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Observations on the histology and photosynthetic performance of "solar-powered" opisthobranchs (Mollusca, Gastropoda, Opisthobranchia) containing symbiotic chloroplasts or zooxanthellae

Heike Wägele[1*], Geir Johnsen[2]

[1] Lehrstuhl für Spezielle Zoologie, Ruhr-Universität Bochum
[2] Trondhjem Biological Station, Institute of Natural History, Norwegian University of Science and Technology

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Abstract

Literature data on diversity of photosynthetic activity in Opisthobranchia are reviewed and new histological data presented on the presence of zooxanthellae in members of the nudibranch clade Cladobranchia. Zooxanthellae are recorded here for the first time in members of the family Arminidae (*Dermatobranchus*) and in the aeolid *Piseinotecus gabinierei.* Although a broad histological survey on Nudibranchia has been performed, only species of the taxon Cladobranchia are reported to house zooxanthellae. A new method to measure photosynthesis is applied to opisthobranchs with chloroplasts and zooxanthellae. With a Pulse Amplitude Modulated Fluorometer (PAM), the chlorophyll *a* fluorescence and corresponding fluorescence yield (electron transfer) of photosynthetically active chloroplasts or zooxanthellae can be analyzed in vivo. This facilitates better understanding of the diversity of zooxanthella and chloroplast uptake (ranging from feeding up to highly evolved forms of symbiosis) in the different opisthobranch clades.

Key words: Opisthobranchia, photosynthesis, chloroplasts, zooxanthellae, symbiosis, Pulse Amplitude Modulated fluorescence

Introduction

Two different functional photosynthetic systems are known from opisthobranch slugs. One of the most striking features, only known from the Sacoglossa, is the incorporation and maintenance of functional chloroplasts in the digestive system (kleptoplasty) after uptake of macroalgal tissue. Kleptoplasty is also common in several mixotrophic species of phytoplankton and ciliates (Jones et al. 1994; Lindholm & Mörk 1989). Whereas members of the 'more primitive' sacoglossans are not able to foster chloroplasts and use them as photosynthetically active units, members of the Placobranchoidea (synonym of Elysioidea, see Jensen 1996) are known to rely on the metabolites of incorporated chloroplasts for many weeks (Hinde & Smith 1972; Clark & Busacca 1978; Clark et al. 1979; Jensen 1996; Williams & Walker 1999; Rumpho et al. 2000).

The second system is more common in opisthobranchs and is widely spread in the Metazoa. It involves the incorporation of single-celled members of the Dinoflagellata, called zooxanthellae, and the use of their metabolites by the hosts for their own needs. For example, tropical coral reef formation relies on such symbioses. Zooxanthellae in opisthobranchs are mainly known from members of the Nudibranchia. Up to now, little is known about the importance of the relationship between the slugs and the algal cells, as well as about the distribution of this phenomenon within the Opisthobranchia, and the uptake, turnover and specifity of the algal species involved. It has been suggested that camouflage offers a selective advantage which favored the retention of algal cells or chloroplasts in the digestive system of sea slugs (Rudman 1987). It also has been suggested that the symbionts enhance the ability of the animals to survive periods of food shortage and allow

***Corresponding author:** Heike Wägele, Ruhr-Universität Bochum, Spezielle Zoologie, Gebäude ND 05/785, D - 44780 Bochum, Germany. Fax: +49(0)234/32-14114; e-mail: Heike.Waegele@ruhr-uni-bochum.de

them to search for and test other food sources (Marín & Ros 1992; Jensen 1997). Finally, the incorporation of chloroplasts may enhance the reproductive output by transfer of photosynthetically fixed carbon into eggs (Crossland & Kempf 1985).

Several morphological and behavioural adaptations can be observed in these slugs (Monselise & Rahat 1980; Clark et al. 1981; Weaver & Clark 1981; Rudman 1981b, 1982a, 1991; Jensen 1997). The branching of the digestive gland provides large surface areas for storage of plastids, and forming dorsal processes (cerata) or wavy notal rims increases the areas for photosynthetic light absorption and utilization. Species containing photosynthetic symbionts often orientate towards the light, whereas aposymbiotic species may avoid light. Avoidance of too high irradiances ('light intensity') may be met by shading the parapodia or crawling away (Rahat & Monselise 1979; Monselise & Rahat 1980; Weaver & Clark 1981).

Whether a given sea-slug contains chloroplasts or zooxanthellae can be detected by measuring the fluorescence originating from photosystem (PS) II, the oxygen evolving site. About 1% of the light absorbed by a photosynthetic symbiont will appear as chlorophyll a (chl a) fluorescence, detected as emitted red light (= fluorescence) from PS II with maximum emission at 685 nm. This is the basis for Pulse Amplitude Modulated fluorescence (PAM) detection of in vivo chl a fluorescence. Chlorophyll a is the final light acceptor molecule during the light harvesting process (Govindjee 1995). About 95% of in vivo fluorescence arise from PS II and its corresponding light harvesting complexes (Butler 1978; Johnsen et al. 1997).

If used properly, chl a - fluorescence can provide information on the identity of the various pigment complexes, excitation energy transfer among them, and on the various electron transfer reactions, specifically of PS II (Govindjee 1995; Johnsen et al. 1997). The quantum yield of chl a fluorescence is related to the rate constants (k's) of various pathways of de-excitation for fluorescence, heat dissipation, energy transfer, quenchers (e.g. photoprotective carotenoids), and photochemistry (Govindjee 1995). This means that chl a that is detached from its respective bonding proteins (e.g. free chl a in a protease active digestive system of a sea slug) will have a fluorescence yield [(absorbed quanta received/quanta emitted (fluorescence)] of approximately 0.3 (30% in vitro) compared to 0.01 (1% in vivo), which means that only approximately 1% of the absorbed quanta ('light') will be emitted as fluorescence in an intact and photosynthetically active cell/chloroplast. In vitro, no losses of fluorescence due to photochemistry will appear and will rise the fluorescence yield significantly.

Using PAM, the kinetics of the different parts of the fluorescence induction curve can be measured (Govindjee 1995; Schreiber et al. 1995). The maximum quantum yield of PS II can be measured after dark acclimation [maximal number of open reaction (oxidized) centers in photosystem II available to process photons] by measuring the fluorescence yield before and after application of a saturation pulse of strong light which closes (reduces) all available reaction centers with photons. The operational quantum yield at a given irradiance (e.g. ambient light), is likewise measured before and after application of a saturation pulse (see "Material and methods").

The operational quantum yield of PS II, φ_{IIe} is defined to approach zero if the chloroplasts are not functionally active due to total or partial digestion by the opisthobranch slugs, or due to very high irradiances which induces closing of all reaction centers of PS II. Plotting photosynthesis versus irradiance (P vs. E curve) shows whether or not the photosynthetic system is functional.

In this paper we demonstrate a fast method to detect the presence of functional photosynthetic zooxanthellae or chloroplasts, and some measurements on photosynthetic activity of selected species are reported and discussed. We also present new histological results on zooxanthellae-bearing opisthobranchs, and a list of investigated species extending the known distribution of microalgae within the Opisthobranchia.

Material and methods

Material was collected for histological investigations (1980–1999) by the first author and colleagues in different areas throughout the world (Table 1). Animals were preserved in formaldehyde/seawater and later embedded in whole or in parts in hydroxyethylmethacrylate. 2,5 µm sections were cut and stained with toluidine blue. Ultrastructure (TEM) was investigated in a few samples. After fixation in glutaraldehyde buffered in cacodylate and further treatment with OsO_4, specimens were embedded in Agar Resin 100. Ultrathin cuts were stained with uranyl acetate and lead citrate, and were investigated in a ZEISS electron microscope.

A Pulse Amplitude Modulated Fluorometer (DIVING PAM, Walz, Germany) was used to measure possible photosynthetic activities in the species marked in Table 1 (column 5). All specimens (one to four individuals per species) were collected on the 11–13 July 1999 in the intertidal zone at Lizard Island, Great Barrier Reef, Australia, except for *Phyllodesmium briareum*, which was collected at 15 m. Animals were kept at 27 °C in dim light (irradiance of ~10 mmol quanta $m^{-2}s^{-1}$) prior to fluorescence and P vs. E measurements to avoid any major differences in light acclimation. Natural sunlight attenuated with different layers (different thickness to attenuate light) of spectrally neutral white polyethylene was used to estimate photosynthetic responses at different irradiances (P vs. E curves).

The fiber optics from the PAM was placed 0.5–1.0 cm from the part of the animal with the highest concentration of chloroplasts/zooxanthellae as indicated by the highest *in vivo* fluo-

rescence. This was done in order to obtain a stable and high signal to noise ratio. The fiber optics detect the fluorescence emitted from the sea slugs before and after the saturation flash (see Equations 1 and 2 below, cf. Govindjee 1995, Schreiber et al. 1995). F_0 is defined as fluorescence measured in dark acclimated tissues and F_0' is measured in actinic (= photosynthetic) light conditions. The signal is obtained by probing the fluorescence using a non-actinic (very low light) light-source obtained from a Light Emitting Diode (LED that is used as pulse modulated probe light) with emission peak at 650 nm, sending light pulses at a frequency of 0.6 kHz. This gives an irradiance of approximately 0.15 mmol quanta m^{-2} s^{-1}, which is too low to induce any photosynthetic activity. F_m (in dark acclimated tissues) and F_m' (light acclimated) is defined as the maximum fluorescence obtained during an 800 millisecond (ms) white light flash from a Halogen lamp (Osram Bellaphot - SL-8/20) with a peak irradiance of ~10,000 mmol quanta m^{-2} s^{-1} (400–700 nm) and with the probe light obtaining data at a frequency of 20 kHz. φ_{IIe} denotes stable charge separation (i.e. electrons generated from light) at PS II (mol charge separation · mol $quanta^{-1}$, cf. Kroon et al. 1993).

The maximum quantum yield of fluorescence for PS II ($\varphi_{IIe\text{-}max}$) is defined as:

$$\varphi_{IIe\text{-}max} = (F_m - F_0)/F_m \text{ (dark acclimated for 15 minutes)} \quad \text{Equation 1}$$

whereas in ambient light, i.e. during photosynthetic activity, the following relationship is used to calculate the operational quantum yield of PS II

$$\varphi_{IIe} = (F_m' - F_0')/F_m' \text{ (in ambient light)} \quad \text{Equation 2}$$

Photosynthesis versus irradiance curves (P vs. E, which indicates non-acclimated photosynthetic responses to different light intensities) were obtained using a gradient from low (4 mmol quanta $m^{-2}s^{-1}$) to high (1000–2000 mmol quanta $m^{-2}s^{-1}$) with 5 minutes incubation time for each irradiance (Equation 2). The specimens were dark acclimated again after the P vs. E experiment. This was done to check if the species regained the maximum fluorescence quantum yield, to ensure that the animals containing zooxanthellae or chloroplasts were not stressed during experiment ($\varphi_{IIe\text{-}max}$) and that zooxanthellae/chloroplasts were functionally active.

The relative electron transfer rate ($\varphi_{IIe} \cdot E$), which denotes photosynthetic rate, was plotted against irradiance (E) and fitted to Equation 3 (Webb et al. 1974) to obtain photosynthetic rate (P^B) and the maximum light utilization coefficient (α^B), and to calculate the light saturation parameter E_k.

$$P^B = P_{max}^B \cdot \left(1 - \exp(-\alpha^B \cdot E/P_{max}^B\right) \quad \text{Equation 3}$$

where P_{max}^B = maximum photosynthetic rate ($\varphi_{IIe} \cdot E$), P^B = photosynthetic rate at a given irradiance ($\varphi_{IIe} \cdot E$), α^B = maximum light utilization coefficient ($\varphi_{IIe} \cdot E \cdot (\mu mol\ quanta\ m^2 s^{-1})^{-1}$), and E_k = light saturation parameter (P_{max}^B/α^B, µmol quanta $m^{-2}s^{-1}$).

To check for surface contamination with other photosynthetic micro-algae, all specimens investigated by PAM were also investigated histologically.

Results

Table 1 lists all species of Opisthobranchia investigated with different techniques. The presence of zooxanthellae is indicated. Fluorescence emission from the slugs was investigated for only few species. Only few sacoglossans have been studied here, since retention and functional kleptoplasty in this group has been very well investigated by several authors (e.g. Marín & Ros 1988, for further references see Table 2).

The presence or absence of zooxanthellae can be seen quite easily by light microscopy, in contrast to chloroplasts, which are much smaller, making identification difficult by light microscopy. Here, the detection of fluorescence emitted from the chloroplasts-containing slug by means of PAM is fast and reliable. Zooxanthellae have only been found in members of the Cladobranchia (see Table 1). However, it has to be emphasized that the number of investigated non-nudibranchs is low. In all investigated species with zooxanthellae, the algae are usually located in the epithelium of the digestive tract.

Within the Dendronotoidea no species with symbiotic zooxanthellae have been found. In the former „Arminoidea“ (see Wägele 1997; Wägele & Willan 2000), zooxanthellae have been found in the digestive glandular epithelium and the lumen of *Dermatobranchus semistriatus* (Figs 1A, B) and in two different undescribed *Dermatobranchus* species (sp.1 and sp. 2) from Australia (Figs 1 C, D). In the latter two, the zooxanthellae are mainly located beneath the notal epidermis and in the dorsal part of the foot. The digestive gland ramifies very often and forms fine tubules in their distal branch ends, with 'carrier' cells (Kempf 1984) containing several zooxanthellae. In *D.* sp. 2 these tiny tubules even reach into the dorsal tubercles of the animal (Fig. 1D). The fine tubules contain a lumen, which is lined completely or partly by the carrier cells. But very often carrier cells are arranged in ribbons, with no evidence of tubules. *D. semistriatus* shows a lower density of zooxanthellae in the digestive glandular epithelium than the other two *Dermatobranchus* species, but the lumen of the digestive gland is filled with them. No fine tubules could be detected in the notal tissue. In the one specimen investigated many zooxanthellae do not look healthy, i.e. the organelles (mainly nucleus and pyrenoid) can not be distinguished very well, indicating disintegration prior to ingestion in the glandular cells (Fig. 1A). Therefore a digestion and not integration seems probable for this specimen.

Within the Aeolidoidea the presence of zooxanthellae in the digestive glandular epithelium is confirmed for the species *Phyllodesmium briareum* and *Pteraeolidia ianthina* from the Indopacific. *Phyllodesmium briareum* exhibits an extremely branched digestive gland. Fine branches with a distinct glandular epithelium containing

Table 1. List of species and families investigated by light microscopy for presence of zooxanthellae. Column 4 (Z) indicates the species, where zooxanthellae have been found. column 5 (P) indicates the species measured with the Pulse Amplitude Modulated fluorometer. *positive photosynthetic activity, o no activity.

Higher Category	Family	Species	Z	P
INCERTAE SEDIS	Acteonidae	*Acteon tornatilis* (Linné, 1758)		
	Hydatinidae	*Hydatina physis* (Linné, 1758)		
CEPHALASPIDEA	Aglajidae	*Chelidonura inornata* Baba, 1949		
	Haminoeidae	*Haminoea antillarum* d'Orbigny, 1841		
	Philinidae	*Philine alata* Thiele, 1912		
	Runcinidae	*Runcina adriatica* Thompson, 1980		
	Cylichnidae	*Scaphander nobilis* Verill, 1884		
SACOGLOSSA	Plakobranchidae	*Elysia expansa* Risso, 1818		*
		Plakobranchus ocellatus van Hasselt, 1824		*
		Thuridilla ratna Marcus, 1965		*
	Polybranchidae	*Cyerce nigricans* Pease, 1866		o
ANASPIDEA	Aplysiidae	*Bursatella leachii* Blainville, 1817		
		Aplysia punctata Linné, 1767		
		Petalifera petalifera (Rang, 1828)		
TYLODINOIDEA	Tylodinidae	*Tylodina perversa* Rafinesque, 1819		
PLEUROBRANCHOIDEA	Pleurobranchidae	*Bathyberthella antarctica* Willan, 1983		
		Berthella stellata (Risso, 1828)		
		Tomthompsonia antarctica (Thiele, 1912)		
NUDIBRANCHIA				
Bathydoridoidea	Bathydoridiidae	*Bathydoris clavigera* Bergh, 1884		
Doridoidea				
	Onchidoridiidae	*Acanthodoris pilosa* (Müller, 1789)		
		Adalaria proxima (Alder & Hancock, 1854)		
		Onchidoris bilamellata (Linné, 1767)		
	Goniodoridiidae	*Ancula gibbosa* (Risso, 1818)		
		Goniodoris castanea Alder & Hancock, 1845		
		Trapania maculata Haefelfinger, 1960		
	Gymnodoridiidae	*Gymnodoris striata* (Eliot, 1908)		
	Polyceridae	*Nembrotha kubayarana* Bergh, 1877		o
		Polycera quadrilineata (Müller, 1776)		
		Roboastra gracilis (Bergh, 1877)		o
		Thecacera pennigera (Montagu, 1815)		
	Trophidae	*Limacia clavigera* (Müller, 1776)		
	Aegiridae	*Aegires albus* Thiele, 1912		
		Notodoris citrina Bergh, 1875		
	Doridiidae	*Archidoris pseudoargus* (Rapp, 1827)		
		Austrodoris kerguelenensis (Bergh, 1884)		
		Discodoris atromaculata (Bergh, 1880)		
		Jorunna tomentosa (Cuvier, 1804)		
		Rostanga pulchra MacFarland, 1905		
	Chromodoridiidae	*Cadlina laevis* (Linné, 1767)		
		Chromodoris westraliensis (O'Donoghue, 1924)		
		Glossodoris atromarginata (Cuvier, 1804)		
		Hypselodoris tricolor Cantraine, 1835		
		Hypselodoris villafranca (Risso, 1818)		
	Dendrodoridiidae	*Dendrodoris nigra* Stimpson, 1855		
		Dendrodoris fumata Rüppell, 1830		
	Phyllidiidae	*Phyllidia flava* Aradas, 1847		
		Phyllidiella pustulosa (Cuvier, 1804)		

Table 1. (Continued).

Higher Category	Family	Species	Z	P
Dendronotoidea	Tritoniidae	*Marionia blainvillea* (Risso, 1818)		
		Tritonia antarctica Pfeffer in Martens & Pfeffer, 1886		
		Tritonia vorax (Odhner, 1926)		
		Tritonia plebeia Johnston, 1838		
		Tritoniella belli Eliot, 1907		
	Dendronotidae	*Dendronotus frondosus* (Ascanius, 1774)		
	Lomanotidae	*Lomanotus vermiformis* Eliot, 1908		
	Dotidae	*Doto coronata* (Gmelin, 1791)		
		Doto floridicola Simroth, 1888		
	Hancockiidae	*Hancockia uncinata* (Hesse, 1872)		
	Tethydidae	*Melibe leonina* Gould, 1852		
	Scyllaeidae	*Scyllaea pelagica* Linné, 1758		
Arminoidea	Arminidae	*Armina maculata* Rafinesque, 1814		
		Armina neapolitana (Delle Chiaje, 1841)		
		Armina tigrina Rafinesque, 1814		
		Dermatobranchus ornatus (Bergh, 1874)		
		Dermatobranchus semistriatus Baba, 1949	Z	
		Dermatobranchus sp. 1	Z	
		Dermatobranchus sp. 2	Z	
	Charcotiidae	*Charcotia granulosa* Vayssière, 1906		
		Pseudotritonia antarctica (Odhner, 1934)		
		Pseudotritonia gracilidens (Odhner, 1944)		
	Dironidae	*Dirona albolineata* MacFarland, 1912		
	Zephyrinidae	*Janolus capensis* Bergh, 1907		
		Janolus cristatus (Delle Chiaje, 1841)		
	Madrellidae	*Madrella ferruginosa* Alder & Hancock, 1864		
Aeolidoidea	Notaeolidiidae	*Notaeolidia depressa* Eliot, 1905		
		Notaeolidia gigas Eliot, 1905		
		Notaeolidia schmekelae Wägele, 1990		
		Notaeolidia subgigas Odhner, 1944		
	Flabellinidae	*Calmella cavolinii* Vérany, 1846		
		Flabellina affinis Voigt, 1834		
		Flabellina babai Schmekel, 1972		
		Flabellina exoptata Gosliner & Willan, 1991		o
		Flabellina falklandica (Eliot, 1907)		
		Flabellina gracilis (Alder & Hancock, 1844)		
		Flabellina pedata (Montagus, 1815)		
	Aeolidiidae	*Aeolidia papillosa* (Linné, 1761)		
		Cerberilla amboinensis Bergh, 1905		
		Protaeolidiella juliae Burn, 1966		
	Facelinidae	*Caloria elegans* (Alder & Hancock, 1845)		
		Cratena peregrina (Gmelin, 1789)		
		Phidiana lottini Lesson, 1831		
	Favorinidae	*Phyllodesmium briareum* Ehrenberg, 1831	Z	*
		Pteraeolidia ianthina (Angas, 1864)	Z	*
	Glaucidae	*Glaucus atlanticus* Forster, 1777		
	Piseinotecidae	*Piseinotecus gabinierei* (Vicente, 1975)		
	Eubranchidae	*Eubranchus exiguus* Alder & Hancock, 1849		
	Tergipedidae	*Cuthona caerulea* (Montagu, 1804)		
		Tergipes tergipes Forskål, 1775		

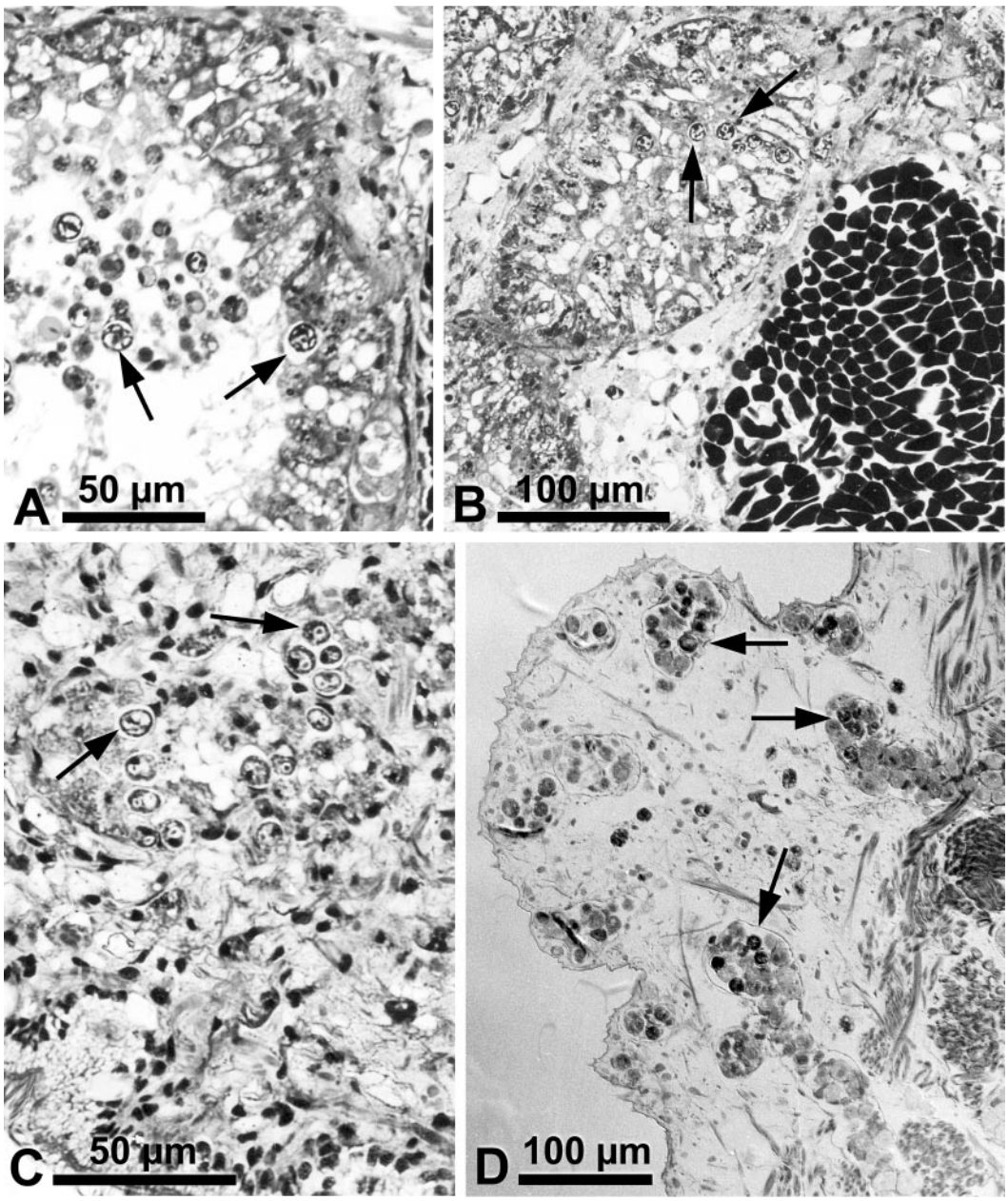
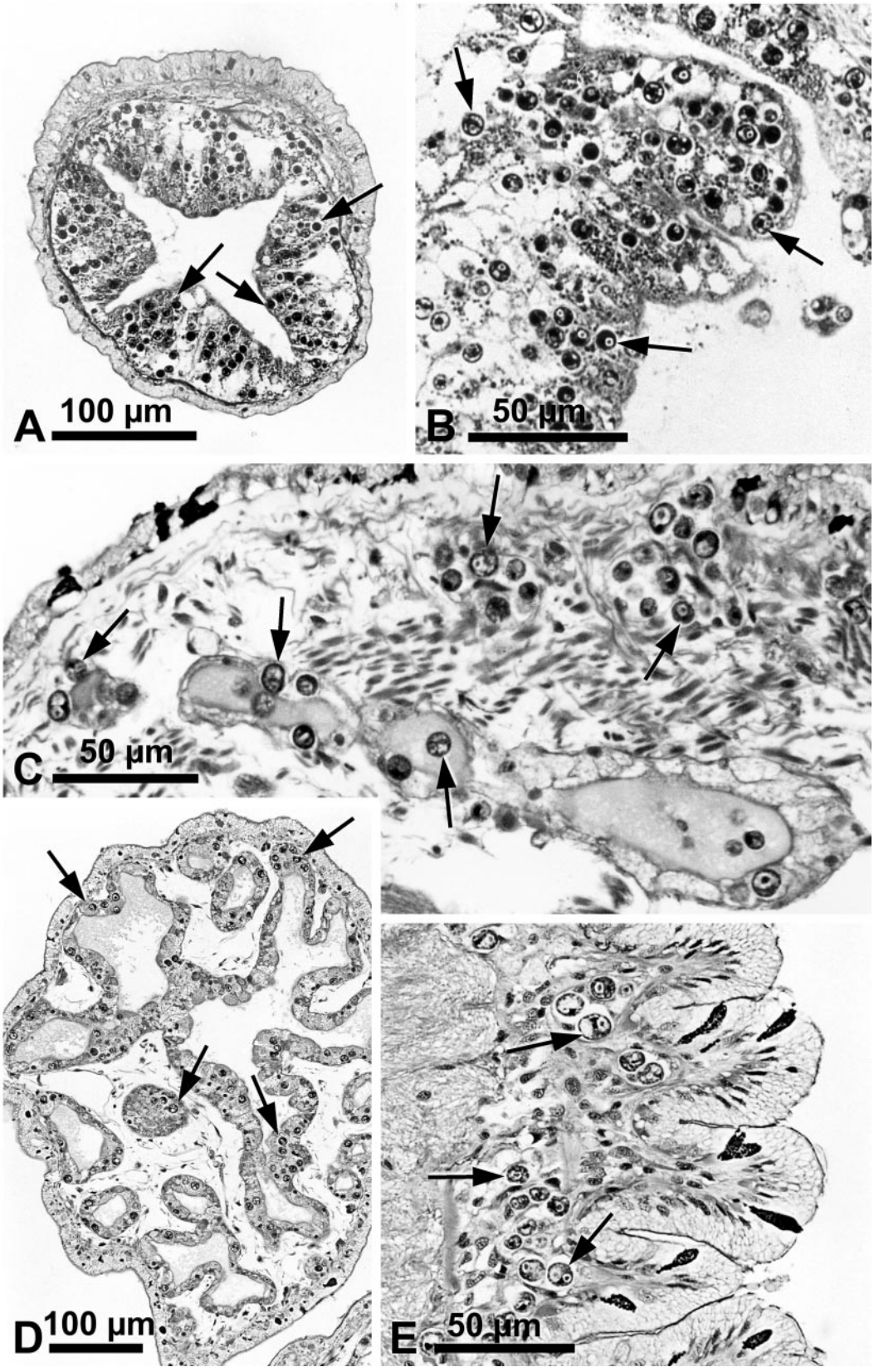


Fig. 1. **A**. *Dermatobranchus semistriatus*. Histological section of digestive gland. Here, only few zooxanthellae are located within the epithelium, but many are lying within the lumen. **B**. *D. semistriatus*. Section through digestive gland with several zooxanthellae located in the epithelium. Black patches at right represent part of a marginal sac. **C**. *Dermatobranchus* sp.1. Zooxanthellae located in fine tubules in the foot. **D.** *Dermatobranchus* sp. 2. Dorsal papillae (dorsal epithelium not preserved) with fine tubules containing zooxanthellae. Arrows: zooxanthellae.

Fig. 2. **A**. *Piseinotecus gabinierei*. Cross section of ceras with digestive glandular epithelium containing zooxanthellae. **B**. *Piseinotecus gabinierei*. Detail of digestive glandular epithelium within the body cavity. **C**. *Phyllodesmium briareum*. Detail of lateral body wall with branch of digestive gland containing several zooxanthellae in the epithelium as well as in the lumen. Note the branch becoming thinner to the left with much smaller epithelial cells. Fine tubules with zooxanthellae are located in the upper part of the figure. **D**. *Phyllodesmium briareum*. Cross section of ceras with highly branched digestive gland. Note the zooxanthellae distributed rather irregularly in the epithelial walls. **E**. *Phyllodesmium briareum*. Longitudinal section through rhinophore with zooxanthellae in fine tubules. Rhinophoral nerve on the left side of picture. Arrows: zooxanthellae. ►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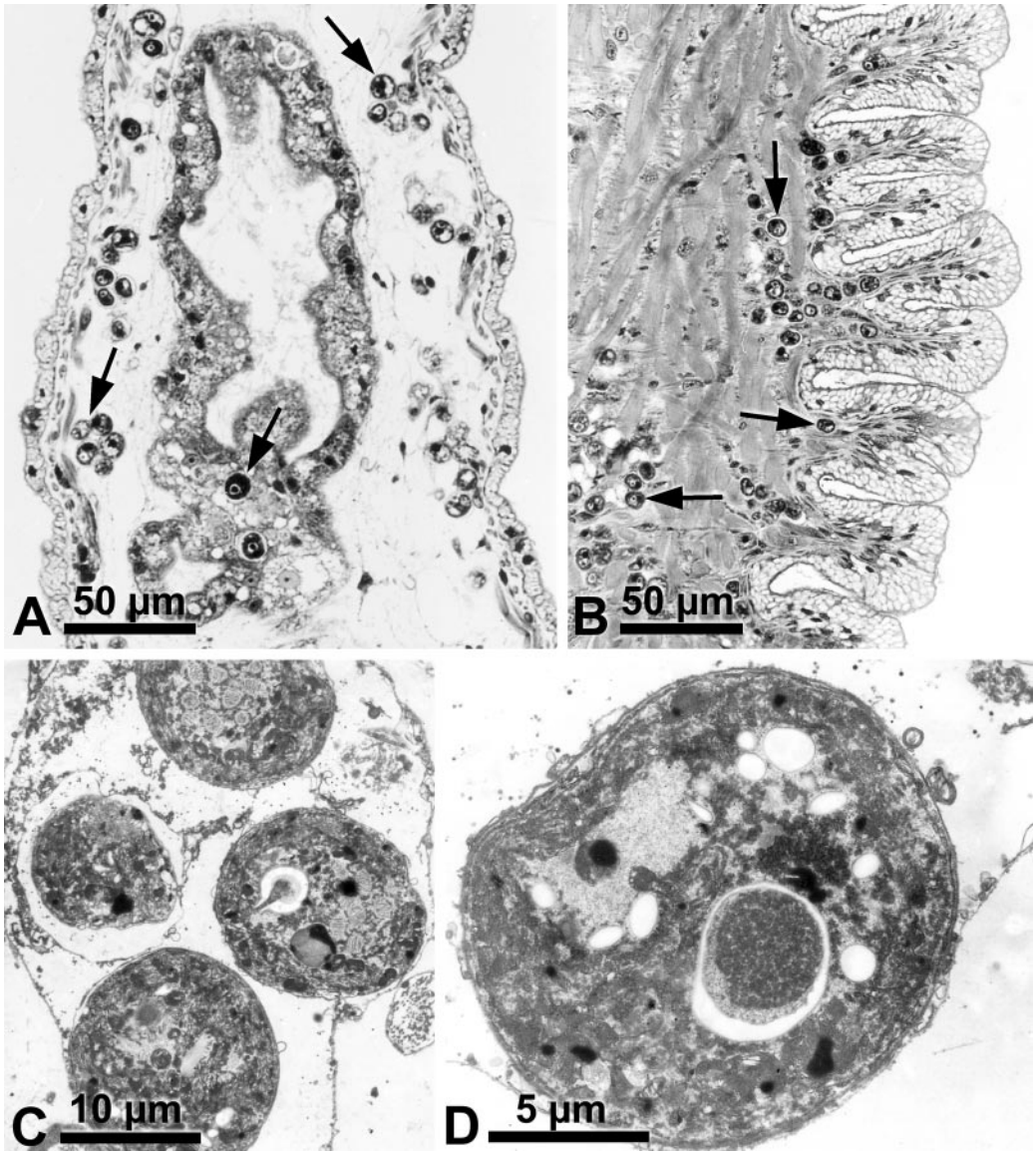

Fig. 3. **A**. *Pteraeolidia ianthina.* Longitudinal section of ceras with main digestive glandular branch and several fine tubules. **B**. *Pteraeolidia ianthina.* Lateral part of notum with folded epithelium and fine tubules and zooxanthellae between the muscles. **C**. *Pteraeolidia ianthina.* Electron microscopic picture of a fine tubule with membrane surrounding the whole structure. **D**. *Pteraeolidia ianthina.* Electrone microscopic picture of one zooxanthella with large pyrenoid. Arrows: zooxanthellae.

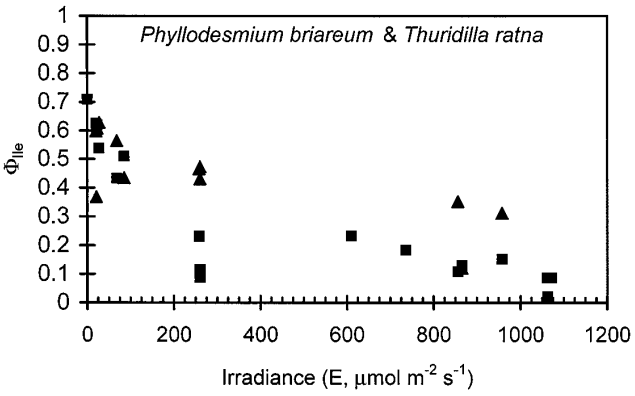

Fig. 4. Operational quantum yield of photosystem II fluorescence (φ_{IIe}) as a function of irradiance (E) for the zooxanthellae containing nudibranch *Phyllodesmium briareum* (squares) and the chloroplast containing sacoglossan *Thuridilla ratna* (triangle). For explanation, see "Material and methods".

zooxanthellae ramify from the main ducts especially in the cerata and in the notal tissue. Within the cerata (Fig. 2D) the main branch forms many of these distinct ramifications, which reach to the epidermis. Zooxanthellae are located in all parts of these branches. Fine tubules, where carrier cells but no distinct glandular cells can be distinguished, are mainly located in the body wall (Fig. 2C), the foot and the rhinophores (Fig. 2E). Transitions of digestive glandular branches with a distinct epithelium into fine tubules with no distinct epithelial cells were observed several times (Fig. 2C).

Pteraeolidia ianthina shows a similar branching of the digestive gland in the cerata, body wall, foot, rhinophores and head. Contrary to *Phyllodesmium*, there is usually only one main duct within the cerata, and many fine tubules where no glandular cells can be distinguished (Fig. 3A). Here, the zooxanthellae are grouped with about three to five cells and surrounded by a narrow

Table 2. Literature records of chloroplast incorporation. Only investigations by ultrastructural methods or by direct measurement of photosynthesis are listed. Only species with retention times greater than 24 hours are included. Column 4 indicates analytical method ("Met") used to detect photosynthetic activity: EM (electron microscopy), O_2 (Winkler method), ^{14}C (carbon fixation), SP spectrophotometry). Dig. gland. epith. = digestive glandular epithelium. Empty spaces in columns signify lack of information in the literature. *Hermaea bifida:* length of chloroplast retention not mentioned.

Species	Authors	Chloroplast location	Met	Foot
SACOGLOSSA				
Plakobranchoidea				
Bosellia mimetica	Marín & Ros (1988, 1989)		EM	*Halimeda tuna*
Elysia atroviridis	Kawaguti & Yamasu (1965)	Dig. gland. epith.	EM	*Codium fragile*
Elysia cauze	Clark et al. (1979)	Dig. gland. epith.	^{14}C	*Caulerpa* species
Elysia chlorotica	Graves et al. (1979)	Dig. gland. epith.	O_2	*Vaucheria*
	Mujer et al. (1996)			
Elysia flava	Marín & Ros (1988)		EM	cf. *Cladophora*
Elysia gordanae	Marín & Ros (1988)		EM	*Cladophora* sp.
Elysia hedgpethi	Greene (1970)	Dig. gland. epith.	^{14}C	
Elysia viridis	Trench & Smith (1970)		^{14}C	*Codium tomentosum*
	Hinde & Smith (1972)		^{14}C	*Codium fragile*
	Trench et al. (1973)		EM	*Codium fragile*
	Marín & Ros (1988)			
Elysia timida	Ros & Rodriguez (1985)		O_2	*Acetabularia acetabulum*
	Marín & Ros (1988, 1989, 1992)		^{14}C	
			EM	
Elysia translucens	Marín & Ros (1988, 1989)		EM	*Udotea petiolata*
Elysia tuca	Waugh & Clark (1986)		SP	
Plakobranchus ianthobapsus	Trench et al. (1970)		^{14}C	
	Greene (1970)		^{14}C	
Plakobranchus ocellatus	Ireland & Scheuer (1979)		^{14}C	*Siphonous algae*
Thuridilla hopei	Marín & Ros (1988, 1989)		EM	*Cladophora vagabunda*
Tridachia crispata	Yonge & Nicholas (1940)	Free in notal tissue in the interior folds		Green algae
	Trench & Smith (1970)		^{14}C	
	Clark & Busacca (1978)			
Tridachiella diomedea	Trench et al. (1970)		^{14}C	
Limapontoidea				
Costasiella lilianae (= *ocellifera*?)	Clark et al. (1981)	Dig. gland. epith.	^{14}C	*Avrainvillea nigricans*
Caliphylla mediterranea	Clark et al. (1990)		^{14}C	
Hermaea bifida	Taylor (1971)		O_2	*Griffithsia flosculosa*
	Marín & Ros (1988)		EM	
Mourgona germaineae	Clark et al. (1990)		^{14}C	
Limapontia depressa	Clark et al. (1990)		^{14}C	

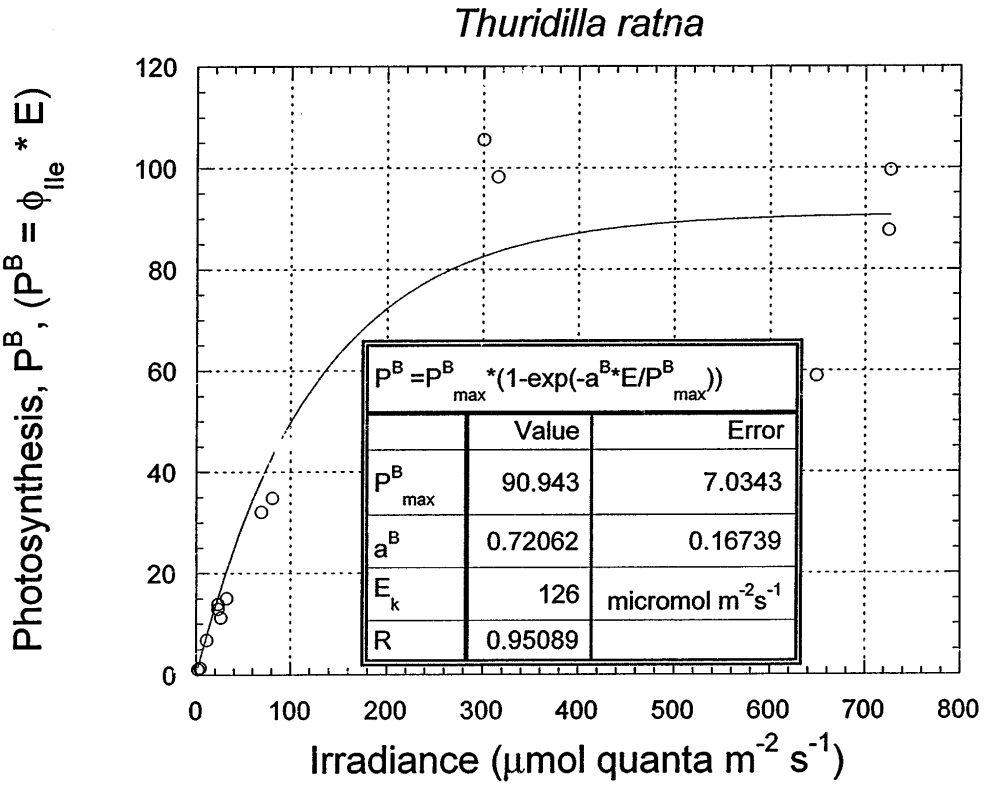

Fig. 5. Photosynthesis, measured as relative electron transfer rate (φ_{IIe} *E), versus irradiance (E) curve for *Thuridilla ratna*. For explanation of symbols, see "Material and methods".

membrane from the carrier cell (Fig. 3C). The carrier cells are lying close together forming continuous ribbons (fine tubules) within the notal tissue. The tubular lumen is visible, but very often it is lined by the carrier cells only on the side facing the outer epithelium. Zooxanthellae are usually not located in the thicker branches of the digestive gland, but mainly in the fine tubules. Besides the cerata, fine tubules can be found in the foot, especially the lateral body wall (Fig. 3B), and in the rhinophores.

Zooxanthellae are also found in the Mediterranean species *Piseinotecus gabinierei*. The zooxanthellae only lie within the digestive glandular epithelium, but are densely packed (Figs 2A,B). The digestive gland does not ramify within the cerata or in the body, and no branches lead to the rhinophores or head. Furthermore, no fine tubules could be detected.

In vivo fluorescence and photosynthetically functional zooxanthellae and chloroplasts were measured in several opisthobranchs. A blindfold test using not only members known to host zooxanthellae or chloroplasts, but also, e.g., members of the Doridoidea, was performed to exclude possible mistakes using the PAM technique. Histological investigation of these specimens also showed that their surfaces did not contain an epiflora of benthic microalgae.

Operational quantum yields and photosynthesis versus irradiance curves for two sacoglossans, *Thuridilla ratna* and *Elysia expansa*, and one nudibranch, *Phyllodesmium briareum*, indicate significant differences in photosynthetic characteristics (Figs 4, 5, 6).

The light saturation parameter (E_k, i.e. the onset of light saturation), of *Thuridilla ratna* (light gradient from 2–900 mmol quanta $m^{-2}s^{-1}$) was only 56% (125 µmol quanta $m^{-2}s^{-1}$) of the corresponding E_k of *Phyllodesmium briareum* of 225 µmol quanta $m^{-2}s^{-1}$ (Fig. 4, 5). The light saturation parameter of *Elysia expansa* (E_k = 55 µmol quanta $m^{-2}s^{-1}$) is significantly lower than in the other sacoglossan examined, and only 24% of the corresponding E_k of *Phyllodesmium briareum* (Figs. 5, 6).

The P vs. E values for *Thuridilla ratna* are mean values for two individuals (one small and one large) (Fig. 5). The differences in $\varphi_{IIe\text{-}max}$ or φ_{IIe} between individuals were not significant. Both sacoglossans were kept at high irradiance (HL) for 15 minutes after the last incubation irradiance (highest E) of the P vs E curve. The $_{IIe}$ changed from 0.09 (start – indication that many reaction centers are closed) to 0.14 (indication that reaction cen-

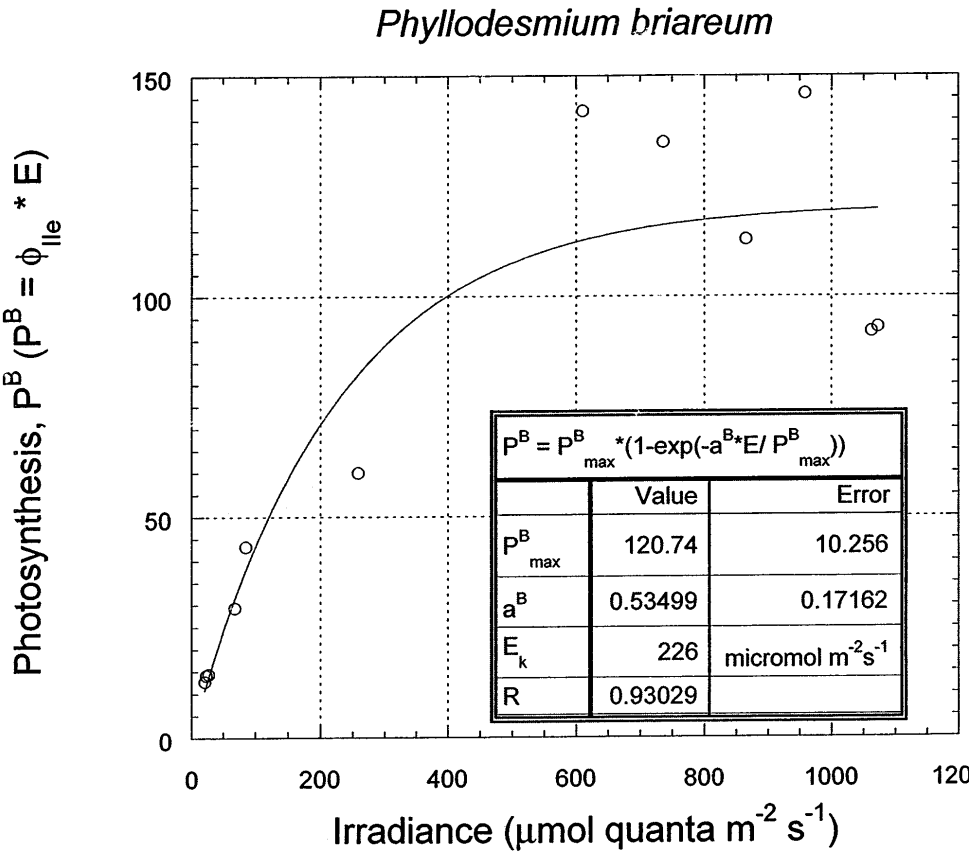

Fig. 6. Photosynthesis, measured as relative electron transfer rate ($\varphi_{IIe} * E$), versus irradiance (E) curve for *Phyllodesmium briareum*. For explanation of symbols, see "Material and methods".

ters start to acclimate to the given high irradiance level) after 15 min in HL exposure at 730 µmol quanta $m^{-2}s^{-1}$ for the larger slug. Correspondingly, the smaller *T. ratna* showed a φ_{IIe} of 0.0 (start) to 0.23 after 15 min incubation time at 830 µmol quanta $m^{-2}s^{-1}$. Both animals were subsequently dark acclimated to test if the light saturated reaction centers (inducing low operational quantum yields) of PS II would re-open (recover) and regain a high maximum quantum yield of PSII $\varphi_{IIe\text{-}max}$, Eq. 1). The observed $\varphi_{IIe\text{-}max}$ were 0.585 and 0.605 for the larger and smaller individual, respectively. This indicates active photoacclimation and photosynthetically functional chloroplasts. A dark acclimated specimen of *Plakobranchus ocellatus* obtained similar $\varphi_{IIe\text{-}max}$ values to those of *T. ratna*, i.e. 0.6.

The photosynthesis versus irradiance characteristics of two individuals (same size) of *Phyllodesmium briareum* showed similar fluorescence yields as a function of irradiance (typical CV of ±1.6–12% of mean yield value at a given irradiance, Fig 6). After subsequent dark acclimation, both individuals of *P. briareum* reached a high $\varphi_{IIe\text{-}max}$ of 0.71, the highest of all opistobranchs examined.

Discussion

Chloroplasts

In Table 2 published records of chloroplast-containing species are listed. Only these cases are included, in which chloroplast activity is verified by experiments, and retention of these organelles inside the animal was longer than 24 hours. These meet the requirements for the levels 5 (medium-term functional retention) and 6 (long-term functional retention) proposed by Clark et al. (1990) for the evolution of 6 steps from non-retention of plastids in Sacoglossa to long-term retention.

Retention of chloroplasts has been reported for many sacoglossans, and several studies have been undertaken to elucidate the importance of this retention (Taylor 1971; Hinde & Smith 1972; Trench et al. 1973; Ireland & Scheuer 1979; Mujer et al. 1996). A retention of func-

Table 3. Literature records of zooxanthellae presence in nudibranchs. Column 4 indicates analytical method ("Met") used to detect photosynthetic activity: H (histological investigation by light microscopy), EM (electron microscopy), O_2 (Winkler method), ^{14}C (carbon fixation), SP spectrophotometry). Dig. gland. epith. = digestive glandular epithelium. Empty spaces in columns signify lack of information in the literature.

Species	Authors	Zooxanthellae-location	Met	Food or potential source of zooxanthellae
NUDIBRANCHIA				
Dendronotoidea				
Doto doerga	Marín & Ros (1991)	Dig. gland. epith.	H	*Aglaophenia pluma*
Doto paulinae	Marín & Ros (1991)	Dig. gland. epith.	H	*Obelia geniculata, Aglaophenia pluma*
Doto rosea	Marín & Ros (1991)	Dig. gland. epith. (only 3–4 days)	H	
Melibe pilosa	Kempf (1984) Crossland & Kempf (1985)	*Symbiodinium microadriaticum:* intracellular	^{14}C	Crustacea
Melibe sp.	Kempf (1984) Crossland & Kempf (1985)	*Symbiodinium microadriaticum:* intracellular	^{14}C	Crustacea
Tritonia sp.	Rudman (1987)			
"Arminoidea"				
Doridomorpha gardineri	Eliot & Evans (1908) Rudman (1982a)	Free in the notal tissue, between muscles, within the visceral cavity, in rhinophores, in epidermis	H	*Heliopora*
Pinufius rebus	Rudman (1981a, 1982a)	Dig. gland. epith.	H	*Porites*
Aeolidoidea				
Aeolidia papillosa	Rousseau (1935)	Dig. gland epith.	H	
Aeolidiella alderi	Marín & Ros (1991) Graham (1938) Navilee (1926, cited in Ros & Rodriguez 1985)	Dig. gland. epith. Intracellular in dig. tract	H	*Metridium, Cereus, Diadumene, Sagatia, Sagatiogeton, Parastephanauge*
Aeolidiella glauca	Rousseau (1934, 1935)	Dig. gland. epith.	H	*Cylista undata* (Anthozoa)
Aeolidiella croisicensis	Rousseau (1935)	Dig. gland. epith.	H	
Aeolidiopsis harrietae	Rudman (1982a)	Dig. gland epith.	H	*Palythoa* (Zoantharia)
Aeolidiopsis ransoni	Rudman (1982a)	Dig. gland. epith. of cerata and body	EM	*Palythoa* (Zoantharia)
Berghia caerulescens	Marín & Ros (1991)	Dig. gland. epith.	H	*Aiptasia mutabilis*
Berghia major	Kempf (1984)	*Symbiodinium microadriaticum:* intracellular		*Boloceroides* (Anthozoa)
Berghia verrucicornis	Carroll & Kempf (1990) Marín & Ros (1991) Kempf (1991)	Dig. gland. epith.	H	*Aiptasiogeton* *Aiptasia pallida*
Catriona maua	Marín & Ros (1991)	Dig. gland. epith.	H	*Ventromma halecioides*
Cuthona caerulea	Marín & Ros (1991)	Dig. gland. epith.	H	*Sertularella, Halecium, Hydrallmania*
Cuthona granosa	Marín & Ros (1991)	Dig. gland. epith.	H	*Podocoryne carnea*
**Dondice parguerensis*	Brandon & Cutress (1985)	Dig. gland. epith.	H?	*Cassiopea xamachana*
Favorinus albus	Rousseau (1935)	Dig. gland. epith.	H	
Phestilla lugubris	Rudman (1982a, 1982b)	Dig. gland. epith.	H	*Porites*
Phestilla panamica	Rudman (1982a)	Dig. gland. epith.	H	*Porites lobata*
Phestilla sibogae	Harris (1973, 1975)	Dig. gland. epith.		*Porites*
Phyllodesmium briareum	Rudman 81991)	Dig. gland. epith.	H	*Briareum* (Alcyonaria)
Phyllodesmium colemani	Rudman (1991)	Dig. gland. epith.	H	*Tubipora musica*
Phyllodesmium crypticum	Rudman (1981b, 1991)	Dig. gland. epith.	EM	*Xenia*
Phyllodesmium guamensis	Avila et al. (1998)	Dig. gland. epith.	H	Species of *Sinularia*
Phyllodesmium hyalinum	Rudman (1981b, 1991)	Dig. gland. epith.	H	*Xenia*
Phyllodesmium longicirrum	Rudman (1981b, 1991)	Dig. gland. epith.	H	*Sarcophyton*
Phyllodesmium macphersonae	Rudman (1981b, 1991)	Dig. gland. epith. which ramifies throughout the body, foot, rhinophores, oral tentacles	H	Not known

Table 3. (Continued).

Species	Authors	Zooxanthellae-location	Met	Food or potential source of zooxanthellae
Phyllodesmium magnum	Rudman (1991)	Dig. gland. epith. ramifying in body wall and foot	H	cf. *Sinularia*
Phyllodesmium pecten	Rudman (1981b, 1991)	Dig. gland. epith.	H	*Xenia*
Pteraeolidia ianthina	Rudman (1982a)	Dig. gland. epith. ans tiny tubules	EM	cf. *Sarcothelia* (Alcyonaria)
	Kempf (1984)	connected with dgl. *Symbiodinium*		*Halocordyle distica* (Hydrozoa)
	Hoegh-Guldberg &	*microadriaticum:* intracellular	O_2,	from the water column
	Hinde (1986)	In elongations of the dig. gland.epith. (hardly visible)	^{14}C	
Spurilla major	Rudman (1982a)	Dig. gland. epith. which ramifies into body wall, rhinophores and oral tentacles	H	
Spurilla australis	Rudman (1982a)	Dig. gland epith. which ramifies into body wall, rhinophores and oral tentacles	H	
Spurilla neapolitana	Fedele (1926)	Dig. gland. epith.	H	*Anemonia, Aiptasia,*
	Rousseau (1935)	Dig. gland. epith.	H	*Aiptasiogeton,*
	Marín & Ros (1991)	Dig. gland. epith., and in the body	H	*Bunodeopsis, Haliplanella, Condylactis*

*It is not definitely stated whether the zooxanthellae are still active or are digested.

tional chloroplasts in the digestive diverticula for less than 24 hours is assumed to be of nutritive value for the slug (Marín & Ros 1988). Jensen (1997) spoke of short-term functional kleptoplasty. This is considered a prerequisite for survival of food shortages and/or to exploring new food resources (Jensen 1997). Unfortunately, it is not proven for many species whether chloroplasts are really photosynthetically active in the digestive tract, or just retained in the digestive gland before degradation occurs.

The efficiency of retention varies greatly among species and seems to depend on the kind of algae consumed. In *Tridachia crispata*, 50% of chloroplasts have been found to still function after 58 days of starvation, whereas in *Oxynoe antillarum* this 50% value was already reached after 15 days, in *Elysia tuca* after 5 days (Clark & Busacca 1978). Hinde & Smith (1972) mentioned a minimum retention time of 3 months for *Elysia viridis*. Recently Mujer et al. (1996) reported an 8-month symbiotic association between *Elysia chlorotica* and the chloroplasts of *Vaucheria litorea*. These authors, and Pierce et al. (1996), were able to demonstrate that such chloroplasts keep the ability to carry out transcriptional and translational processes, which implies that some regulatory function – normally provided by the nucleus of the plant cell – has to be taken over by the nuclear genes of the slug or is contained in the genome of the kleptoplastids (Mujer et al. 1996).

Recently, Rumpho et al. (2000) published an update on chloroplast symbiosis in Sacoglossans. We would like to refer to their review for further information on this group.

In our study we investigated four members of the Sacoglossa and could identify a photosynthetic activity in *Plakobranchus ocellatus*, *Elysia expansa* and *Thuridilla ratna*. This is in accordance with investigations on other species of these genera (see Table 2). The absence of any photosynthetic activity in *Cyerce nigricans* shows that chloroplasts taken in as food are not incorporated but digested.

Zooxanthellae

The presence of symbiotic algae in members of the Aeolidoidea (Nudibranchia) has been known for nearly a century, but it was Rousseau (1934, 1935) who described unicellular algae as symbionts in opisthobranchs in detail. Several authors have described zooxanthellae in the digestive system of aeolids without realizing them to be separate organisms, e.g. in *Aeolidiella glauca* (Hecht 1885, cited in Hoffmann 1939), or only assumed that certain cells are symbiotic algae, e.g. in *Doridomorpha gardineri* (Eliot & Evans 1908). The number of known species fostering unicellular algae has since increased considerably (see Table 3). It was Rudman (1981a) who first described the zooxanthellae of a non-aeolid species, *Pinufius rebus*, and assumed that they are taken from their prey. Especially Rudman's investigations (1981a, b; 1982a, b; 1991) on several nudibranchs with zooxanthellae elucidated the biological factors of

these symbioses. It still is unclear how many species of the dinoflagellate genus *Symbiodinium* Freudenthal, 1962 (= *Gymnodinium*) are involved in symbiotic relationships with nudibranchs. Hoegh-Guldberg & Hinde (1986) identified a species in *Pteraeolidia ianthina* as not being distinguishable morphologically from *Symbiodinium microadriaticum* Freudenthal, 1962. Marín & Ros (1991) also assume that the dinoflagellates they identified in several nudibranchs belong to this species. On the other hand, Blank & Trench (1985) identified at least four different species in cnidarians on the basis of chromosome numbers and physiological behaviour.

Starvation tests with several zooxanthellae-bearing nudibranchs (*Berghia major, Pteraeolidia ianthina* and *Melibe pilosa*) in constant light or constant darkness (Kempf 1984) showed the importance of zooxanthellae for slug survival under food shortage conditions. Whereas Kempf was not able to find out whether the carbon fixed by the zooxanthellae was transported to the slugs' cells, or whether the nutrients became available only after digestion of the zooxanthellae, Hoegh-Guldberg & Hinde (1986) discussed the translocation of the fixed photosynthetic substances (probably glycerol) to the host, *P. ianthina*. Hoegh-Guldberg et al. (1986) mentioned that nearly twice as much as the slug's total carbon budget could be transferred by the zooxanthellae in summertime.

Zooxanthellae are usually located within cells of the digestive glandular epithelium (Table 3). Very often, the digestive gland ramifies and the branches spread below the epidermis, especially below parts exposed to the sunlight. This is very obvious in *Phyllodesmium briareum*. In *Pteraeolidia ianthina* and the arminid *Pinufius rebus*, the algae are described to be located within kinds of tubules which have their origin in the digestive gland, and which ramify throughout the body and even reach within the rhinophores and oral veil (Kempf 1984; Rudman 1981a, 1982a). The 'tubules' were identified in the present study as carrier cells arranged (partly) around tubules, but very often only agglomerated into a ribbon-like structure. Rudman (1982a) mentioned for *P. ianthina* that zooxanthellae are exclusively located within these tubules. Here we basically confirm his findings, although a few algal cells are also located within the digestive glandular epithelium.

Rudman (1991) described the different arrangements of zooxanthellae in the various species of *Phyllodesmium*. *P. briareum* is a species highly adapted to symbiosis with photosynthetically active zooxanthellae. Our findings confirm the results of Rudman on this species, with one exception. Rudman described the zooxanthellae as situated only in the peripheral parts of the digestive glandular branches whereas in our specimen, the dinoflagellates could be found throughout the digestive glandular epithelia. Fine tubules are described by Rudman (1991) for several species of *Phyllodesmium (P. macphersonae, P. colemani, P. briareum, P. magnum* and *P. longicirrum*), indicating that this is an adaptation to zooxanthellae symbiosis. Avila et al. (1998) described a similar arrangement of zooxanthellae in the branched digestive gland within the cerata of *Phyllodesmium guamensis*, and fine tubules in the cerata, but no tubules in the rest of the body.

The findings of zooxanthellae in *Piseinotecus gabinierei* is new, and rather astonishing since this species feeds on the hydrozoan *Eudendrium ramosum* (Cattaneo-Vietti et al. 1990). Whether these zooxanthellae are photosynthetically active or only present due to ingestion, has to be clarified by future investigations. Although *Aeolidia papillosa* has been reported to house zooxanthellae (Rousseau 1935; L. Schmekel, pers. comm. 2000), these findings could not be confirmed in our material.

Several members of the Dendronotoidea belonging to the genera *Doto*, *Melibe* and *Tritonia* are known to house zooxanthellae (see Table 3). In this study, we investigated additional species of these genera but none of them contained algae.

Some species which formerly were placed within the paraphyletic Arminoidea (see Wägele & Willan, 2000) are demonstrated to contain zooxanthellae. They belong to the family Arminidae. Whereas the three investigated species of *Armina* did not show any zooxanthellae in their digestive tract, the digestive glandular epithelium of the three species of *Dermatobranchus* (*D. semistriatus, D.* sp.1 and *D.* sp.2) were densely packed with the algae, and the presence of fine tubules in the two unnamed *Dermatobranchus* species indicates a possible symbiotic function similar to the findings in *Phyllodesmium* and *Pteraeolidia*. Rudman (1982a) described the arrangement of the digestive gland in another arminoid, *Doridomorpha gardineri*. This species, which is not closely related to the Arminidae, shows a similar arrangement of the glandular branches and the presence of tubules in the body wall, as described here for the *Dermatobranchus* species. The presence of tubules in muscle tissue is noted here for *Dermatobranchus*, but also for *Pteraeolidia ianthina* and *Phyllodesmium briareum*.

Up to now only members of the Cladobranchia are known to host zooxanthellae. No dorid species has been found in literature or our own investigations, to house algae. The symbiotic relationships appear to depend on the hosts' food sources. Only nudibranchs that feed on zooxanthellae-bearing cnidarians have the ability to obtain the algae. Exceptions seem to be *Melibe pilosa* and an unidentifed *Melibe*, which feed on small invertebrates, especially crustaceans, but which are described to house zooxanthellae (Kempf 1984). It is not clear how the species 'capture' the algae. One specimen of *Melibe leonina* investigated in this study did not show any

zooxanthellae (Table 1). The presence of zooxanthellae in *Piseinotecus gabinierei* is peculiar, since the hydrozoan prey (*Eudendrium ramosum*) is not known to house zooxanthellae. It can not be excluded that uptake of zooxanthellae happens by chance while feeding on prey which is colonized by organisms housing dinoflagellates, e.g. Foraminifera. Alternatively the range of the nudibranchs' prey may be wider than known. In the Mediterranean, *Aglaophenia* species are known to house zooxanthellae (A. Svoboda, pers. comm. 2000).

Wägele & Willan (2000) have shown that the Arminidae are a basal group within the Cladobranchia, and Kolb & Wägele (1998) demonstrated the most basal position of *Dermatobranchus* within the Arminidae. Future measurements, especially on members of the Arminidae, Zephyrinidae and Dendronotoidea, might bring a much better understanding of the role of consumed zooxanthellae and the evolution of symbioses within the Cladobranchia. If photosynthesis is demonstrated for the groups mentioned above, we can assume that symbiosis is an old functional achievement of the Cladobranchia and has not evolved independently, as Rudman (1982a, 1987) assumed.

Photosynthetic activity

The confirmation of zooxanthellae (and also chloroplasts) in the digestive tract by histological means does not necessarily imply that the incorporated algae are used as 'solar panel energy cells'. This has already been emphasized by Rudman (1982a). Degradation or digestion of the cells can be tested for by keeping animals in an aquarium for a longer period of time prior to preservation and further histological investigations. Photosynthetic activity can be measured by O_2 production using the Winkler method (Ros & Rodriguez 1985), by applying an oxygen electrode (Hoegh-Guldberg & Hinde, 1986), or with ^{14}C-incubation (e.g. Crossland & Kempf 1985). Contrary to these time-consuming methods which also stress the animals involved, the Pulse Amplitude Modulated Fluorometer quickly and reliably detects whether or not the animals contain chl a (degraded and non degraded), and allows measurements of photosynthetic activity of chloroplasts and zooxanthellae without hurting or even disturbing the animals.

We have tested several nudibranchs for photosynthetic activity. Amongst these were members of the Doridoidea (*Chromodoris*, *Nembrotha*) and Aeolidoidea (*Flabellina exoptata*, *Pteraeolidia ianthina* and *Phyllodesmium briareum*). Only the last two showed photosynthetic activity. These data confirm the former investigations on *P. ianthina* (Hoegh-Guldberg & Hinde 1986) and the assumption of Rudman (1991) that *P. briareum* is a species with a highly evolved photosynthetic symbiotic relationship.

Some precautions should be taken: chl a fluorescence is easy to detect but is difficult to measure correctly and to interpret. A lot of methodological pitfalls and limitations are known, especially regarding the use of the saturation pulse intensity and duration (Kroon et al. 1993; Govindjee 1995). The correct use of probe light and to avoid stressing the animals and zooxanthellae/chloroplasts during saturation pulse measurements are very important. Accurate estimations of minimal and maximal fluorescence yields in dark and in light (F_0 and F_m, F_0' and F_m') may be difficult to obtain and the user should take into consideration that the quantum yield of fluorescence φ_F is related to the different rate constants (k's) of various pathways of de-excitation. These rate constants comprise fluorescence (F), heat dissipation (H), excitation energy transfer (T), quenching of fluorescence (Q, e.g. photoprotective carotenoids and acidity inside chloroplasts), and photochemistry (P, cf. Govindjee 1995):

$$\varphi_F = \frac{k_F}{k_F + k_H + k_T + k_Q + k_P} \qquad \text{Equation 4}$$

Future investigations need to include the chl a-specific absorption coefficients for the zooxanthellae/chloroplasts, their corresponding fraction of light utilized by PS II and the spectral composition of the light to calculate photosynthesis in absolute units (Johnsen et al. 1997). These non-destructive fluorescence and bio-optics based measurements should be compared with conventional oxygen and ^{14}C measurements.

Differences in E_k (light saturation parameter) indicate differences in photo-acclimational status and species-specific differences (Figs 5–6). Such differences can be used as valuable information for understanding and modeling photosynthesis and growth characteristics in opisthobranchs. In general, low E_k values indicate shade-acclimated zooxanthellae or chloroplasts (i.e. opisthobranchs collected below 20 meters depth), whereas high E_k values indicate bright light acclimated systems (animals collected near surface). Species with similar maximum photosynthetic rates but with different E_k's indicate that the maximum light utilization coefficients (initial slope of the P vs. E curve) are different (Figs 5–6). In the present study the interpretation and comparison of the E_k values may be biased since we have different sizes of the slugs and different density of chloroplasts and zooxanthellae inducing intra- and intercellular self-shading (= package effect, see Johnsen et al. 1997). This may explain the relatively high E_k value (225 µmol quanta $m^{-2}s^{-1}$) obtained from *P. briareum* containing high density of zooxanthellae (collected at 15 m depth, Fig. 6) relative to E_k values of 55 and 125 µmol quanta $m^{-2}s^{-1}$ in the chloroplast-containing *E. expansa* and *T. ratna*, respectively (Fig. 5).

Contrary to histological investigations, PAM can easily help distinguish species with symbiotic zooxanthellae from those just ingesting and digesting zooxanthellae. Therefore future investigations on many species mentioned to have zooxanthellae (e.g. *Dondice parguerensis*, see Brandon & Cutress 1985, *Phestilla panamica*, see Rudman 1982b, *Dermatobranchus semistriatus*, this study, see Table 3) will clarify their function for the slugs. Future investigations with PAM should be done under different light and food conditions as a function of time. This information should be done in combination with histological, pigment signature (chemotaxonomy of "sun panel" pigments) and bio-optical measurements (indicating light harvesting and utilization from 400–700 nm, i.e. the spectral region of visible light). This will elucidate the evolution and function of zooxanthellae and chloroplast symbiosis, respectively. The study of role of fine tubules and other morphological and behavioural adaptations in these seaslugs may benefit by a combined use of all the mentioned techniques. Key environmental variables such as light regime (irradiance, spectral composition of light and day length), temperature, salinity, nutrients, microelements (copper, iron, zinc etc.), harmful chemicals (e.g. run-off from agriculture, oil spills) and toxins (e.g. from algal blooms) will alter the physiology of the host, zooxanthellae, or chloroplasts. These changes can easily be assessed by a combined information using histological measurements (long term responses from weeks to months), bio-optical measurements (midterm responses from minutes to days) and PAM (short term responses from microseconds to minutes).

The effect of runoff from mainland agricultural industry, such as the herbicide Dichlorophenyl dimethyl urea (DCMU), an efficient PS II inhibitor (cf. Johnsen *et al*. 1997), can easily be detected by the use of PAM, as well as its influence on zooxanthellae-bearing invertebrates, such as sea slugs and coral reefs that are depending on photosynthetic symbionts.

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