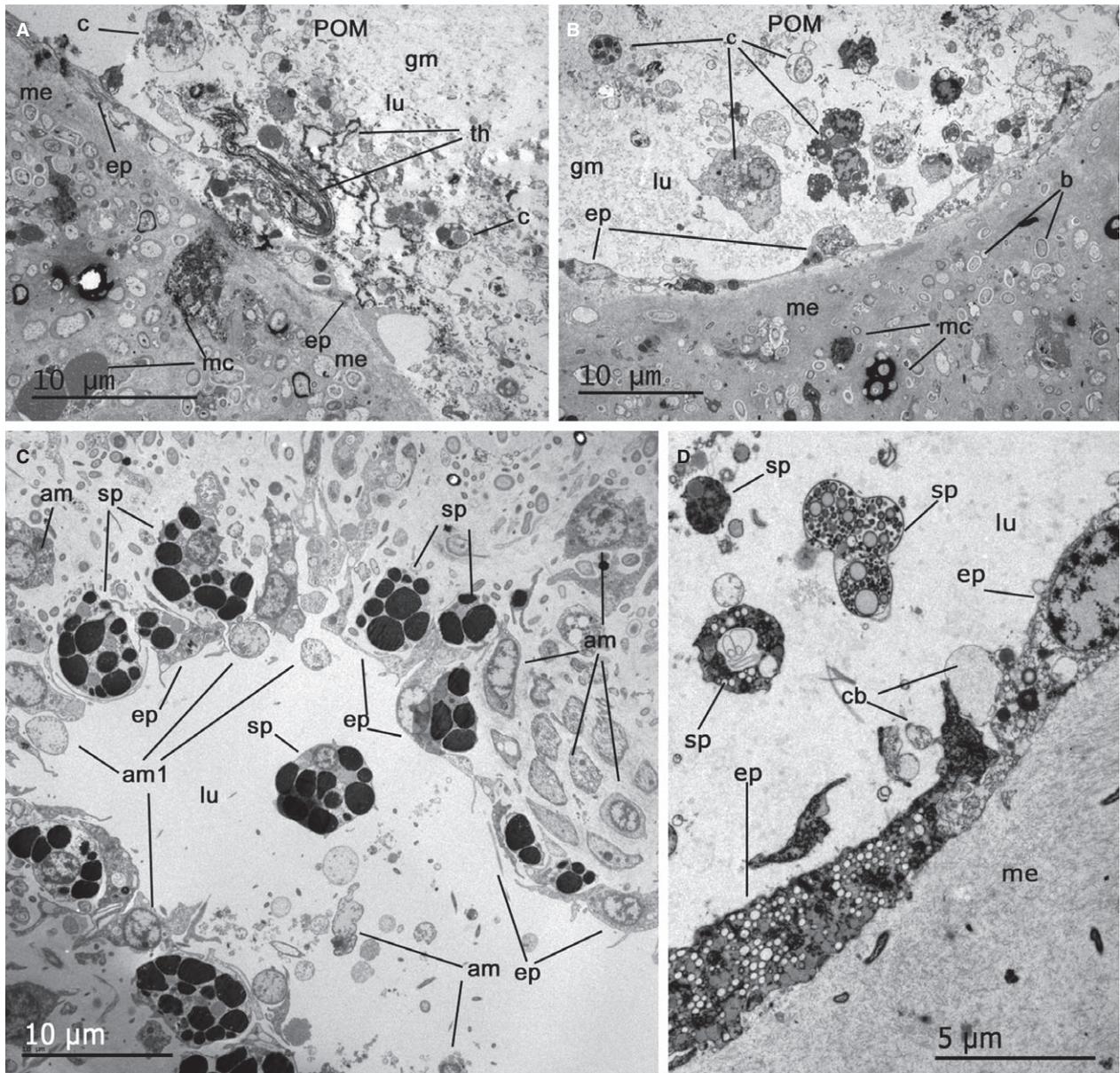


Fig. 2. General transmission electron microscope views of particulate organic matter (POM) located in the lumen of the outgoing canals. (A and B): Lumen (lu) of aquiferous canals of *Thymosia* sp. containing cells (c) of several sizes, along with thread-like or membrane-like structures (th) and an amorphous gel-like matrix (gm). Note the canal wall made up of epithelial cells (ep, endopinacocytes). Abundant symbiotic bacteria (b) and various mesohyl cells (mc) occur in the sponge mesohyl (me). (C): Lumen (lu) of aquiferous canal of *Aplysina cavernicola* containing large and small cells (sp, spherulous cells; am, archaeocyte-like cells) that have been released. Note also the accumulation of archaeocyte-like and spherulous cells under the epithelium (ep) of the canal, as well as migration of some archaeocyte-like cells (am1) throughout the epithelium. (D): Lumen (lu) of aquiferous canals of *Protosuberites epiphytum* containing remains of spherulous cells (sp). Note that the epithelial cells (ep) lining the canal contain abundant microvesicles and are exocytosing portions of their cytoplasm (i.e. cb, cytoplasm residual bodies; me, mesohyl).

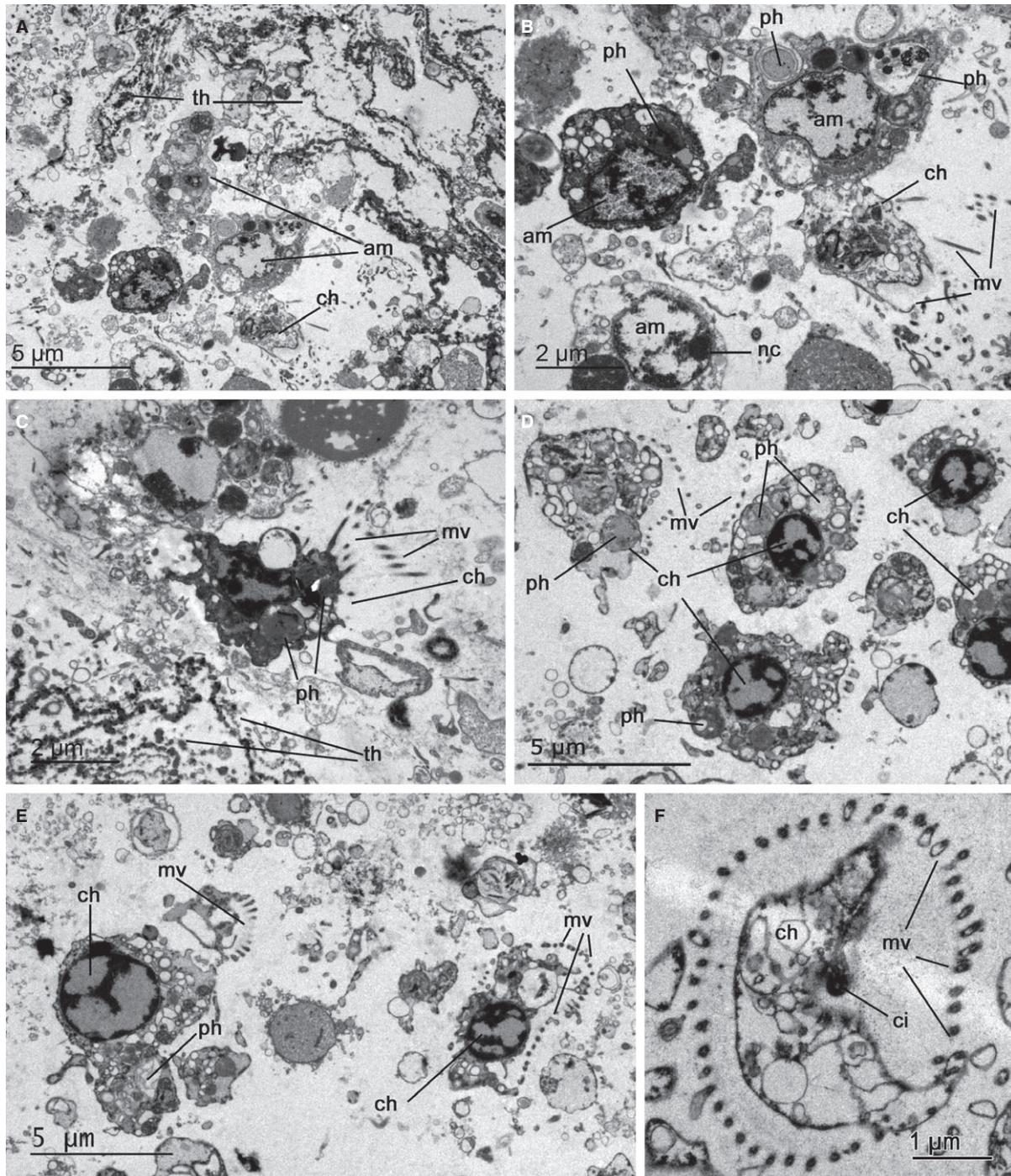


Fig. 3. Views of apoptotic choanocytes and archaeocyte-like cells shed to the outgoing canals. (A): Abundance of thread-like or membrane-like (th) structures, along with remains of archaeocyte-like cells (am) and choanocytes (ch) in *Thymosia* sp. (B): Close-up of (A), showing in greater detail one choanocyte (ch) – easily identifiable by its sectioned collar of microvilli (mv) – along with three archaeocyte-like cells (am) that still contain phagosomes (ph) in the cytoplasm and traces of the nucleolus (nc) in the nucleus. (C): Another example of a discarded choanocyte (ch) in *Thymosia* sp., showing the microvilli collar (mv) and phagosomes (ph) in an electron-dense (i.e. blackened = apoptotic) cytoplasm. Note also the occurrence of thread-like material (th) in the particulate organic matter (POM). (D and E): Discarded choanocytes (ch) in *Protosuberites epiphytum* showing their collar of microvilli (mv) and abundant phagosomes (ph) in the cytoplasm. Note also the occurrence of other cellular remains contributing to the POM of the canal. (F): Detail of a choanocyte (ch) of *P. epiphytum* in transverse section at the level of the cilium (ci) and the collar, which consists of a high number (49) of microvilli (mv).

Table 1. Contribution (%) of the most representative cell types (C1 = choanocytes, C2 = spherulous and granular cells, C3 = archaeocyte-like and unidentifiable cells) to the particulate organic matter in the canals of each studied sponge species and to the total count, after pooling data for all four sponge species.

Cell type	<i>Protosuberites epiphytum</i>	<i>Thymosia</i> sp.	<i>Dictyonella marsilli</i>	<i>Aplysina cavernicola</i>	Total (%)
C1	52.0 ± 11.3	46.6 ± 11.7	00.0	00.0	24.7 ± 26.3
C2	27.3 ± 9.6	43.3 ± 13.4	89.2 ± 6.4	90.5 ± 8.0	62.6 ± 29.8
C3	20.7 ± 9.9	10.1 ± 07.4	10.8 ± 6.4	09.4 ± 8.0	12.7 ± 08.8

and provided with a Gatan module for acquisition of digital images.

Analysis of ultrastructure data

Although the POM in the outgoing canals consists to a large extent of remnants of degraded cells whose origin cannot be easily retraced to the original cell type, entire apoptotic cells also occur. The occurrence of recognizable cell categories may serve to approximately quantify the relative contribution to the outgoing POM of the most abundant cell types in different sponge species. This approach provides only a descriptive snapshot into the cellular process of POM production and is by no means intended to quantify POM production over time or the energetics associated to the POM outflow. Neither do these cell-count data necessarily reflect the average cellular composition of POM over a daily cycle. Rather, the main objective was to document the various cell types contributing to the outgoing POM and to investigate whether the cellular mechanisms of POM production may vary substantially across species.

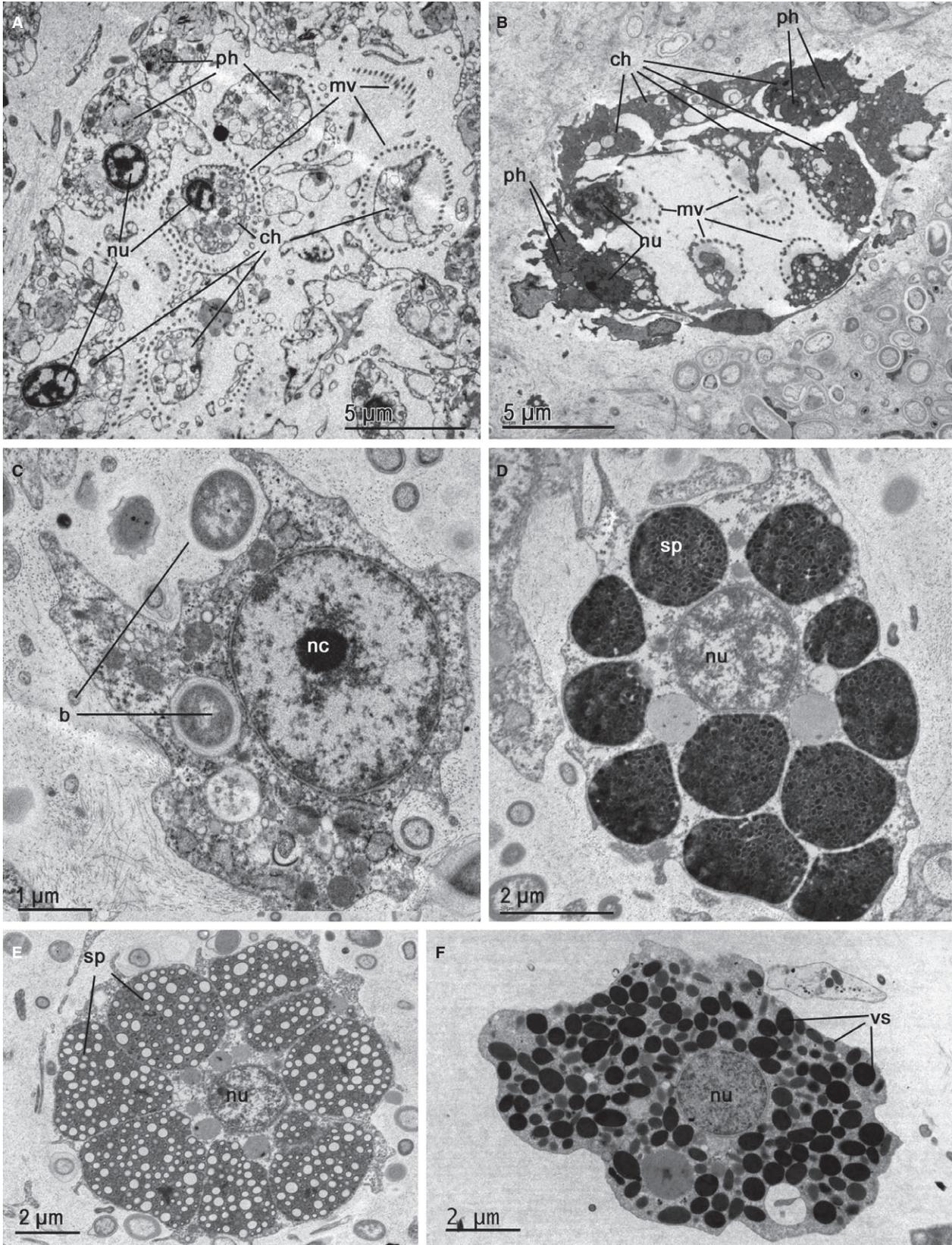
To this aim, sets ($n = 6$ per species) of 25 cells in the outgoing water canals were randomly chosen, counting the number of cells assignable to each of the three following categories: C1 = choanocytes; C2 = mesohyl cells charged with inclusions (*i.e.* spherulous and granular cells); and C3 = archaeocyte-like cells or unidentifiable cells that were either partially degraded or sectioned at a plane that did not allow reliable identification. Although the contribution (%) of the C1, C2 and C3 cell categories to the POM was intended to be calculated from counting six sets of 25 cells each (making a total of 150 counted cells per species), in two of the sponge species (*Dictyonella marsilli* and *Aplysina cavernicola*) the total number of

cells in the small aquiferous canal area seen in an ultra-thin section did not always reach the intended value, but ranged from 10 to more than 25. Therefore, the contribution percentages for *D. marsilli* and *A. cavernicola* were calculated from counting unequal sets ($n = 6$), ranging from 10 to 25 cells, and making a total of 94 counted cells in *D. marsilli* and 112 in *A. cavernicola*. These counts were used to estimate the total contribution of C1, C2 and C3 cellular categories to POM in each of the species and, by pooling data ($150 + 150 + 94 + 112 = 506$ cell counts) across sponge species, in the global set of sampled sponge individuals. Multi-factor analyses of these data were specifically avoided as 'cell type' values (C1, C2 and C3) within a given species may not be strictly independent from each other. Therefore, between-species differences in the contribution (%) of a given cellular category were examined using a one-way analysis of variance (ANOVA) on arcsine-transformed data followed by Student–Knewman–Keuls (SNK) *a posteriori* tests to identify the group(s) responsible for the differences.

Results

The microscopy study revealed that many of the outgoing aquiferous canals contained organic particles (POM) in all four sponge species (Fig. 1E and F). The POM contained an amorphous gel-like material (Fig. 1E and F) probably resulting from cytoplasm lysis, along with thread-like and membrane-like structures (Figs 1E, F, 2A and 3A) resembling the leftovers of digestion. There were choanocytes, but also diverse cells with inclusions (Fig. 2A–D) and phagosomes (Fig. 3B–E) like those that commonly occur in the internal mesenchyme-like tissue (mesohyl) of the sponge body. The prevailing cellular

Fig. 4. Features of cells living in the sponge mesohyl before becoming outgoing particulate organic matter. (A and B): Choanocyte chamber in *Protosuberites epiphytum* and *Thymosia* sp., respectively. Choanocytes (ch) show the nucleus (nu), the collar of microvilli (mv) and phagosomes (ph). (C): Archaeocyte of *Thymosia* sp., showing the nucleolus (nc) and engulfing mesohyl bacteria (b). (D and E): Late and early stages of a spherulose cell, respectively, of *Aplysina cavernicola*, showing the nucleus (nu) and their characteristic inclusions, known as spherules (sp). Note that during cell maturation, the membrane-bound subspherules contained in the spherules become more electron-dense. (F): Section through the nucleus (nu) plane of a granular cell of *Dictyonella marsilli*. The membrane-bound inclusions (vs) are always electron-dense and much smaller than those of spherulous cells.



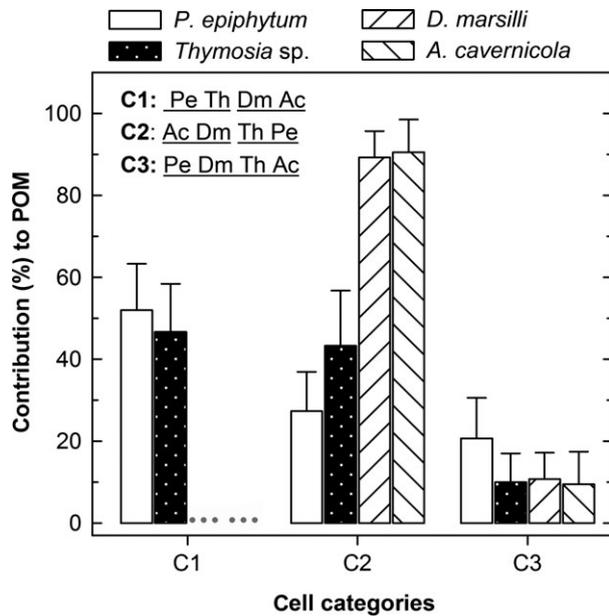


Fig. 5. Summary graph of cell contributions to the outgoing particulate organic matter (POM). Average (\pm SD) of contribution (%) by different cellular categories (C1 = choanocytes, C2 = spherulous and granular cells, C3 = archaeocyte-like and unidentifiable cells) to the POM in the canals of the different sponge species. In the upper left-hand corner, the results of the one-way ANOVAs and the *a posteriori* tests examining between-species differences in each cell category are given. Contributions of the cells in the different species (Pe, *Protosuberites epiphytum*; Th, *Thymosia sp.*; Dm, *Dictyonella marsilli*; Ac, *Aplysina cavernicola*) are arranged in decreasing order of magnitude; groups of underlined letters indicate non-significant differences between pairs of means according to the *a posteriori* tests.

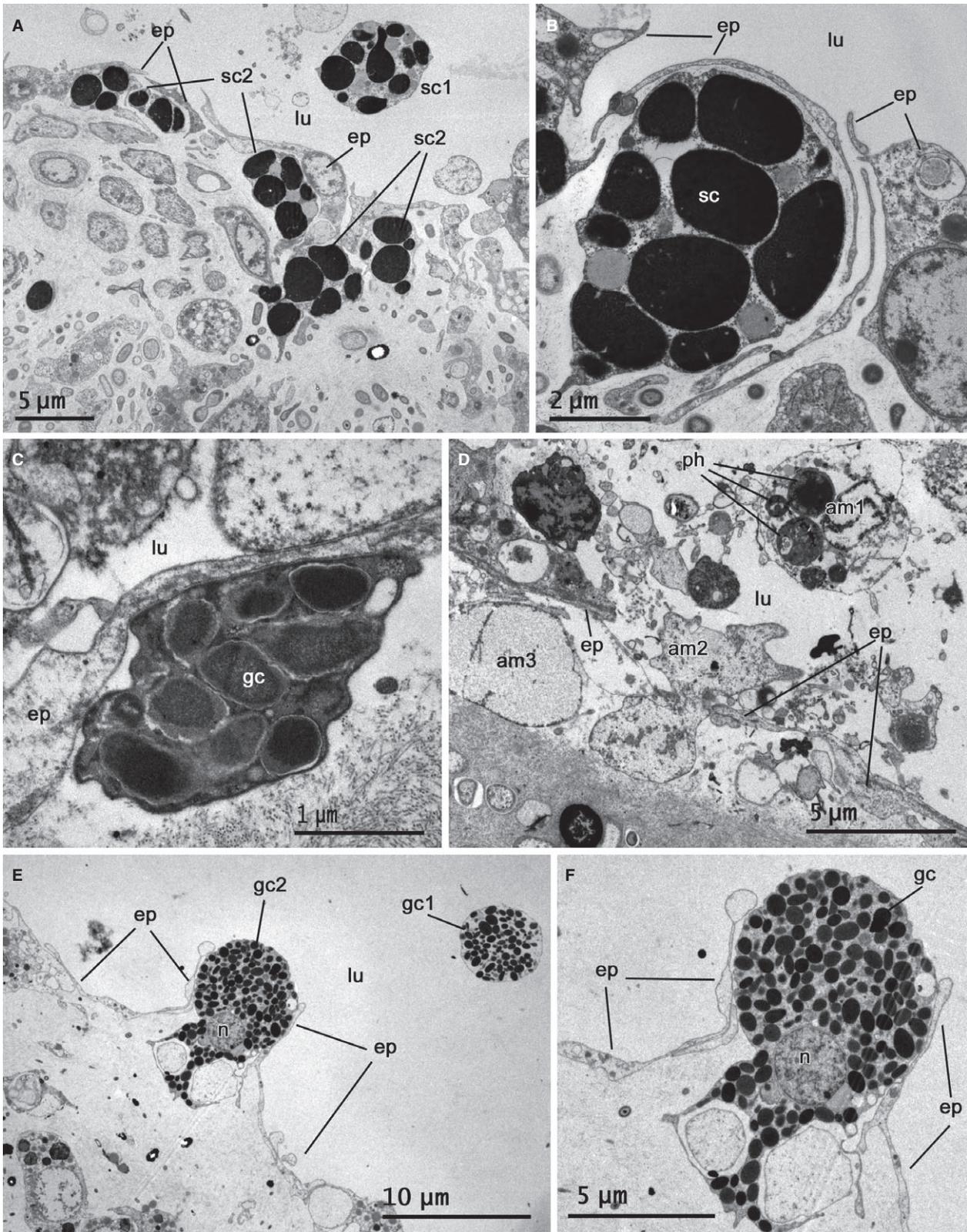
composition of the POM varied among species (Table 1), with the contribution from the choanocytes ranging from 0% to about 52%. In the snapshot provided by this TEM study, choanocytes were not present in the canals of *Dictyonella marsilli* and *Aplysina cavernicola*, but they did occur in those of *Protosuberites epiphytum* and *Thymosia sp.* (Fig. 3), contributing about 50% of the POM cells in these two sponge species (Table 1). Interestingly, despite the presence of discarded choanocytes in the outgoing canals of these two species, their choanosomes were

highly organized, showing abundant choanocyte chambers (Figs 1E, F and 4A, B). Discarded choanocytes, often containing vesicles of incompletely digested particulate food (phagosomes), were easily recognizable from their small size (about 2 μ m), a blackened apoptotic chromatin and a distinctive collar of microvilli (Fig. 3). The collar was characterized by a remarkably high number (>45) of microvilli in *P. epiphytum* (Fig. 3F).

Among the mesohyl cells of the outgoing POM, archaeocyte-like cells were rare (under 10% on average), although they were more common in *Thymosia sp.* than in the other species. These cells were easily identifiable because of their large size, a nucleolate nucleus and cytoplasmic inclusions (Fig. 3A and B) similar to those of their counterparts in the mesohyl (Fig. 4C). Spherulous cells (Figs 2C, D and 4D, E) and/or granular cells (Fig. 4F and G) were recognized in the POM of all species because of their characteristic inclusions. The vesicles of spherulous cells were larger and consisted of masses of membrane-bound subspherules, which are very obvious and electron-clear at early cell stages, becoming more electron-dense with cell age (Fig. 4D and E); the inclusions of the granular cells were smaller and somewhat similar to the subspherules of the late-stage spherulous cells (Fig. 4F). The cells with inclusions made a contribution to the POM from moderate (27%) to significant (90%), depending on the species (Table 1).

A tentative quantification derived from pooling cell counts across the individuals of all sponge species suggests that the cells with inclusions (*i.e.* C2 = spherulous and granular cells) accounted for about 62% of the cells in the POM at the moment of the tissue sampling, while choanocytes (C1) globally accounted for about 25% (Table 1). The low global contribution of choanocytes derives from the fact that they were abundant (about 50%) in the POM of two species, but absent in the POM of the two others. A one-way ANOVA (Fig. 5) of the cell counts in Table 1 corroborated marked between-species differences in choanocyte contribution ($df = 3$, $F = 57.8$, $P < 0.001$) and *a posteriori* SNK tests confirmed that the choanocyte contributions in *P. epiphytum* (46.6%) and *Thymosia sp.* (52%) were not significantly different. The ANOVA ($df = 3$, $F = 33.8$, $P < 0.001$) of the C2 category

Fig. 6. Migration and squeezing of a mesohyl cell through non-sealing epithelia. (A): Spherulous cell (sc1) released to the lumen (lu) of a canal of *Aplysina cavernicola* and accumulation of several other spherulous cells (sc2) underneath the canal epithelium (ep) immediately prior to release. (B): Detail of a spherulous cell (sc) of *A. cavernicola* pushing against the epithelial cells (ep) of the canal to find their way out to the lumen (lu). (C): Detail of a granular cell (gc) of *Thymosia sp.*, also pressing against the epithelial cells (ep) of the canal. (D): Archaeocyte-like cell (am1) containing several phagosomes (ph) that have been released to the lumen (lu) of a canal in *Thymosia sp.* Another archaeocyte-like cell (am2) is squeezing between the non-sealing epithelial cells (ep), while a third cell (am3) occurs right underneath the epithelium, ready for subsequent migration. (E): Granular cell (gc1) released into the lumen (lu) of a canal in *Dictyonella marsilli*. Note another granular cell (gc2) with a visible nucleus (n) squeezing between the epithelial cells (ep). (F): Detail of the squeezing granular cell (gc) in panel D, which is pushing through the non-sealing junction between epithelial cells (ep) to find their way out to the lumen of the canal.



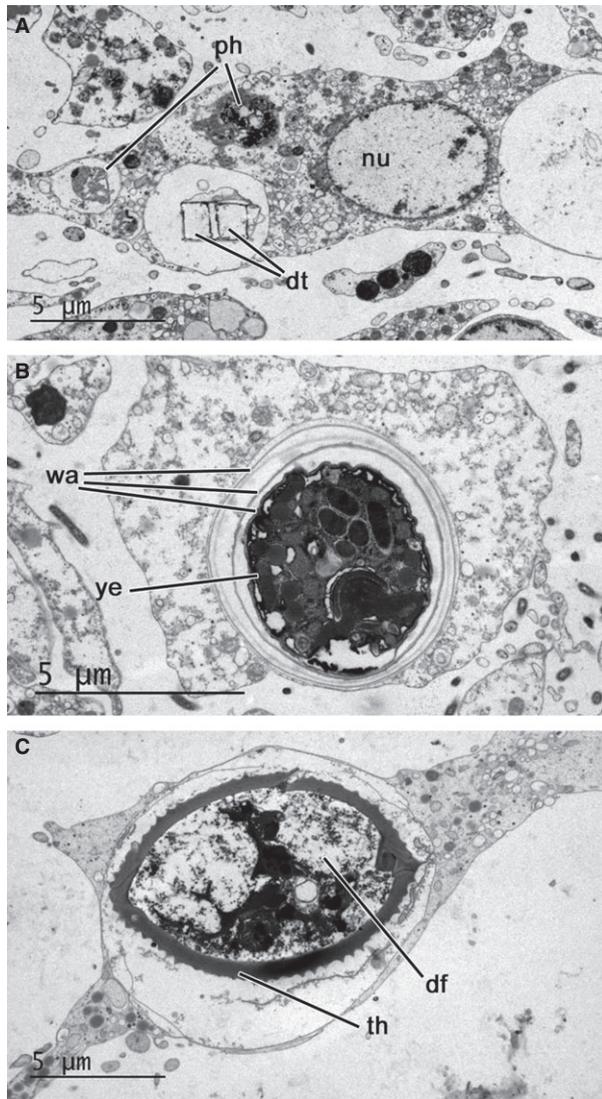


Fig. 7. Examples of refractory digestive leftovers in archaeocytes. (A): Archaeocyte sectioned at the nucleus (nu) level and showing diverse phagosomes (ph) in the cytoplasm. Note that one of the phagosomes contains remains of a two-cell diatom chain (dt), with the silica frustule persisting after digestion of the organic cell components. (B): Archaeocyte with a large phagosome containing a yeast (ye) that has a multilayered chitin wall (wa), which is known to be quite resistant to sponge digestion (Maldonado *et al.* 2010). (C): Archaeocyte with a large phagosome containing a dinoflagellate (df) with thick cellulose theca (th) that remains unaltered even when most of the inner organic cell content has already been digested.

and the *a posteriori* tests concluded that the contributions of the cells with inclusions in *A. cavernicola* (90.5%) and *D. marsilli* (89.2%) were not significantly different from each other, but significantly larger than those in *Thymosia* sp. (43.3%) and *P. epiphytum* (27.3%), which in turn were not statistically different from each other. A low contribution by archaeocyte-like and unidentified cells

(averaging collectively $12.7 \pm 8.8\%$) was noticed in all sponges (Fig. 5), with non-significant between-species differences ($df = 3$, $F = 2.6$, $P = 0.075$).

The pathway through which the mesohyl cells (*i.e.* spherulous cells, granular cells and archeocytes) ended in the lumen of the aquiferous canals to contribute to the POM was also documented. In all studied sponges, cells with inclusions (Figs 2C and 6A–C), along with archaeocyte-like cells and other unidentified cells (Fig. 6D), often with signs of cytoplasm decay, appeared to accumulate underneath the epithelium of the excurrent canals, where they squeezed through the non-sealing inter-cellular junctions to find their way into the lumen of the canals (Fig. 6D–F). Therefore, busy traffic of diverse mesohyl cells through the epithelium of the canals contributed to producing the outgoing POM. This process was evident in all four studied species, but it was more intense in *Thymosia* sp. and *A. cavernicola*. Archaeocyte-like cells appeared to be engaged in digestion and elimination of refractory leftovers (Fig. 7), while spherulous and granular cells appear to be involved in excretion of metabolic by-products (see Discussion).

In addition to the shedding of complete mesohyl cells into the outgoing canals, another process producing POM was discovered. It involved discharge of different cytoplasmic components not entire cells. In *P. epiphytum* and *Thymosia* sp., the excurrent canals contained, beside choanocytes and mesohyl cells, many anucleated cytoplasmic bodies and isolated groups of membrane-bound vesicles (Fig. 3A–D). Although some of these elements might have escaped from discarded mesohyl cells after plasmalemma breakage, active traffic of cytoplasmic inclusions through the mediation of the monolayered epithelium lining the excurrent canals was documented in these two sponges (Figs 8 and 9). Mesohyl cells charged with diverse inclusions accumulated at the mesohyl side of the epithelial cells (endopinacocytes) of the canals (Fig. 8A and B). The inclusions of the mesohyl cells were released into the narrow mesohyl space between them and the endopinacocytes (Fig. 8A–C) through two mechanisms: either passageways transiently opened in the plasmalemma of the mesohyl cells (Fig. 8C) or plasmalemma exocytosis (Fig. 8A). The inclusions released into the mesohyl adjacent to the epithelium by these mechanisms were readily incorporated by the endopinacocytes, the cytoplasm of which became heavily charged with inclusions (Fig. 8D). These acquired inclusions were finally discharged into the lumen of the canals as POM, by means of the endopinacocytes, which were able to extrude portions of their cytoplasm content (Fig. 8A, B and E), shedding cytoplasmic bodies charged with different amounts of vesicles and inclusions (Fig. 8A, E and F). Likewise, the endopinacocytes lining the canals of

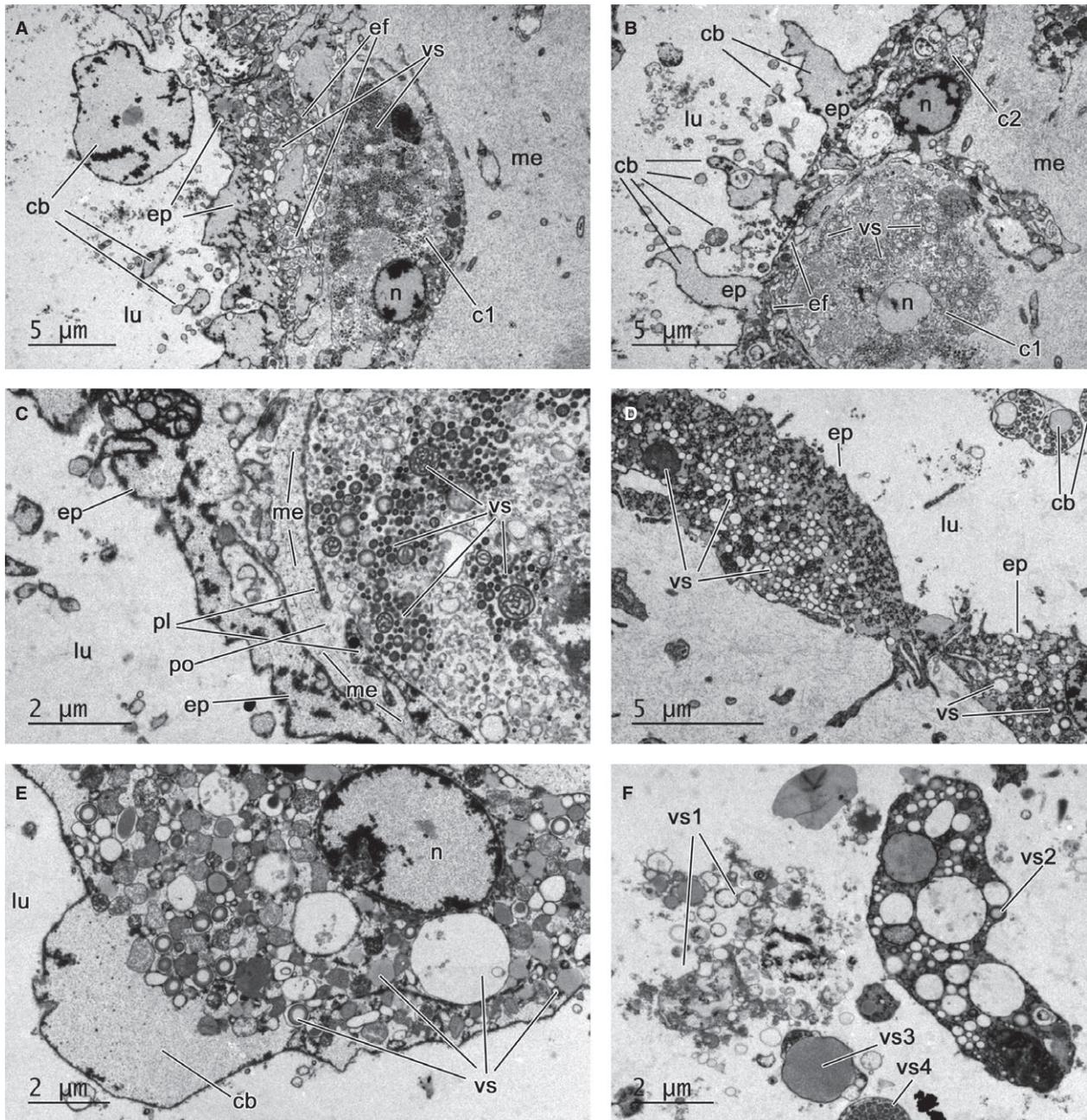


Fig. 8. Release of cytoplasmic bodies and vesicles from epithelial cells in *Protosuberites epiphytum*. (A and B): General view of cells (c1, c2) with inclusions that have migrated through the mesohyl (me) to contact the mesohyl side of the canal epithelium (ep). These cells with inclusions, having also a patent, large nucleus (n), are exocytosing their vesicles (vs) to the narrow mesohyl space (ef, exocytosis front) left between them and the epithelial cells (ep). Note also that the epithelial cells (ep) are incorporating the released vesicles from the exocytosis front (ef) in the mesohyl. On the lumen (lu) side of the canal, the epithelial cells are extruding part of their cytoplasm content in the form of cytoplasmic bodies (cb), contributing to the outgoing particulate organic matter (POM). (C): Detail of the passageway (po) in the plasmalemma (pl) with inclusions, through which the vesicle content (vs) is discharged to the narrow mesohyl band (me) left between the epithelial cell (ep) of the canal and the cell with inclusions. (D): Detail of epithelial cells (ep) charged with vesicles (vs) previously transferred from the cells with inclusions. Note the occurrence in the canal lumen (lu) of released cytoplasmic bodies (cb) charged with vesicles similar to those occurring in the epithelial cells. (E): Detail of the nucleus (n) and the vesicle (vs) content in the cytoplasm of an epithelial cell, which has also started extruding a cytoplasmic body (cb) to the canal lumen (lu). (F): Detail of different vesicle types (vs1, vs2, vs3, vs4) released to the lumen of a canal by the epithelial cells and becoming part of the outgoing POM.

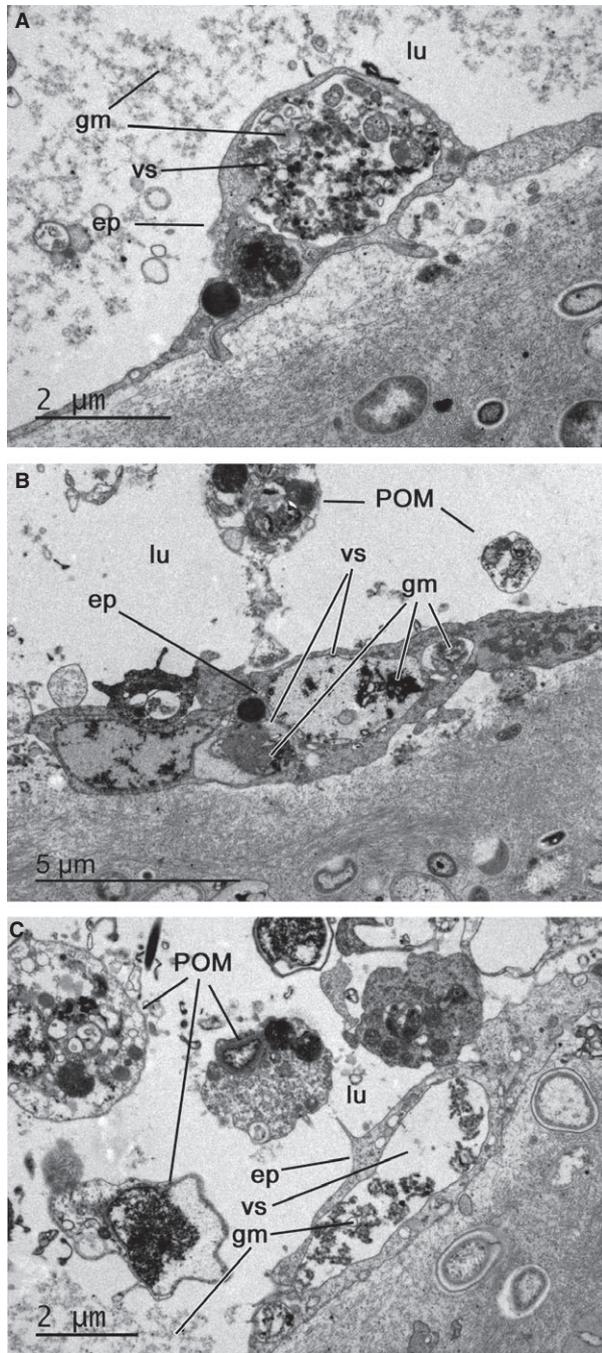


Fig. 9. Epithelial cells of the outgoing canals of *Thymosia* sp. (A–C): Epithelial cells (ep) showing large vesicles (vs) charged with a granular material (gm) similar to the one also found in the adjacent lumen (lu) making part of the outgoing particulate organic matter (POM).

Thymosia sp. contained large vesicles charged with amorphous material similar to the detrital POM occurring in the canal lumen (Fig. 9), suggesting the mediation of these epithelial cells in the production of POM. Nevertheless, unlike in *P. epiphytum*, images of the suggested

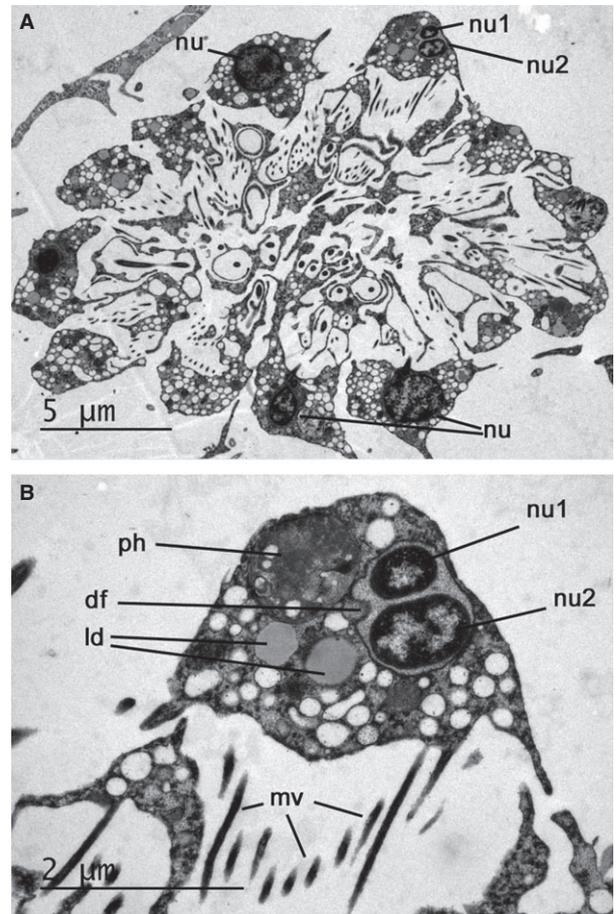


Fig. 10. Example of choanocyte mitosis in *Axinella polyoides*. (A): General view of a choanocyte chamber showing 13 sectioned cells, four of them at the nucleus level (nu). Note that only one of the cells is engaged in mitosis, having duplicated the genetic material of the nucleus (nu1, nu2). (B) Detail of the dividing choanocyte, which has duplicated its chromatin (nu1, nu2) and is starting the division furrow (df) of the nuclear membrane. A large phagosome (ph), lipid droplets (ld) and the microvilli (mv) of the collar are also clearly visible.

cytoplasm extrusion by the endopinacocytes were never captured.

Discussion

The TEM approach reveals that the outgoing POM through which sponges fuel oligotrophic food webs results from more complex cellular processes than mere choanocyte renewal. The squeezing of entire cells with inclusions (spherulous, granular and archaeocyte-like cells) into the excurrent canals and the extrusion of membrane-bound inclusions mediated by the endopinacocytes appears to contribute notably to the outgoing POM. In two of the studied species, discarded choanocytes were not seen in the outgoing canals. Nevertheless,

these observations and the quantification of the relative contribution to the POM from the different cell types must be taken as approximate and tentative, because if choanocyte renewal happens to be a very rapid synchronous process, the current approach would underestimate the global choanocyte contribution; it would be necessary to sample the sponge tissue at high frequency for several days to capture in full the putative pulses of cell renewal. Therefore, the current report that choanocytes do not contribute to the POM in *Aplysina cavernicola* and *Dictyonella marsilli* should be interpreted cautiously, because pulses of choanocyte contribution, if any, to the POM could have escaped this sampling, which described the cellular situation within a relatively narrow time window. Therefore, the results of this study should not be interpreted as evidence that choanocytes do not contribute to the POM of some species. Rather, the findings should be interpreted as solid evidence that cell categories other than choanocytes and that cellular processes other than epithelial renewal also contribute significantly to the production of sponge POM.

In the studied specimens of *Protosuberites epiphytum* and *Thymosia* sp., choanocyte chambers were always well formed and functional, with no sign of choanocyte division or replacement, despite the adjacent outgoing canals being filled with POM (Fig. 1E and F). These facts suggest that POM production may not necessarily be linked to dramatic, extensive pulses of choanocyte proliferation and shedding. Furthermore, during the last decade, I have investigated by TEM the cytology of not only the four species herein reported, but also that of 13 additional species with diverse phylogenetic affinities (i.e. *Petrosia ficiformis*, *Aplysina aerophoba*, *Ircinia fasciculata*, *Ircinia variabilis*, *Chondrosia reniformis*, *Chondrilla nucula*, *Hymeniacion perlevis*, *Crambe crambe*, *Cymbaxinella damicornis*, *Axinella polypoides*, *Diplastrella bistellata*, *Eurypon major* and *Corticium candelabrum*). Although that research has involved examination of hundreds of choanocyte chambers, surprisingly only one case of choanocyte division has been noticed, in *Axinella polypoides* (Fig. 10). More importantly, only one of the choanocytes in the chamber was engaged in mitosis, while the remaining choanocytes in that same chamber and in the adjacent chambers showed no sign of division. This indirect evidence suggests that choanocyte renewal, whenever it happens in this species, takes place gradually and asynchronously at the chamber level, rather than in dramatic, extensive pulses. These direct cytological observations strongly contrast with previous BrdU labeling studies concluding very frequent and extensive choanocyte renewal (de Goeij *et al.* 2009; Alexander *et al.* 2014). Nevertheless, it has to be considered that so far no control procedure has been performed to assess whether the

DNA chemical labeling by BrdU at a final concentration of 50 μM may be responsible for part of the cell shedding, for increasing the natural ratios of cell division or for other side effects. As we currently know little about the dynamics of sponge cell populations, choanocyte nuclear division (i.e. proliferation) cannot be reliably equated to choanocyte shedding.

The detected migration of mesohyl cells into the canals appears to be related to the elimination of digestive leftovers (egestion and defecation) and metabolic by-products (excretion), two basic physiological functions only rarely investigated in sponges (Vacelet 1967; Willenz 1983; Wolfrath & Barthel 1989; Martinand-Mari *et al.* 2012). As sponges lack organ systems to collect and evacuate products from intra-cellular digestion and metabolism in the deep mesohyl, these waste products are stored in cells that subsequently enter into the outgoing flow, contributing to the POM that exits the sponge. Archaeocyte-like cells, known to have intense phagocytic activity, appear to be engaged in digestion and elimination of refractory leftovers (Fig. 7), while spherulous and granular cells appear to be involved in excretion of metabolic by-products. Although many aspects of the physiology of sponges still remain poorly understood, it is clear that many physiological processes of the sponges are based on the ability of these organisms to maintain substantial cell and metabolite traffic through their simple epithelia. Extrusion of spherulous cells through the epithelia of the aquiferous canals of *A. cavernicola* has previously been documented by Vacelet (1967), who first suggested that it could be a way to eliminate excretory products. Likewise, spherulous cells heavily charged with inclusions have been reported to leave the body of the non-feeding larva of the sister sponge species *Aplysina aerophoba* (Maldonado 2009). The larva is a lecithotrophic life-cycle stage unable to incorporate particulate food but able to generate metabolic excreta (Jaekle 1995). Therefore, spherulous cells are concluded to be involved in elimination of metabolic by-products that are not related to the digestive process.

In addition to the shedding of complete cells into the outgoing canals, another process generated POM through the participation of the endopinacocytes. The fact that the epithelial cells of the outgoing canals participate actively in the production of POM is noteworthy, but it is not an entirely new finding. Kilian (1952) observed achaeocytes and endopinacocytes releasing blister-like exocytotic bodies into the excurrent canals. Willenz & Van de Vyver (1986) also documented the release of vesicles by pinacocytes. The extrusion of cytoplasm and vesicles through the formation of cytoplasmic bodies by the endopinacocytes closely resembles the process described for the spermatocytes during the initial stages of sponge

spermatogenesis (Riesgo *et al.* 2007; Riesgo & Maldonado 2009), through which the spermatocytes get rid of their cytoplasm content in order not to contaminate the egg with cytoplasmic determinants at the time of pronucleus transference.

The squeezing of entire cells with inclusions (spherulous, granular and archaeocyte-like cells) into the excurrent canals and the extrusion of membrane-bound inclusions mediated by the endopinacocytes appear to contribute notably to the outgoing POM. In the absence of detailed studies on vesicle content, it is assumed that the energetic content of these mesohyl cells – charged with excretion by-products and digestive leftovers – is lower than that of the choanocytes. It is worth noting that many of the discarded choanocytes and some archaeocyte-like cells were charged with phagosomes containing undigested food. Consequently, these cells are expected to contribute greatly to the POM transfer of energy to the following steps in the trophic chain. As water pumping and food ingestion are energetically costly processes, it is intriguing that choanocytes that are about to be discarded keep engulfing and start digesting pieces of particulate food that will never contribute to the sponge energy balance because these cells will readily be discarded as POM.

Conclusions

This study provides novel information to understand how the ecologically relevant sponge DOM-to-POM recycling demonstrated by de Goeij *et al.* (2013) is generated within sponges. The production of the outgoing POM fueling oligotrophic food webs has here been shown to result from cellular processes far more complex than initially anticipated. The composition of the outgoing POM varies notably among sponge species, consisting of several kinds of cells, with variable contributions from choanocytes. Digestive and excretory processes continuously produce POM and must be taken into account. As refractory leftovers of digestion and excretion contain little energy, the contribution of sponge POM to the energy budgets of the trophic chain in a given habitat will depend upon the ratios of dissolved *versus* particulate carbon in the diets of the dominant sponges, and also upon the ratios of mesohyl cells with refractory contents *versus* choanocytes with phagosomes containing food to be digested or undergoing digestion. Therefore, the results of this study allow several predictions relevant to the energetics of reefs and other oligotrophic environments to be made: (i) POM is generated by sponges irrespective of whether the carbon source is dissolved or particulate; (ii) where the contribution of refractory leftovers of digestion and metabolic by-products to the POM outflow is high, there should be a weak connection between DOM input and

POM output; and (iii) where the contribution of choanocytes is high, the POM produced should have a comparatively high energetic value. Consequently, the importance of sponge POM production in the energy budget of a given oligotrophic food web will depend upon both the specific composition of the sponge community and the average ratio of dissolved to particulate carbon in the diet of the dominant sponges, both factors that vary from habitat to habitat.

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