

# Trophic strategies in dinoflagellates: how nutrients pass through the amphiesma

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## Summary

Dinoflagellates, the protists of high ecological relevance, possess a very complex cell covering, the amphiesma. In this article, we review the available information about the structure and role of the amphiesma and discuss how nutrients overpass this barrier, focusing on membrane transport, micropinocytosis and receptor-mediated endocytosis. The hypothesized role of the pusule, a unique membrane organelle with unknown functions, in dinoflagellate nutrition is discussed.

**Key words:** amphiesma, dinoflagellates, endocytosis, membrane transporters, nutrient uptake, pusule

## Introduction

Dinoflagellates are unicellular eukaryotic organisms playing a crucial role in marine ecosystems as one of the main groups of primary producers. They are represented mainly by free-living planktonic forms, but some of them belong to benthic, parasitic or symbiotic species (Gómez, 2012). For example, members of the genus *Symbiodinium* are widely known for their endosymbiotic relationships with various invertebrates, including reef-building corals (Baker, 2003). Dinoflagellates are often the causative organisms of harmful algae blooms, or red tides (Richlen et al., 2010; Glibert et al., 2012; Telesh et al., 2016; Skarlato and Telesh, 2017). Some of them produce potent toxins which can be a reason of human poisoning if accumulated in fish and shellfish (Wang, 2008). Despite the ecological significance of dinoflagellates as primary producers, they are also important as a heterotrophic component of

food webs. Nearly half of the dinoflagellate species are obligate heterotrophs lacking chloroplasts, and many phototrophic chloroplast-containing species are in fact mixotrophs which can use dissolved organic compounds (osmotrophy) and/or prey cells (phagotrophy) as additional nutrient sources (Stoecker, 1999; Jeong et al., 2005; Matantseva and Skarlato, 2013; Pechkovskaya et al., 2017). Diverse trophic strategies of dinoflagellates are often considered as a key factor defining their success in marine habitats. At the same time, nutrition of these protists is not sufficiently studied from the standpoint of cell biology. Little information has been acquired since the time the explicit review by Schnepf and Elbrächter (1992) was published. Here we provide a review of the available information about cellular and molecular aspects of dinoflagellate nutrition, i.e. direct nutrient transport into a cell mediated by membrane proteins, evidences for receptor-mediated endocytosis and micropinocytosis, and

consider how these processes can be reconciled with the complex cell covering of these organisms.

#### AMPHIESMA AND THE ROLE OF AMPHIESMAL VESICLES

Dinoflagellates along with ciliates and apicomplexans belong to the clade Alveolata and are characterized by a similar structure of the cell covering. In general, the cell covering of these protists consists of a continuous plasma membrane and flattened single-membrane vesicles (alveoli) localized underneath. These vesicles are called an inner membrane complex (IMC) in apicomplexans or alveolar sacs in ciliates. In dinoflagellates, the entire cell covering, including a plasma membrane and alveoli, is termed amphiesma, and alveoli – amphiesmal vesicles, or sacs. Based on morphology of the cell covering, dinoflagellates are separated into two groups: armored (thecate) and naked (athecate). Amphiesmal vesicles of armored species contain rigid thecal plates built of cellulosic material, while amphiesmal vesicles of naked species lack them. Recently, it was shown that armored dinoflagellates have a monophyletic origin and had developed from an athecate ancestor (Orr et al., 2012). In some taxa, amphiesmal vesicles contain fibrous layer (pellicle) involved in the rearrangement of the cell covering and/or can be underlined by microtubules (Pozdnyakov and Skarlato, 2012).

The role of amphiesmal vesicles as a structure providing rigidity to the cell covering is obvious. In addition, alveoli may be involved in cell signaling, membrane trafficking and storage of various ions and molecules, but these functions have not been confirmed experimentally in the case of dinoflagellates. However, there is some information concerning functioning of homologous membrane compartments in ciliates.

The cortical sacs of ciliates function as a large  $\text{Ca}^{2+}$  store resembling terminal parts of sarcoplasmic reticulum in myocytes (Stelly et al., 1991). Calcium mobilization from the cortical sacs triggers the store-operated  $\text{Ca}^{2+}$  entry and subsequent cellular responses, e.g. trichocyst exocytosis. Indeed, a composition of proteins of the sarcoplasmic reticulum involved in  $\text{Ca}^{2+}$  regulation is similar to that in alveolar sacs of ciliates. For instance, alveolar sacs contain the sarcoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase (SERCA) and  $\text{Ins}(1,4,5)\text{P}_3$  receptor (Plattner, 2014).

Similar to alveolar sacs of ciliates, amphiesmal vesicles of dinoflagellates may function as  $\text{Ca}^{2+}$  stores.

This hypothesis is supported by the fact that ecdysis, an initial process of amphiesmal rearrangement, is  $\text{Ca}^{2+}$ -dependent (Tsim et al., 1997; Berdieva et al., 2018). In the process of ecdysis, amphiesmal vesicles fuse with each other and a cell loses motility. Then a cell sheds its plasma membrane, thecal plates and the outer amphiesmal vesicle membrane, while the inner amphiesmal vesicle membrane becomes a new plasma membrane (Pozdnyakov and Skarlato, 2012). Ecdysis occurs during the life cycle of many dinoflagellates and can be induced by stress, e.g. mechanical perturbation (centrifugation) or application of chemical agents, such as a cellulose synthesis inhibitor, 2,6-dichlorobenzonitrile (Morrill, 1984; Morrill and Loeblich, 1984; Sekida et al., 2001; Pozdnyakov et al., 2014). The new cell covering emerges in a relatively short time. The appearance of juvenile amphiesmal vesicles is observed 15 min after ecdysis in *Scrippsiella hexapraecingula* (Sekida et al., 2001) and 75 min – in *Heterocapsa niei* (Morrill, 1984). In 2 hours, a cell usually restores motility.

Robust experimental evidences for amphiesmal vesicles operating as intracellular stores of certain chemical compounds are absent; nevertheless, there are some indirect observations corroborating this assumption. In the study of the ultrastructure of the naked dinoflagellate *Prosoaulax lacustris*, Calado and colleagues (1998) observed cytoplasmic vesicles fusing with (and probably releasing their filling into) the amphiesmal vesicles. However, the nature of this filling was not determined.

The presence of amphiesma must complicate nutrient transport into a cell, because cytoplasm is separated from the plasma membrane by two additional membranes in the major part of the cell surface. Thus, to reach cytoplasm, nutrients have to cross three membranes, as well as thecal plates in the case of armored species.

#### MEMBRANE TRANSPORT OF DISSOLVED NUTRIENTS

Nutrient transport across the plasma membrane can be active or passive. Active transport is carried out in the direction against the gradient of chemical or electrochemical potential and requires the energy input, while passive transport involves movement of substances along these gradients. There is a great diversity of specific transporters, ion channels and porins participating in the nutrient transport in living organisms (Chrispeels et al., 1999; Saier, 2000; Schubert et al., 2017). For instance, in plants, nitrate transporters NRT2 and NPF, ammonium

transporter AMT and urea transporter DUR3 play a crucial role in the nitrogen assimilation. In addition, aquaporins of the NIP (nodulin 26-like intrinsic protein) and PIP (plasma membrane intrinsic proteins) subfamilies can facilitate uptake of some nutrients, such as urea (Gaspar et al., 2003; Wallace and Roberts, 2005).

Little is known about the composition of plasma membrane proteins responsible for the nutrient transport in dinoflagellates. Overall, these protists are likely to express an extremely wide range of proteins involved in the transport of micro- and macronutrients into a cell, especially considering their mixotrophic lifestyle and remarkable ability to use different inorganic and organic nutrient sources (Lee, 2008; Zhao et al., 2017). Nevertheless, specific data on the spectrum of such proteins are still scarce. Most of the information on this account was obtained by the analysis of genomic and transcriptomic data by means of bioinformatics. Several transporters involved in the uptake of nitrogen, such as nitrate transporters of the families NRT2 and NPF, have been found in the transcriptomes of *Prorocentrum minimum*, *Karenia brevis*, *Lingulodinium polyedrum*, *Alexandrium tamarense*, *Symbiodinium* sp.; moreover, ammonium transporters AMT have been identified in *K. brevis* and *Amphidinium carterae*, and urea transporters DUR3, as well as aquaporins MIP – in *P. minimum* (Morey et al., 2011; Dagenais Bellefeuille and Morse, 2016; Matantseva et al., 2016; Lauritano et al., 2017; Pechkovskaya et al., 2017). Experimental data are even more limited. It has been shown that the expression of NRT2.1 protein in *L. polyedrum* does not depend on the nitrogen source and time of the light/dark cycle (Dagenais Bellefeuille and Morse, 2016).

However, the question of cellular localization of the nutrient transport systems remains open. If they are localized only on the plasma membrane, nutrients still have to overcome the barrier of the amphiesmal vesicles to reach cytoplasm. This can be achieved in several potential ways, some of which were discussed by Schnepf and Elbrächter (1992) and shown in figure 1. First, nutrients can pass through the sutures between the amphiesmal vesicles. However, according to transmission electron microscopy (TEM) observations, in armored dinoflagellates these sutures appear too tight leaving no space even for small molecules to pass (Dodge and Crawford, 1970; Hoppenrath and Leander, 2008). In naked species, the amphiesmal

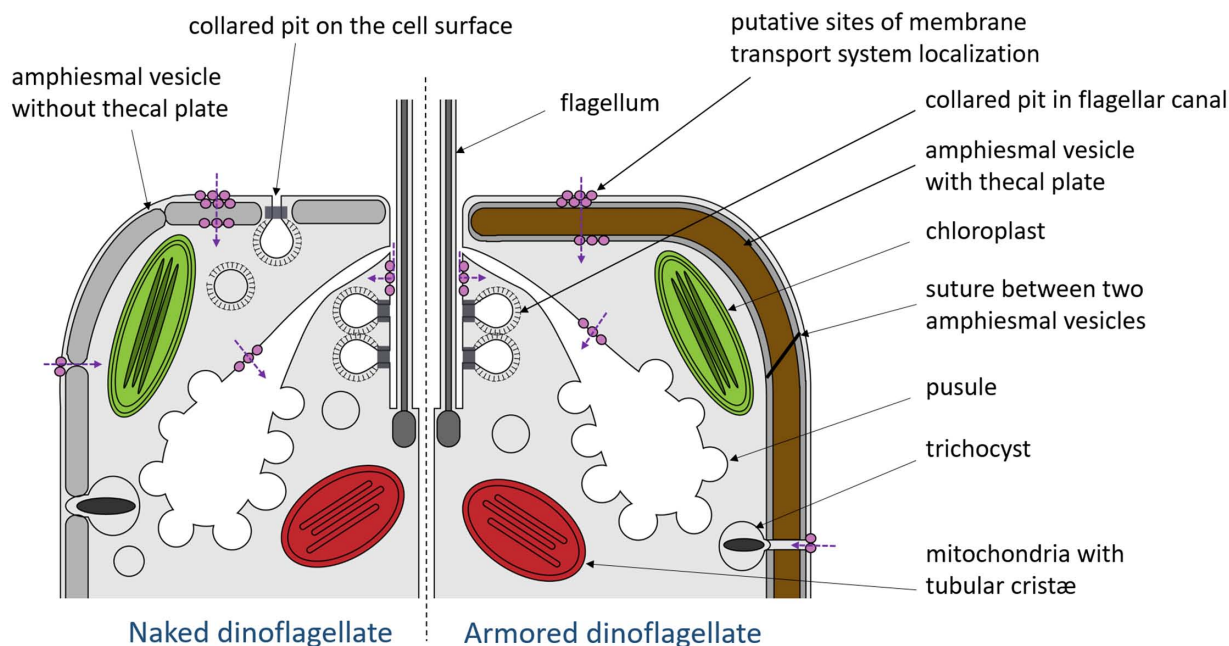
vesicles also lie very close to each other (Dodge and Crawford, 1970). Alternatively, the same sets of transporters could be present not only on the plasma membrane, but also on the outer and inner membranes of the amphiesmal vesicles. It is assumed that thecal plates do not represent a serious obstacle for small molecules and ions, because they bear thecal pores (Klut et al., 1989; Hoppenrath and Leander, 2008). According to TEM images, there is a possibility, that the outer and inner amphiesmal membranes fuse in the thecal pores forming membrane pipes or veins (Morrill and Loeblich, 1983). Thus, a thin cytoplasmic layer between the plasma membrane and outer amphiesmal vesicle membrane is linked with central cytoplasm, and nutrients can easily pass through (Spector, 1984). However, it is commonly observed that the pores are often locked by tricho- and mucocysts (Hoppenrath et al., 2013). In addition, it is reasonable to suggest that membrane transporters are localized mainly in the regions that lack amphiesmal vesicles: flagellar canal and the pusule (Fig. 1).

As mentioned above, most dinoflagellates are heterotrophs or mixotrophs and can utilize organic substances as a nutrient source. Small molecules, such as urea and free dissolved amino acids, are transported by the membrane proteins. But many molecules are too big for that and thus can be captured only by endocytosis, in particular, by receptor-mediated endocytosis or micropinocytosis.

#### WHERE ENDOCYTOSIS IS POSSIBLE?

Cell covering of Alveolata is complex and, therefore, all three alveolate groups (Apicomplexa, Ciliata and Dinoflagellata) have evolved permanent cytostomes and/or fixed endocytic sites. In sporozoa, a big group of apicomplexan parasites, these sites are called micropores and represent tiny plasma membrane invaginations in the IMC openings. The number of micropores can vary depending on the life cycle stage. In some species there is only one endocytic site, while there are two or more micropores scattered sparsely on the cell surface in the others (Scholtyssek and Mehlhorn, 1970). Interestingly, a micropore-like structure was observed in the parasitic dinoflagellate *Hematodinium* sp. (Appleton and Vickerman, 1996).

Most ciliates, for example *Paramecium* and *Tetrahymena*, have a permanent cytostome by which the food particles are engulfed. In addition, these organisms are capable of clathrin- and dynamin-



**Fig. 1.** Schematic representation of the possible endocytosis sites and hypothetical localization of membrane transporters in naked and armored dinoflagellates.

mediated (receptor-mediated) endocytosis (Wiejak et al., 2004; Elde et al., 2005). The process is characterized by the appearance of tiny pits coated with clathrin on the cytoplasmic surface. These structures are often termed collared pits due to the presence of the electron dense protein complex around the pit neck. In ciliates, numerous clathrin-coated collared pits are observed on the bottom of the plasma membrane sockets, termed parasomal sacs. Apparently, the parasomal sacs also play a role of the docking sites for recycling endosomes (Allen and Fok, 2000).

According to the transcriptomic data, the clathrin-dependent endocytic pathway may be also relevant for the dinoflagellate nutrition. In particular, this assumption was made in the study of *Alexandrium catenella* (Zhang et al., 2014). However, there is only one site of active endocytosis, which is the flagellar canal, where collared pits are commonly observed in dinoflagellates. Collared pits in the region of flagellar canal have been described in both naked and armored species, for example, in *Amphidinium rhynchocephalum* (Farmer and Roberts, 1989), *Gymnodinium nolleri* (Ellegaard and Moestrup, 1999), *Woloszynskia limnetica* (Roberts et al., 1995) and *Ceratium furcoides* (Roberts, 1989).

In naked dinoflagellates, collared pits can also be found on the cell surface. A number of pores between

amphiesmal vesicles were detected in *Amphidinium carterae* and *Prosoaulax lacustris* (Klut et al., 1989; Calado et al., 1998). Some of these openings served as the sites of trichocyst extrusion and the other represented plasma membrane invaginations ending with collared pits, which means that in these species endocytosis may potentially occur not only in the region of flagellar canal. In addition, it was shown that some cytoplasmic vesicles fused with the plasma membrane and presumably released their content via the pores between amphiesmal vesicles. However, it was noted that collared pits and fusing vesicles were rather rare than usual on the cell surface (Calado et al., 1998).

#### THE PUSULE

The pusule is a cellular organelle which is only found in dinoflagellates. In general, it represents a system of membrane vesicles, tubules and sacs connected with the flagellar canal by a permanent opening. A similar structure was found in *Cystodinium bataviense*, the dinoflagellate lacking flagella, but no direct contact with external environment was observed (Timpano and Pfiester, 1985). The pusule is usually surrounded by the vacuolar system and mitochondria. Dodge (1972) developed a classification of pusules based on TEM



**Table 1.** Classification of pusules according to J.D. Dodge (1972).

Type of pusule	Subtype	Organisms
Pusule with vesicles	a) Simple type: vesicles opening directly into flagellar canal	Marine organisms: <i>Amphidinium carterae</i> , <i>A. rhynchocephalum</i> , the symbiotic stage of <i>A. klebsii</i> , <i>Akashiwo sanguinea</i>
	b) Pusule with collecting chamber which branches from the flagellar canal	Marine organisms: <i>Amphidinium herdmanii</i> , <i>Warnowia pulchra</i> , <i>Karlodinium veneficum</i>
	c) Pusule with internal collecting chamber	Freshwater organism: <i>Gymnodinium australe</i>
	d) Complex tubular pusule with vesicles	Freshwater organism: <i>Woloszynskia coronata</i>
Pusules constructed of tubules or sack only	a) Simple tubular pusule	Marine and freshwater organisms: <i>Lingulodinium polyedra</i> , <i>Triplos furca</i> , <i>Borghiella tenuissima</i> , <i>Ceratium furcoides</i> <i>Prosoaulax lacustris</i> (Calado et al., 1998).
	b) Tubular pusule with invaginations	Marine organisms: <i>Kryptoperidinium foliaceum</i> , <i>Scrippsiella acuminata</i> , <i>Peridinium depressum</i> , <i>Heterocapsa rotundata</i> , <i>Cachonina hallii</i> , <i>Heterocapsa triquetra</i>
	c) Sack pusule (Fig. 2)	Marine organisms: <i>Prorocentrum balticum</i> , <i>P. minimum</i> , <i>P. nanum</i> , <i>P. micans</i> , <i>P. dentatum</i> , <i>Peridinium cinctum</i> (Calado et al., 1999).
No pusule		<i>Aureodinium pigmentosum</i> , <i>Alexandrium tamarense</i> , <i>Goniodoma pseudogoniaulax</i> , <i>Oxyrrhis marina</i> , <i>Katodinium glandulum</i> , <i>Crypthecodinium cohnii</i> , <i>Symbiodinium microadriaticum</i> , <i>Amphidinium chattonii</i> .

microphotographs of 40 dinoflagellate species from both marine and freshwater environments. He specified two main groups: (1) pusules consisting of vesicles or a collecting chamber, surrounded by vesicles, and (2) tubular or sack pusules (Table 1). As an example, ultrastructure of the sack pusule of *P. minimum* is shown in Fig. 2.

The function of the pusule is not completely understood. Table 2 summarizes the knowledge available to date. A widespread hypothesis suggests that the pusule plays an important role in osmoregulation. This organelle can shrink and swell thus resembling a contractile vacuole (CV) of freshwater protists, such as ciliates and amoebas. However, there is no experimental proof of this suggestion so far. Dodge (1972) noticed that the most complex pusular system observed in dinoflagellates was found in the freshwater species *Woloszynskia coronata*, but this organelle is well developed in most of the marine species too. Interestingly, the increased salinity (28–34 ppt NaCl) leads to the enlargement of the pusule in *Prorocentrum micans* (Klut et al., 1987), suggesting that this organelle rather functions as a cellular “kidney” excreting the excess of salt and waste molecules, but not the excess of water as it happens in the case of CV. The size of the pusule also increases with increasing temperature, but it does not depend on pH in the range from 6.0 to 8.0 or presence/absence of light (Klut et al., 1987). Interestingly, disorganized bacteria, membranes, amorphous and fibrillar

material are often observed in the lumen of flagellar canal and the pusule, allowing to assume that big nondigested particles may also be excreted via this region (Klut et al., 1987; Calado et al., 1999). In addition, Loeblich and colleagues suggested that the pusule might be implicated in mucilage excretion in *Prorocentrum* sp. (Loeblich et al., 1979).

There are the alternative hypotheses of the pusule function. A curious idea was proposed by Morrill and Loeblich who inferred that the pusule might be a membrane source during the cell division (Morrill and Loeblich, 1984). Since rebuilding of the cell covering after ecdysis requires comparable amount of new membrane material, the pusule can support this process as well.

Furthermore, the pusule is likely to be involved in the dinoflagellate nutrition. First, the region where phagocytosis and uptake of big molecules takes place is restricted to the sulcus and flagellar canal. Some dinoflagellates have evolved special feeding organelles, such as peduncle, lobopodia, and pallium (Schnepf and Elbrächter, 1992). Second, it is probable that the pusule also plays a role in the nutrient uptake being an important site of the endocytosis and membrane transporter localization. Klut and colleagues (1987) treated *P. micans* and *A. carterae* with ferritin or horseradish peroxidase (HRP). They have shown that these molecules are accumulated in the pusule and flagellar canal of both species. Vacuolar canaliculi, as well as some vesicles surrounding the pusule, also contained HRP



Table 2. Possible functions of the pusule.

Function	Pros	Cons	Experimental evidence
Osmoregulation (contractile vacuole style)	1) Changes the volume (Cachon et al., 1983) 2) The most complex pusule system is in the freshwater dinoflagellate <i>Woloszynskia coronata</i> (Dodge, 1972)	1) Present in marine species (Dodge, 1972) 2) No regular contraction	no
Excretion/osmoregulation	1) Disorganized bacteria found near the sack pusules (Calado et al., 1999) 2) High salinity (28 -34 ppt NaCl) leads to the enlargement of pusule (Klut et al., 1987) 3) Mitochondria positioned close to a pusule are more affected by Cd <sup>2+</sup> treatment. Destroyed mitochondria might be transported to the pusule area for excretion (Soyer and Prevot, 1981).		yes
Mucilage excretion	Appearance of fibrillar material in the lumen of a pusule (Loeblich et al., 1979).		no
Ingestion of molecules and ions entry	1) Uptake of horseradish peroxidase, cationized ferritin, and lectins via the flagellar canal and the pusules was shown by TEM in <i>P. micans</i> and <i>A. carterae</i> (Klut et al., 1987). 2) Mitochondria positioned close to a pusule are more affected by Cd <sup>2+</sup> treatment (Soyer and Prevot, 1981)		yes
Flotation apparatus	Larger dinoflagellates usually have a more extensive vacuolar system than smaller ones (Dodge, 1972).	There is no correlation between the size of a cell and the size of a pusule (Dodge, 1972).	no
Membrane source for cell division	Hypothesized (Morrill and Loeblich, 1984)		no

essential for ecdysis in *Prorocentrum minimum* (Dinophyceae, Prorocentrales). *Phycol. Res.* doi: 10.1111/pre.12214.

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