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Solar powered phycozoans

Herbivore sacoglossans with photosynthetic chloroplasts

Thesis for the degree philosophiae doctor

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Norwegian University of Science and Technology
Faculty of Natural Sciences and Technology
Department of Biology



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Perfer et obdura; dolor hic tibi proderit olim (Ovid)

Preface

This thesis evolved from the NFR funded strategic university programme MODTEQ where a submersible model of a Pulse Amplitude Modulated fluorometer was used to detect the photosynthetic performance in macro algae (kelp forest), zooxanthellate corals and solar powered sea slugs. Collaboration between Geir Johnsen and Heike Wägele resulted in that Ingo Burghardt and I were to investigate solar powered opisthobranchs from two different functional angles; Ingo was to look at zooxanthellae (photosynthetic endosymbiotic dinoflagellates) in nudibranchs and I was to look at chloroplasts in sacoglossans. In regard of methodology and preliminary investigations of solar powered sea slugs, very little was known on how zooxanthellae or photosynthetic organelles (chloroplasts) function in opisthobranchs. The aim of this thesis was therefore to further develop bio-optical methodology to identify and describe the photosynthetic functionality of chloroplasts derived from macroalgae in *Sacoglossa*.

The work on this thesis has been carried out at Trondhjem Biological Station, Department of Biology, Norwegian University of Science and Technology. Financial support has been contributed by the Norwegian Research Council, NFR 153790/120 through the MARE programme. My supervisors have been Professor Geir Johnsen (TBS, NTNU) and Professor Heike Wägele (Institut für Evolutionsbiologie und Ökologie, Der Rheinischen Friedrich-Wilhelms-Universität Bonn).

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during two lengthy field trips to the outer parts of the Great Barrier Reef. I am also grateful to Torkild Bakken and a handful of other nudiphile divers during collection of sacoglossans in Norwegian waters. Last but not least, my sincere thanks to staff and colleagues at Trondhjem Biological Station.

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List of papers

- I Burghardt I, Evertsen J, Johnsen G, Wägele H (2005) Solar powered seaslugs – Mutualistic symbiosis of aeolid Nudibranchia (Mollusca, Gastropoda, Opisthobranchia) with *Symbiodinium*. *Symbiosis* 38:227-250

- II Evertsen J, Burghardt I, Johnsen G, Wägele H (2007) Retention of functional chloroplasts in some sacoglossans from the Indo-Pacific and Mediterranean. *Mar Biol* 151:2159–2166

- III Evertsen J, Johnsen G (2008) *In vivo* and *in vitro* differences in chloroplast functionality in the two North Atlantic sacoglossans (Gastropoda, Opisthobranchia) *Placida dendritica* and *Elysia viridis*. *Mar Biol* (submitted)

- IV Evertsen J, Johnsen G, Tvedt K (2008) *In vivo* and *in vitro* characteristics of photosynthetic chloroplasts retained in some sacoglossans (Gastropoda, Opisthobranchia) from Lizard Island, Great Barrier Reef. (manuscript)

Papers are referred to in the text by their respective numbers.

List of definitions, symbols and units

PAM	Pulse Amplitude Modulated fluorometer
HPLC	High Precision Liquid Chromatography
LM	Light Microscopy
TEM	Transmission Electron Microscopy
E	Irradiance ($\mu\text{mol photons m}^{-2} \text{ s}^{-1}$), 400-700 nm
PSII	Photosystem II
RC _{PSII}	Reaction Centre bonded to PSII
LHC _{PSII}	Light Harvesting Complexes bonded to PSII
PSU	Photosynthetic Unit (composed of photosystem I, PSII and their LHC)
Φ_F	Quantum yield of chl <i>a</i> fluorescence (Equation 1)
Q _A	Primary quinone acceptor in PSII
F ₀	Ground fluorescence in dark acclimated chloroplasts (all RC _{PSII} are open)
F _m	Maximum fluorescence in dark acclimated chloroplasts (all RC _{PSII} are closed)
Φ_{PSII}	Maximum quantum yield of chl <i>a</i> fluorescence from PSII in dark acclimated chloroplasts (Equation 2) ($\text{mol e}^- \text{ mol photons}^{-1}$)
F ₀ '	Ground fluorescence under actinic light conditions
F _m '	Maximum fluorescence under actinic light conditions
Φ_{PSII} '	Operational quantum yield of chl <i>a</i> fluorescence from PSII under actinic light conditions (Equation 3) ($\text{mol e}^- \text{ mol photons}^{-1}$)
RFC	Retention ability of Functional Chloroplasts (Equation 4)
ETR	Electron Transfer Rate (Φ_{PSII} ' * E)
P	Photosynthesis, measured as ETR (Equation 5) in P vs. E curves
α	Light utilisation coefficient (Φ_{PSII} ' * \bar{a}_{PSII})
P _{max}	Maximum photosynthetic rate (Φ_{PSII} ' / q τ)
E _k	Light saturation parameter = P _{max} / α ($\mu\text{mol photons m}^{-2} \text{ s}^{-1}$)
\bar{a}_{PSII}	Light absorbed by PSII and LHC _{PSII} ($\text{m}^2 \text{ mg (chl a)}^{-1}$)
q	[chl <i>a</i>] per PSU ($\text{mg chl a mol (PSU)}^{-1}$)
τ	Minimum turnover time of electrons in PSU (h)
LL	Low Light (0-400 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$)
ML	Medium Light (400-1000 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$)
HL	High Light (>1000 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$)
TOB	Thylakoid Organising Body

1. Introduction

“Experimental and quantitative studies on several algal-invertebrate symbiosis have suggested or shown directly that these associations possess a variety of interacting systems that stabilize and perpetuate them. It is becoming evident that host-algal symbiont interactions occur at many levels of biological organisation. Among some contemporary workers in algal-invertebrate symbiosis there has been a shift away from “reciprocal benefit”, the “who does what for whom”, and the catalog-categorization approaches to symbiosis research. There is a growing emphasis on studies probing the underlying mechanisms allowing or causing animals and algae to form stable entities that thrive and persist through time. We are becoming aware that algal-animal symbioses have unique biological identities. The association is an organism that makes its living in a particular way, in a particular ecological context. [] I wish to introduce the term *phycozoan* (*phyco*, seaweed, *zoa* animal) to denote the compound organism resulting from the intimate association of algae and animals.”

Pardy – Phycozoans, phycozoology, phycozoologists?

Here, Pardy (1983) addresses the association between algal cells, organelles and invertebrates, which together form a solar powered organism. In the marine environment, solar powered organisms are quite common; the best known are tropical octocorals harbouring zooxanthellae (dinoflagellate microalgae), but phycozoans occur among a wide range of other invertebrates as sponges, foraminiferans, cnidarians, molluscs and tunicates (van Oppen et al 2005). This thesis is a product of investigating phycozoan entities represented by solar powered opisthobranchs; the association between zooxanthellae and carnivorous nudibranchs, and between macroalgal chloroplasts and herbivore sacoglossans, with emphasis on the latter association.

The solar powered nudibranchs incorporate zooxanthellae, whole algal cells where the majority belong to the genus *Symbiodinium*, which is usually divided into eight clades (Pochon et al 2006). Solar powered nudibranchs are mainly found in æolid taxa (Rudman 1981, Kempf 1984, Hoegh-Guldberg & Hinde 1986, Rudman 1991, Wägele & Johnsen 2001, Paper I). Nudibranchs are carnivorous opisthobranchs that in adult stages completely have lost any shell-like structures (Wägele & Willan 2000), where term “æolid” refers to the æolidoidean group of nudibranchs that have finger-like extremities dorsally called “cerata”. Cerata are blood-filled tubes which contains extensions of the digestive gland and which in most æolid nudibranchs contain kleptocnids (the storage of cnidocysts for defence, see Martin (2003)). The process of storing cnidocysts in the cerata and using them for defence is common within the æolid nudibranchs (Greenwood 1988), and might have been a precondition leading to the ability to also retain functional zooxanthellae in similar ways

(Rudman 1981). The zooxanthellae are kept inside the cells of the digestive gland, originating from the food of the nudibranch, usually hydroids or soft corals (Kempf 1991, Paper I). The benefit of retaining zooxanthellae is the photosynthetic products, which allow the nudibranchs to tolerate starving conditions for several weeks. In Paper I, nudibranchs like *Pteraeolidia ianthina* (Figure 1a in this thesis), were observed to be effective in retaining zooxanthellae; after 71 days of starvation, the photosynthetic activity (see Chapter 2.2) had not decreased significantly. Other nudibranchs belonging to the genus *Phyllodesmium*, showed varying degrees of photosynthetic activity decreasing as function of time during starvation (Paper I, Burghardt et al 2008). The first example indicates that the zooxanthellae in *P. ianthina* are able to divide and keep a stable number of zooxanthellae in the nudibranch digestion. Studies on zooxanthellae in corals have shown that a typical doubling time of zooxanthellae *in hospice* is low compared to free living dinoflagellates, and is in the order of 70–100 days (Hoegh-Guldberg & Hinde 1986). Free living dinoflagellates may often obtain a doubling time every third to tenth day depending on irradiance (Johnsen & Sakshaug 1993). The second example where the photosynthetic activity decreases as a function of time in species of *Phyllodesmium*, indicates either that the retained zooxanthellae are not able to divide or renew their numbers at the same rate as they are digested, or that the functionality of the zooxanthellae is arrested (Paper I, Burghardt et al 2008). Zooxanthellae are intact algal cells, which should be able to repair themselves and photoacclimate (the ability to adjust their photosynthetic activity to changing light climate, see Chapter 2.3). However, species of *Phyllodesmium* are highly adapted to their prey, soft corals, and are in many cases cryptic when feeding on the coral because their cerata mimic the coral polyps (Paper I, Burghardt & Gosliner 2006). This can indicate a dependence to one particular soft coral species (Paper I), and thus an independence of zooxanthellae; if the nudibranch is living most of its lifecycle on one coral, the need to tolerate long periods of starvation is not important. In contrast, *P. ianthina* has been observed to retain zooxanthellae from at least four different clades (Loh et al 2006), indicating a wide range of prey since cnidarians are not known to host more than one clade (Toller et al 2001). However, one prey organism may be the stinging hydroid genus *Aglao phenia*, which is known to contain zooxanthellae (Song & Lim 2001), because *P. ianthina* also retain kleptocnids that can deliver a bad cnidocyst sting if touched, even to humans (pers. obs). However, most *P. ianthina* were not found near any prey organisms, which indicates that this is a very mobile nudibranch which will benefit from tolerating long periods searching for food.

With the association between zooxanthellae and nudibranchs as a background, this thesis will look into the functionality of algal chloroplasts retained by sacoglossans. The sacoglossans are herbivore opisthobranchs incorporating photosynthetic chloroplasts in their digestive cells after ingesting macroalgal cytoplasm (Jensen 1997). Like the nudibranchs, the sacoglossans are opisthobranchs where the presence of hard shell-like structures is highly reduced. However, based on the presence or absence of a rudimentary shell-like structure, the Sacoglossa can be classified into two subgroups: the Oxynoacea which have an internal rudimentary shell, within which the digestive gland also is completely contained, comprising of the families Oxynoidae, Juliidae and Volvatellidae. And the non-shelled Plakobranchea, divided into the parapodia-bearing Plakobranchoidea and the cerata-bearing Limapontioidea (Jensen 1996, see Figure 1b). The Plakobranchoidea have a pair of flat wing-like appendages (parapodia) which are folded up dorsally (see Figure 1c and d). The digestive gland ramifies into numerous small tubules which also expand into the parapodia. Plakobranchoidea (=Elysiidae) is the largest family with about 120 species (Wägele 2004). The other families are Bosellidae and Platyhedylidae. The Limapontioidea have cerata dorsally (except *Limapontia*, the only genus in the cerata-bearing group which does not have cerata), which are fused with the digestive gland (in only two genera, *Cyerce* and *Sohgenia*, does the digestive gland not connect with the cerata). It comprises the families Polybranchiidae, Hermaeidae and Limapontiidae.

Sacoglossans are almost exclusively feeding on coenocytic and siphonous macroalgae (Jensen 1980), the exception being species which feed on seagrasses, the eggs of other opisthobranchs and diatoms (Jensen 1993). The sacoglossan feeding habit is adapted for suctorial feeding of algal cytoplasm, with a modified uniseriate radula used for puncturing algal cells. This has led to most sacoglossans feeding on algae with a large cytoplasm to cell volume with numerous nuclei (coenocytic) where the cell also may contain transverse cell walls (siphonous). Coenocytic algae have multinucleate cells but lack cross-walls. In macroalgal physiological literature the term siphonous has been synonymous to coenocytic, however, siphonous algae have a tubular multinucleate thallus (Grant & Borowitzka 1984). Thus all siphonous algae are coenocytic (e.g. the ulvophycean Bryopsidales), but not all coenocytic algae are siphonous (e.g. the rhodophyte *Griffithsia*). There are also large-celled, non-coenocytic algae such as *Acetabularia*, which in some respect resemble coenocytes. After puncturing the algae, the sacoglossans extract the algal cytoplasm with a muscular pharyngeal pump. The digestive system is adapted to a fluid diet, with large internal surface area for absorption and a reduced

intestine (Taylor 1968). The digestive gland tends to be highly ramified (digestive gland tubules extends throughout the body), particularly in sacoglossans that retain functional chloroplasts for long time periods (Paper II). They are also able to sequester secondary metabolites used defensively in mucus secretions (Marin & Ros 2004). The retention of photosynthetic chloroplasts is not an ability unique to sacoglossans, but is also common in several mixotrophic species of phytoplankton (Gast et al 2007), ciliates (Dolan & Perez 2000) and foraminiferans (Lee 1998).

The close associations between sacoglossans and their feeding habits have led to suspicions of co-evolution between the slugs and the algae, however, no evidence in this respect have yet been presented (Jensen 1997). Instead, speciation and diet radiation have been widespread, indicating intense competition for food sources and habitat exploitation. This is evident in that the oxynoceans only feed on the algal genus *Caulerpa*. The diet of sacoglossans seem to have radiated out from this food source independently in the plakobranchoid and limpaontoid sacoglossans to comprise coenocytic and siphonous algae mostly within the chlorophyte macroalgal group Bryopsidales (*Caulerpa*, *Codium*, *Bryopsis* etc) and other siphonous groups within xanthophytes and rhodophytes (Jensen 1997).

The ability to retain chloroplasts in the digestive gland in the sacoglossans has been named as “chloroplast symbiosis” (Hinde & Smith 1974), “chloroplast farming” (Hinde 1980), “kleptoplasty” (Waugh & Clark 1986, Clark et al 1990) or “chloroplast retention” (Marin & Ros 1992). The problem with these definitions is that they either stretch the relationship between chloroplast and slug into one being between two autonomous entities (symbiosis, here understood as “an association, for significant proportions of the life cycles, of individuals that are members of different species”, as defined by Margulis (1981)), or omit the functional aspect of photosynthetic chloroplasts altogether. The term “kleptoplasty” seems to be more fitting denoting retention of functional chloroplasts in the Sacoglossa. The presence of chloroplasts in the sacoglossan digestion system observed by histological methods (Kawaguti & Yamasu 1965, Taylor 1968) gave the first suspicion of “symbiotic” organelles. But, observations alone can not expose the functionality of the chloroplasts, and in the 1970’s investigations were conducted on regarding photosynthetic activity (^{14}C incorporation, oxygen production based methods), chloroplast division, and chloroplast synthesis of lipids, proteins and pigments inside the sacoglossans digestive cells. The association between an algal organelle and animal host does not seem to fall within the general concept of symbiotic

relationships, since it is likely that the chloroplasts in the sacoglossan digestive system can not divide (Trench & Olhorst 1976), nor synthesise any e.g. chlorophyll *a* (chl *a*) or galactolipids (Trench & Smith 1970, Trench et al 1973a and b, Trench et al 1974). In this regard, the lifespan of the chloroplasts in the digestive cells of sacoglossans was observed to last up to a few weeks, and Clark et al (1991) proposed that six types of retention of chloroplasts could be attributed to sacoglossans, from retention of non-functional (digested) chloroplasts to retention of chloroplasts that fix ^{14}C or show high concentrations of chl *a* for more than one week.

These investigations brought forward the need to find a reliable method which could measure the photosynthetic activity in solar powered opisthobranchs. The first aim of this thesis was therefore to implement the *vivo* bio-optic methodology using PAM (Pulse Amplitude Modulated Fluorometry), introduced by Wägele & Johnsen (2001) to detect photosynthetic activity in opisthobranchs, but not developed further. Paper I-IV uses the PAM to develop ways to investigate the functionality of the zooxanthellae and chloroplasts when retained by the opisthobranchs. However, the main aim of this thesis is to investigate how chloroplasts remain functional in the digestive cells of the sacoglossans. Chloroplast functionality in sacoglossans is here defined as the ability to carry on photosynthesis in the sacoglossan digestion system. Chloroplast functionality can thus be studied in regard of

- 1) **Chloroplast lifespan;** how are the chloroplasts stored in the sacoglossan digestion system, and how long are the chloroplasts able to continue photosynthesis in the sacoglossans? What affects the chloroplast photosynthetic processes (chloroplast integrity, electron transfer rate in photosynthesis based on PAM measurements)?

Paper II and III

- 2) **Chloroplast autonomy;** are the chloroplasts able to divide and repair themselves (measuring changes in photosynthetic parameters, histological observations)? Are the chloroplasts able to acclimate to changes in irradiance (changes in pigment composition, function and photosynthetic parameters)?

Paper III and IV

- 3) **Chloroplast origin;** which algae/plants are potential donors of functional chloroplasts to sacoglossans and how do we trace them (pigment composition, chemotaxonomy, chloroplast ultrastructure)?

Paper IV

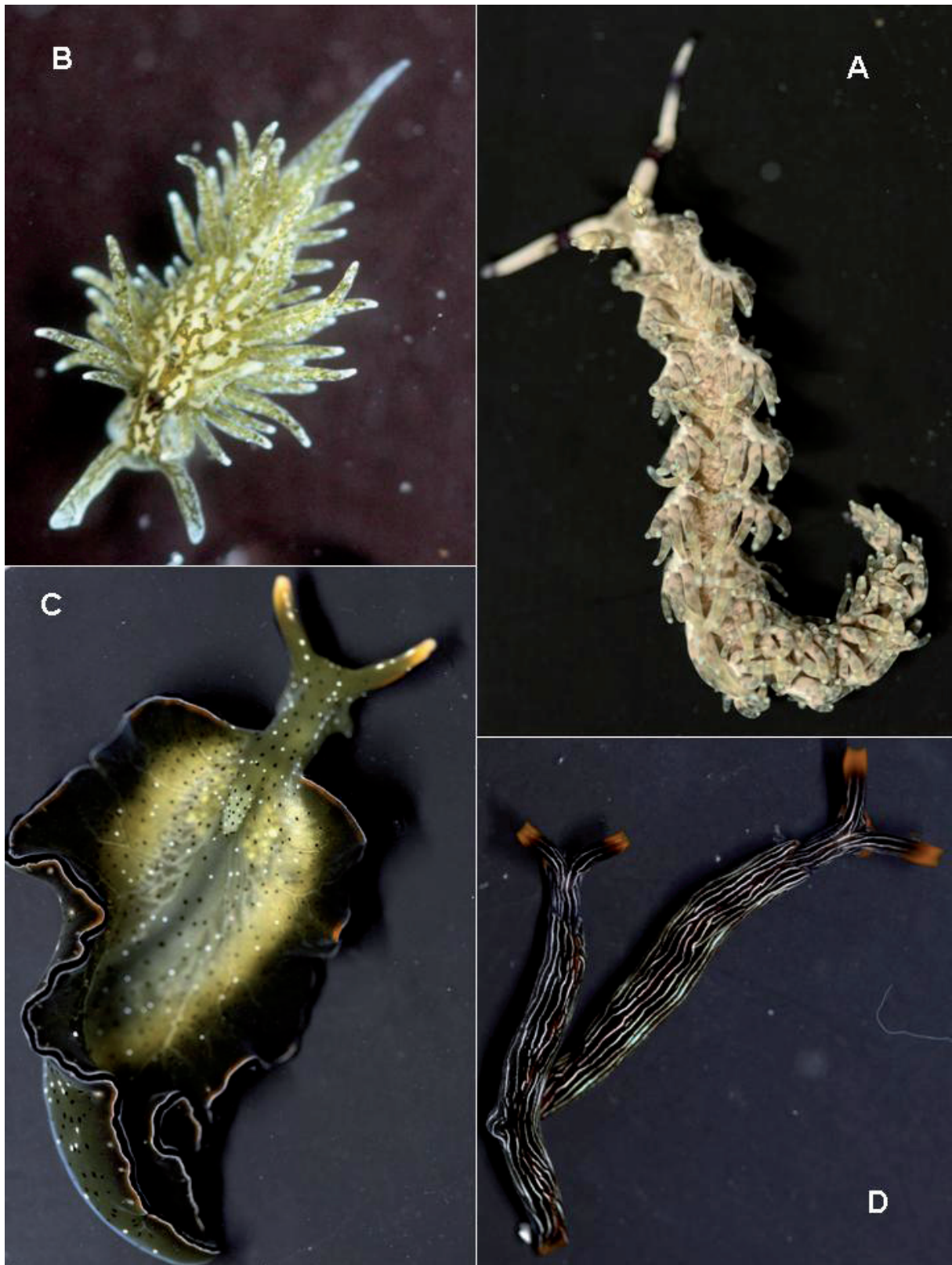


Figure 1. Solar powered opisthobranchs.

A. *Pteraeolidia ianthina* from Lizard Island, Great Barrier Reef, the most highly evolved nudibranch which hosts zooxanthellae. Specimen 60 mm in length.

B. *Placida dendritica* from Mausundvær in Norway, a limapontoid sacoglossan with cerata. This species does not retain functional chloroplasts. Specimen 15 mm in length.

C. *Elysia ornata* from Lizard Island, a plakobranchoid sacoglossan with unfolded parapodia. Specimen 50 mm in length.

D. *Thuridilla ratna* from Lizard Island, another plakobranchoid sacoglossan with convoluted parapodia. Specimens 20 mm in length. All photos by Geir Johnsen.

2. *In vivo* methods – Pulse Amplitude Modulated Fluorometry and chloroplasts

2.1 *In vivo* chl *a* fluorescence

The measurement of *in vivo* chl *a* fluorescence has been extensively applied to studies of phytoplankton physiology and growth in both laboratory and field setting (Owens 1991, Govindjee 1995). The apparent simplicity of conducting fluorescence measurements as a window to the physiological state of an algae providing information on pigment complexes, their excitation energy transfer among them and the energy transfer reactions in photosystem II (PSII) (Govindjee 1995), has brought this method into the realm of opisthobranchs using PAM (Wägele & Johnsen 2001, Paper I-IV).

In a functional chloroplast, or zooxanthellae, light is absorbed by the light harvesting antenna pigments, and the absorbed energy can take one of three possible pathways: energy can be accepted by reaction centres in PSII (RC_{PSII}) and be used to drive photosynthesis (photochemistry), secondly, excess energy can be dissipated as heat, or it can be re-emitted as chl *a* fluorescence. About 95% of *in vivo* fluorescence arises from PSII, the oxygen evolving site, and its corresponding light harvesting complexes (LHC_{PSII}) (Butler 1978, Johnsen et al 1997). These three processes are in constant competition, so that any increase in the efficiency in one will decrease the other (Equation 1). The quantum yield of chl *a* fluorescence (Φ_F), which is the number of photons emitted as fluorescence relative to the number of photons absorbed, is therefore directly related to the rate constants (*k*'s) of various pathways of de-excitation for fluorescence (F), photochemistry (P), and heat dissipation (H) (Govindjee 1995)

$$\Phi_F = k_F / (k_F + k_P + k_H) \quad (\text{Eq. 1})$$

When chloroplasts and zooxanthellae are functional, here defined as being able to perform photosynthesis, Φ_F is low because photochemistry efficiently uses electrons. When irradiances reach saturating levels, meaning that most RC_{PSII} are closed due to Q_A (primary quinone acceptor in PSII) being reduced, Φ_F may reach as high as 1-3%. When RC_{PSII} are saturated with high light or by adding DCMU, (dichlorophenylmethylurea, an electron transport inhibitor) does *in vivo* Φ_F go as high as 3%. If the chloroplasts are non-functional, here understood as digested by the sacoglossans, chl *a* and associated pigments will be

detached from their apo-proteins (*in vitro*), resulting in that PSII and the RC_{PSII} will be non-functional, and energy may be re-emitted up to 30% Φ_F (autofluorescence) and 70% heat (Figure 2).

If we want to investigate functional chloroplasts and zooxanthellae any further, we have to measure the kinetics of chl *a* fluorescence, here defined as a variable fluorescence effected from RC_{PSII}'s in an open state (ground fluorescence, F_0) to a closed state (maximum fluorescence, F_m) observed during illumination (Figure 3). This is called fluorescence induction, and has been widely used as a tool for studying photosynthetic processes in PSII (Krause & Weis 1991, Govindjee 1995). A PAM has the ability to measure the fluorescence kinetics before and after applying a saturating pulse of light that will close all functional RC_{PSII}. The duration of the saturation pulse (0.8 seconds used in our setup) is long enough to close all RC_{PSII}, but short enough to avoid chl *a* fluorescence quenching processes. To measure the photosynthetic electron transfer rate in functional chloroplasts, the maximum quantum yield of chl *a* fluorescence from PSII (Φ_{PSII} , Eq. 2) can be acquired from dark acclimated chloroplasts (this means that a maximum number of RC_{PSII} are open (oxidised) and ready to process photons) by measuring F_0 before and F_m during a saturating pulse of strong light ($>10\ 000\ \mu\text{mol photons m}^{-2}\ \text{s}^{-1}$) which closes (reduces) all RC_{PSII} with photons (Q_A is reduced, see Figure 2). The operational quantum yield of chl *a* fluorescence (Φ_{PSII}' , Eq. 3) at actinic (inducing photosynthesis) irradiances is likewise measured before and after the application of a saturation pulse (Figure 3). The quantum yield of PSII ($\text{mol e}^- \text{mol photons}^{-1}$, either maximum or operational) therefore approaches zero if all RC_{PSII} are non-functional (Butler 1978, Dau 1994).

Chloroplasts/zooxanthellae acclimated in darkness:

$$\begin{aligned} \text{Maximum quantum yield of chl } a \text{ fluorescence} &= \Phi_{PSII} \text{ (mol e}^- \text{ mol photons}^{-1}\text{)} \\ &= F_m - F_0 / F_m \end{aligned} \tag{Eq. 2}$$

Chloroplasts/zooxanthellae acclimated in actinic light conditions:

$$\begin{aligned} \text{Operational quantum yield of chl } a \text{ fluorescence} &= \Phi_{PSII}' \text{ (mol e}^- \text{ mol photons}^{-1}\text{)} \\ &= F_m' - F_0' / F_m' \end{aligned} \tag{Eq. 3}$$

Equations 2 and 3 can thus be used to detect photosynthetic activity in chloroplasts and zooxanthellae (Paper I-IV). Further, Φ_{PSII} as a function of time, can be used to measure the

lifespan of functional chloroplasts and zooxanthellae (Paper I and Chapter 2.2), and Φ_{PSII} as function of irradiance can reveal changes in photosynthetic parameters (Paper I and Chapter 2.3).

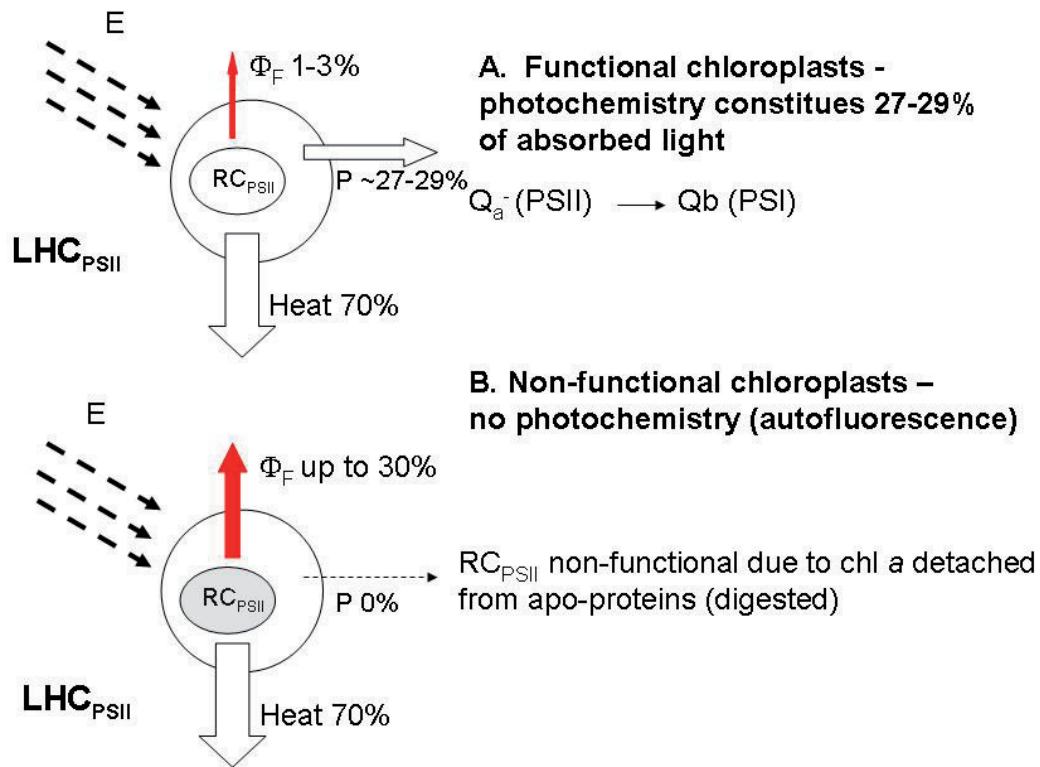


Figure 2. *In vivo* chl *a* fluorescence (Φ_F).

A. In functional chloroplasts or zooxanthellae which are able to do photosynthesis (P), ~27-29% of the absorbed light may be used in the photochemical process, while approximately 70% is lost as heat (thermal decay), and about 1-3% is emitted as chl *a* fluorescence, of which ~95% originate from PSII.

B. In non-functional chloroplasts or zooxanthellae, which are destroyed by the sacoglossans digestion, RC_{PSII}'s are non-functional due to chl *a* and other pigments being detached from its apo-proteins or digested, and Φ_F may increase up to 30%, resulting in autofluorescence.

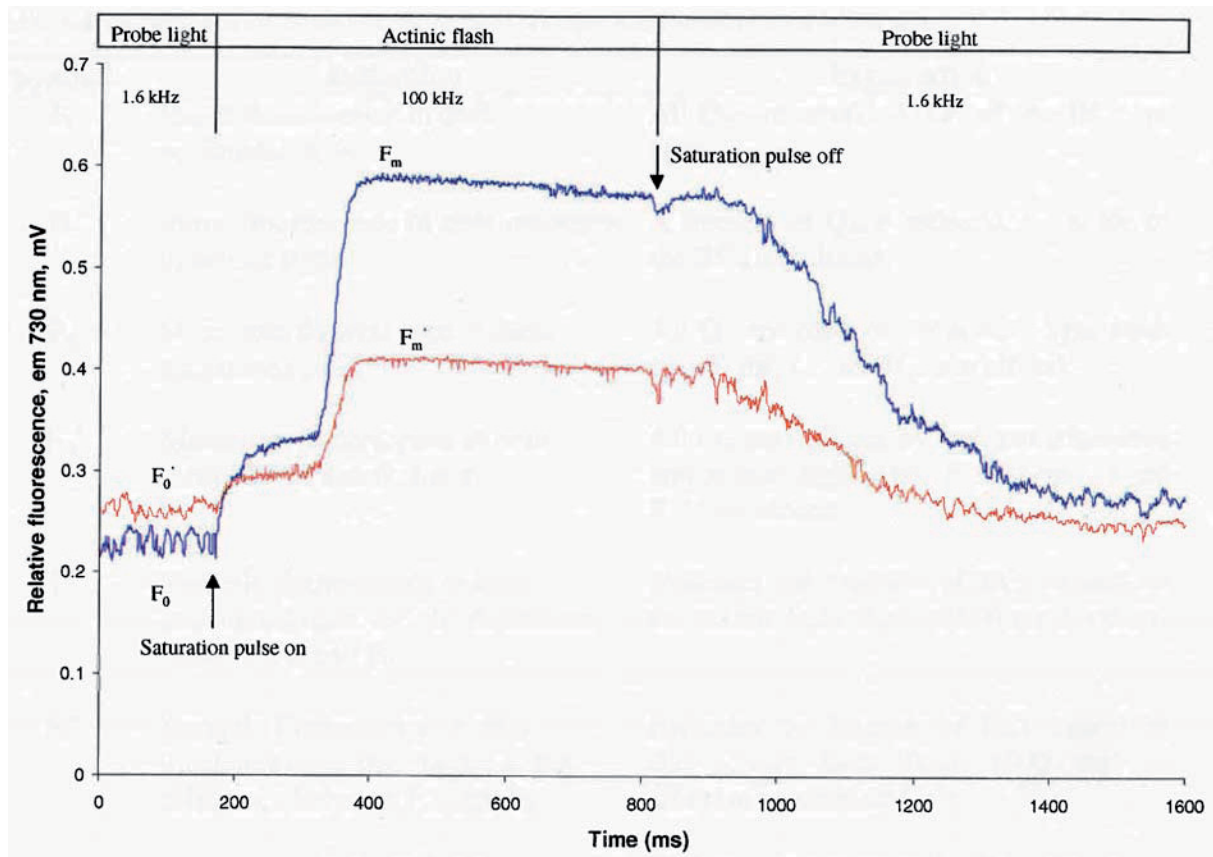


Figure 3. A PAM fluorescence induction curve as measured in chloroplasts or zooxanthellae. Chloroplasts incubated for 15 minutes in darkness have all functional RC_{PSII} open, and the probe light of the PAM measures the initial fluorescence (F_0). After applying a saturating flash saturating all RC_{PSII} ($\sim 10\,000\ \mu\text{mol photons m}^{-2}\ \text{s}^{-1}$ for 0.8 seconds) the maximum fluorescence is obtained (F_m), and the quantum yield of chl a fluorescence (Φ_{PSII} , see Eq. 2) can be estimated. Under actinic irradiance levels, where the chloroplasts in this example have been incubated at $50\ \mu\text{mol photons m}^{-2}\ \text{s}^{-1}$ for 5 minutes, the F_0 signal will rise to F_0' level and approach F_m' when all RC_{PSII} are saturated with photons, used to calculate an operational quantum yield of chl a fluorescence Φ_{PSII}' (Eq. 3) which always is lower than Φ_{PSII} . Induction curve copied with permission from Johansen (2002).

2.2 Photosynthetic activity of chloroplasts in sacoglossans as a function of time

The simplest way to characterise the functionality of chloroplasts or zooxanthellae in the digestive cells of opisthobranchs, is to estimate their photosynthetic lifespan. The functional state of incorporated chloroplasts or zooxanthellae and their survival time in the digestive cells of opisthobranchs has previously been measured by methods such as ^{14}C incorporation, chl *a* concentration and oxygen evolution measurements (references in Paper I and II). However, chl *a* concentration does not indicate chloroplast or zooxanthellae functionality, because the presence of chl *a* does not discriminate between photosynthetically active chl *a* or chl *a* detached from apo-proteins in LHC_{PSII} or digested algal material resulting in autofluorescence from chl *a* (Figure 2). Likewise the use of oxygen electrodes and ^{14}C incorporation are highly affected by respiration from the slug, together with the presence of chloroplasts, mitochondria, and microbes (Falkowski & Raven 1997). Instead, it is more practical to ascribe chloroplast and zooxanthellae functionality to photosynthesis and the vitality of PSII measured by a PAM, which is relatively insensitive to respiration (Hancke et al 2008). If photosynthetic chloroplasts or zooxanthellae are present, the slugs ability to retain functional (photosynthetic) chloroplasts or zooxanthellae can be estimated as photosynthetic activity as a function of time. This can be achieved by measuring Φ_{PSII} in slugs kept under starving conditions (no new chloroplasts are introduced from food algae) over a period of days to months (Paper I and II). For sacoglossans, the Retention ability of Functional Chloroplasts is defined as the RFC, which is estimated by plotting Φ_{PSII} as a function of time designating the lifespan of the chloroplasts as a number of days (Paper II, Figure 4 in this study). The daily decrease in Φ_{PSII} can be estimated from a regression line fitted to a plot of Φ_{PSII} as a function of time. By dividing the initial Φ_{PSII} (measured before any sacoglossans are put under starving conditions) by the daily decrease of Φ_{PSII} (estimated from the slope of the regression line) the RFC is expressed as a number of days (Paper II):

$$\text{RFC (days)} = \text{initial } \Phi_{\text{PSII}} / \text{decrease in } \Phi_{\text{PSII}} \text{ day}^{-1} \quad (\text{Eq. 4})$$

A decline in Φ_{PSII} as a function of time will occur because some RC_{PSII} become photosynthetically incompetent due either to inefficient excitation transfer from LHC to RC_{PSII} , impairment of primary photochemistry, or disruption of electron transport between Q_A and the plastoquinon pool (Kolber and Falkowski 1993). Down regulation and closure of

RC_{PSII} is firstly attributed to chloroplasts not being able to repair themselves (they gradually degrade from age), and secondly from photodamage (here understood as the light induced damage resulting in turnover of the D1 protein subunit of the reaction centre of PSII when the rate of photon absorption by PSII antenna exceeds the use of the absorbed energy in photosynthesis (Demmig & Björkman 1987)). All chloroplasts that perform oxygenic photosynthesis experience photodamage to PSII under strong light (Adir et al. 2003, Mohanty et al 2007). Chloroplasts in a normal algal cell environment are able to repair PSII after photodamage (e.g. D1 repair, see Chapter 2.3). But, since chloroplasts in the digestive cells of sacoglossans are not able to neither divide nor synthesise pigments, lipids or membrane proteins (Paper III), the decrease of Φ_{PSII} as a function of time will be a result of a balance between chloroplasts degrading from age, photoacclimation status, and photodamage to PSII modified by key environmental variables (e.g. irradiance and temperature). It must be pointed out that sacoglossans studied in laboratory conditions with stable light and temperature conditions, have shown surprisingly long RFC values. This is explicit for *Elysia timida* and *Elysia viridis*, both kept under low light conditions not inducing high levels of photodamage (Paper II and III).

For the nudibranchs, which retain zooxanthellae, Φ_{PSII} was used as an indication of the ability of the zooxanthellae to renew their numbers in the digestive system of the slugs (Paper I). Φ_{PSII} as a function of time in starving nudibranchs, can be investigated with a period of darkness; zooxanthellae under dark conditions switch to a heterotrophic state which is a burden to the nudibranch. The dark period therefore leads to a reduction in Φ_{PSII} as a function of time, because the slug has started to digest its zooxanthellae. If the zooxanthellae are able to recover when brought back into light, the Φ_{PSII} should increase as a function of time.

Observations on the RFC in different sacoglossans in this thesis can be summed up in Figure 4. Based on Clark et al (1990) and the results in Paper II-IV, our knowledge of the lifespan of chloroplasts in sacoglossans can be classified into eight different levels (Table 1). Level 1–3 represents sacoglossans with non-functional chloroplasts which are digested when they are phagocytosed into the digestive cells of the sacoglossans. As shown for *Placida dendritica* in Paper III, no intact chloroplasts are observed after ingestion, but several degradation stages of chloroplasts under digestion are present (Chapter 3.2), and this species can be classified under level 3. When measured with a PAM, only autofluorescence will be detected (Figure 1, level 1-3 in Table 1), or as in *Ercolania kencolesi*, the Φ_{PSII} signal may be present for some time

(days) but at such low signals it can not be attributed to photosynthesis but indicating digested chloroplasts (see also Chapter 3.2). Level 4–8 represents sacoglossans with functional chloroplasts. Intact chloroplasts can be observed surrounded by the phagosome, and the initial Φ_{PSII} is usually the same as for the food algae (Paper III). Level 4 comprises sacoglossans where the chloroplasts remain photosynthetically active for less than one day, and when measured with a PAM, the chloroplasts will become non-functional within 24 h ($\Phi_{\text{PSII}} \sim 0$). Level 5, 6, 7 and 8 comprises sacoglossans retaining functional chloroplasts from 1-7 days, from 7-30 days, from 30-90 days and for more than 90 days respectively. Most species investigated in this study belong to level 5 and 6 (Figure 4). Only three species are observed to retain functional chloroplast for more than three months (Level 8); *E. viridis*, *Plakobranthus ocellatus* and *Elysia chlorotica* (Table 1). However, *E. viridis* has varying RFC's because light- and temperature conditions in different experiments vary greatly (see references in Table 1). *P. ocellatus* retains functional chloroplasts from the chlorophyte *Caulerpa* (Paper IV), and *E. chlorotica* retains functional chloroplasts from the chromophyte *Vaucheria* (Rumpho et al 2006). Because these two latter sacoglossans retain chloroplasts from so different algal groups (see Table 2), it is difficult to compare the functionality of the chloroplasts. Since the decrease of [chl *a*] as a function of time in sacoglossans, which does not separate functional chl *a* from chl *a* detached from its apo-proteins, is used to estimate the RFC in *Elysia hedgepethi* and *Plakobranthus ianthobapsus* (Greene 1970), and *Elysia cause*, *Elysia tuca*, *Oxynoe antillabrum*, and *Tridachia crispata* (Clark & Busacca 1978) in Table 1, these results must be considered cautiously.

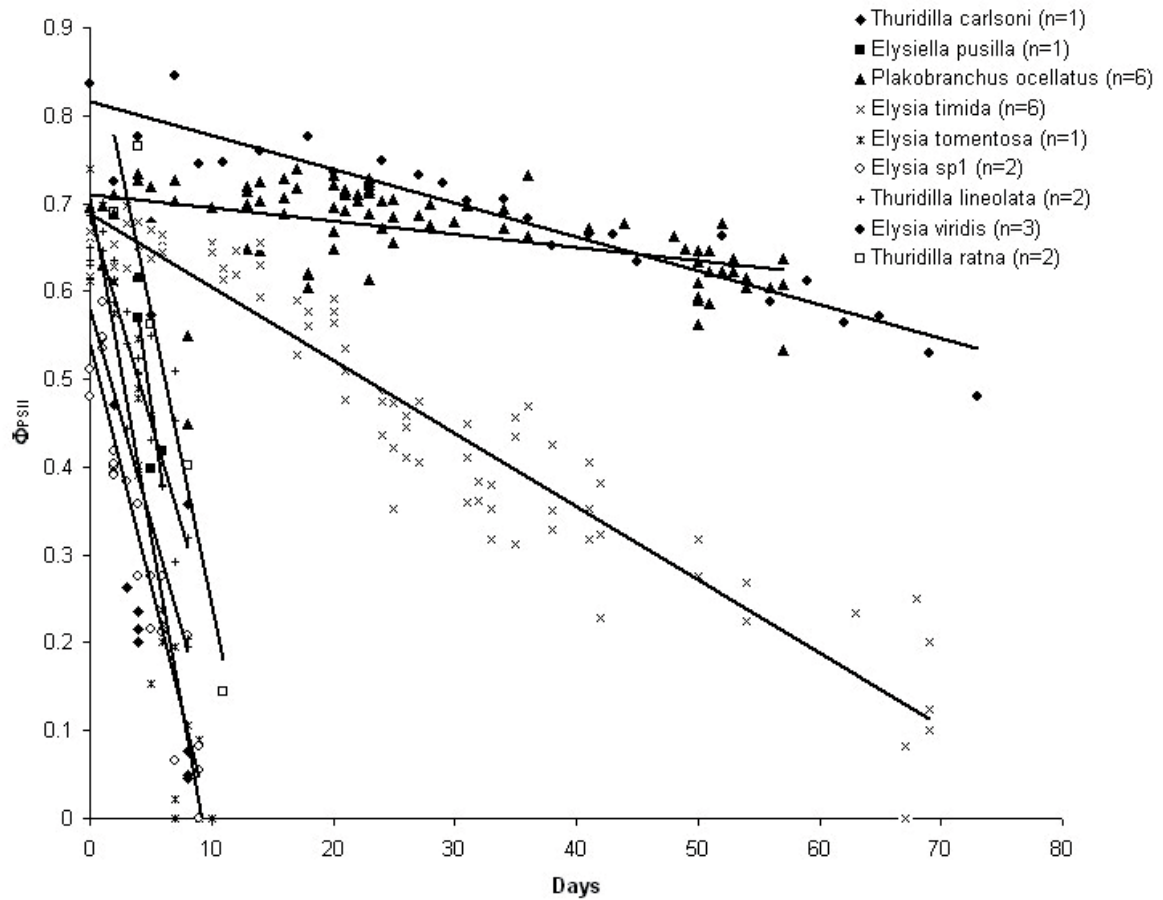


Figure 4. The decrease of Φ_{PSII} as a function of time (RFC, Eq. 4) for nine sacoglossans, obtained from Norway (*E. viridis*), the Mediterranean (*E. timida*), Indonesia (*E. tomentosa*) and Australia (the remaining species) used in this thesis (Paper II-IV). Their respective RFC numbers are given in Table 1. The regression lines are estimated from the plots by the least square method. The point of intersection of the regression line on the y-axis (Φ_{PSII}) designates the initial Φ_{PSII} which divided by the slope of the regression line (the daily decrease of Φ_{PSII}) gives RFC as a function of days. n denotes number of specimens examined.

Table 1. The new classification scheme of retention abilities of functional chloroplasts in sacoglossans (RFC) as suggested in Paper II, and further summed up from Paper III and IV in this study. The table indicates that the majority of sacoglossans retaining functional (photosynthetic) chloroplasts belong to the Plakobranchoidea. $\mu\text{g chl a/g fw}$ denotes the concentration of chl *a* per gram fresh weight of slug.

Sacoglossan	RFC	Method	Reference	Level	Functionality	Time
<i>Ascobulla ulla</i>		14C	Clark et al (1990)	1	Direct digestion	0
<i>Elysia catulus</i>		14C	Clark et al (1990)	2	Non-functional	<2 h
<i>Elysia evelinae</i>		14C	Clark et al (1990)			
<i>Ercolania coerulea</i>		14C	Clark et al (1990)			
<i>Placida dendritica</i>		PAM	Paper III	3	Non-functional	>24 h
<i>Ercolania kencelesi</i>		PAM	Grzymbovski et al (2007)			
<i>Alderia modesta</i>	<1	14C	Clark et al (1990)	4	Functional	<24 h
<i>Hermaea cruciata</i>	<1	14C	Clark et al (1990)			
<i>Elysiella pusilla</i>	2	PAM	Evertsen (2006)	5	Functional	1-7 days
<i>Mourgona germainea</i>	3-7	14C	Clark et al (1990)			
<i>Caliphylla mediterranea</i>	7	14C	Clark et al (1990)			
<i>Bosellia mimetica</i>	>7	14C	Clark et al (1990)			
<i>Elysia ornata</i>	8-18	PAM	Paper IV	6	Functional	1-4 weeks
<i>Elysiella pusilla</i>	10	PAM	Paper II			
<i>Elysia sp.1</i>	10	PAM	Paper II			
<i>Elysia hedgepethi</i>	10	14C	Greene (1970)			
<i>Elysia hedgepethi</i>	11	$\mu\text{g chl a/g fw}$	Greene (1970)			
<i>Elysia tomentosa</i>	10	PAM	Paper II			
<i>Thuridilla carlsoni</i>	10	PAM	Paper II			
<i>Elysia thompsoni</i>	11	PAM	Evertsen (2006)			
<i>Thuridilla undula</i>	12	PAM	Evertsen (2006)			
<i>Thuridilla ratna</i>	12-14	PAM	Paper IV			
<i>Thuridilla lineolata</i>	15	PAM	Paper II			
<i>Elysia cauze</i>	23	$\mu\text{g chl a/g fw}$	Clark & Busacca (1978)			
<i>Elysia sp.2</i>	25	PAM	Evertsen (2006)			
<i>Elysia tuca</i>	29	$\mu\text{g chl a/g fw}$	Clark & Busacca (1978)			
<i>Oxynoe antillabrum</i>	36	$\mu\text{g chl a/g fw}$	Clark & Busacca (1978)	7	Functional	1-3 months
<i>Elysia viridis</i>	42	14C	Hinde & Smith (1972)			
<i>Plakobranchus ianthobapsus</i>	43	14C	Greene (1970)			
<i>Elysia timida</i>	45	14C	Marin & Ros (1992)			
<i>Elysia australis</i>	~56	14C	Hinde (1980)			
<i>Elysia viridis</i>	62-87	14C	Hinde & Smith (1975)			
<i>Costasiella lilliana</i>	65	14C	Clark et al (1981)			
<i>Elysia timida</i>	80	PAM	Paper II			
<i>Elysia viridis</i>	~84	14C	Trench et al (1973b)			
<i>Tridachia crispata</i>	84	$\mu\text{g chl a/g fw}$	Clark & Busacca (1978)			
<i>Plakobranchus ianthobapsus</i>	108	$\mu\text{g chl a/g fw}$	Greene (1970)	8	Functional	>3 months
<i>Elysia viridis</i>	55-273	PAM	Paper III			
<i>Plakobranchus ocellatus</i>	97-277	PAM	Paper IV			
<i>Elysia chlorotica</i>	280	O2	Paper IV			
<i>Plakobranchus ocellatus</i>	335	PAM	Paper II			

2.3 Photosynthetic activity of chloroplast in sacoglossans as a function of photoacclimation

When irradiance increases, the photosynthetic activity in PSII in terms of generating electrons in light (Φ_{PSII}), decreases until the RC_{PSII} start to be light-saturated; no matter how much higher the irradiance gets, the photosynthetic activity can not work any more efficiently. At low irradiances, below the light saturation parameter E_k (see below), the photosynthesis response to irradiance is light limited and the photosynthetic activity is linearly proportional to irradiance. As irradiance increases ($>E_k$), the photosynthetic activity becomes increasingly non-linear until reaching saturation. Φ_{PSII} (Eq. 3) as function of the incident irradiance (E , $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) is therefore defined as the relative electron transfer rate ($\text{ETR} = \Phi_{\text{PSII}} * E = (\text{mol e}^- \text{mol photons}^{-1}) \mu\text{mol photons m}^{-2} \text{s}^{-1}$). Since ETR here denotes how much electrons are generated relative to the amount of photons absorbed as a function of the incident irradiance, ETR can be used to generate photosynthesis versus irradiance curves (P vs. E). A commonly used model for the relationship between photosynthetic activity (P) and irradiance (E) is provided by Webb et al. (1974)

$$P(\text{ETR}) = P_{\text{max}}(1 - \exp(-\alpha * E/P_{\text{max}})) \quad (\text{Eq. 5})$$

The relationship between photosynthesis and irradiance demonstrates a predictable shape with the P vs. E curve. The photosynthetic parameter α is denoted as the light utilisation coefficient (Sakshaug et al 1997) and is related to Φ_{PSII} multiplied by photons absorbed by PSII ($\alpha = \Phi_{\text{PSII}} * \bar{a}_{\text{PSII}} (\text{m}^{-2} \text{mg chl } a^{-1})$). This means that at the light limited part of the P vs. curve, photosynthetic (ETR) response is proportional to irradiance. Ultimately, photosynthesis becomes light-saturated (P_{max}) and stays the same despite higher irradiances. P_{max} denotes the maximum photosynthetic rate, and can be described as the ratio of Φ_{PSII} divided by the concentration of chl a per photosynthetic unit (q) and the minimum turnover time for processing photons (τ) ($P_{\text{max}} = \Phi_{\text{PSII}} / q \tau$) (Dubinsky et al 1986, Sakshaug et al 1997). E_k is the ratio between P_{max}/α and is called the light saturation parameter ($\mu\text{mol photons m}^{-2} \text{s}^{-1}$) (Kroon et al. 1993, Sakshaug et al. 1997). Photoacclimation is the algal response to variations in light regime, and chloroplasts can photoacclimate by changing the composition of photosynthetic and photoprotective pigments, adjusting photosynthetic parameters, changing enzymatic activities involved in photosynthesis and respiration, and by changing cell volume, respiration rates and chemical composition (Johnsen & Sakshaug 1993). Low light (LL)

acclimated chloroplasts have more light harvesting pigments (Chapter 3.1), an α up to 5 times higher than HL acclimated chloroplasts. Paper III indicates that when sacoglossans are allowed to ingest and retain functional chloroplasts from food algae kept at the same LL and temperature conditions, Φ_{PSII} and α remain the same as for the food algae, but P_{max} and therefore also E_k increases 2-6 fold. Since both α and P_{max} comprise Φ_{PSII} , the former parameter can not explain the increase in P_{max} and E_k . Since the pigment composition remains unchanged (Chapter 3.1, Paper III), α and q should not either change. The increase in E_k may therefore be attributed to an increase of τ , leading to a decreased turnover time of e^- , indicating that chloroplasts in the digestive cells of sacoglossans experience higher irradiances than in the food algae (see also Chapter 3.3). This indicates that chloroplasts in the digestive cells of sacoglossans may have a partially arrested photoacclimation status; they can not divide nor synthesise new pigments, but may partially be able to adjust their turnover time of electrons to higher irradiance levels.

The limited functionality of the chloroplasts in the digestive cells of sacoglossans is further demonstrated when Φ_{PSII} , α and P_{max} are monitored as a function of time under sacoglossan starving conditions (no fresh chloroplasts are ingested). Here, Φ_{PSII} , α and P_{max} decreased as a function of time similarly (Paper III) indicating a reduction in the number of functional RC_{PSII} of the PSU. A lack of functional D1 proteins in PSII, is related to a decrease in Φ_{PSII} when the PSU (mainly the D1 repair cycle) is not repaired (Björkmann & Demmig 1987, Vasilikiotis & Melis 1994). The corresponding decrease in α and P_{max} support the observations that the chloroplasts can not synthesise pigments, lipids or membrane proteins (Paper III and references therein). This indicates that chloroplasts in the digestive cells of sacoglossans will age (Figure 4, Table 1).

How fast the chloroplasts degrade in the sacoglossans, is linked to the chloroplasts aging as pointed out in Chapter 2.2 and above, and to the photoacclimation status of the chloroplasts, and photodamage to PSII. Photodamage may occur at daytime and is closely related to the requirement to repair PSII, which is linked to the D1 protein. The D1 protein has a rapid turnover *in vivo*, and this turnover increases with increasing light intensity (Baker & Bowyer 1994). This implies that if the chloroplasts in the digestive cells of the sacoglossans have a limited functionality, recovery of PSII activity will be lowered if the chloroplasts are kept at high irradiances exceeding their E_k .

Plakobranchnus ocellatus is well adapted to retaining functional chloroplasts. This species is found burying in shallow sand flats, and this burying habit could be one way to regulate the light conditions its chloroplasts are exposed to at high irradiances. This may explain the high RFC values up to 10 months observed for this sacoglossan (Paper II, Paper IV). In most cases, LL acclimated chloroplasts are found in this slug (Figure 5), which indicates that this sacoglossan mostly feed on shade acclimated algae. But staying in low light conditions may result in low photosynthetic activity. The observed solution to this problem is to let the digestive gland tubules branch off throughout the slug body, or aggregate them dorsally, bringing more light to the chloroplasts and increasing the E_k in the chloroplasts in sacoglossans (Chapter 3.2, Paper II). Plakobranchoid sacoglossans also have the option to unfold their parapodia (Figure 1c) in low light conditions, thus supplying the chloroplasts with more light. Sacoglossans with HL acclimated chloroplasts (as exemplified by *Thuridilla ratna* in Figure 6), can tolerate a wider range of irradiances. However, exposing the retained chloroplasts to high irradiances will only increase the rate at which D1 is depleted, resulting in a decrease of RFC. This can also be exemplified by *Elysia ornata*, which has been found in a wide variety of habitats and with both LL and HL acclimated chloroplasts. The result is RFC values varying from 1-2 weeks (Paper IV). As seen in Figure 4 in this study, most sacoglossans seem to not retain functional chloroplasts for more than two weeks, and it might be that most sacoglossans are opportunistic, here understood as having the ability to feed on a wide range of algal food from a wide range of habitats.

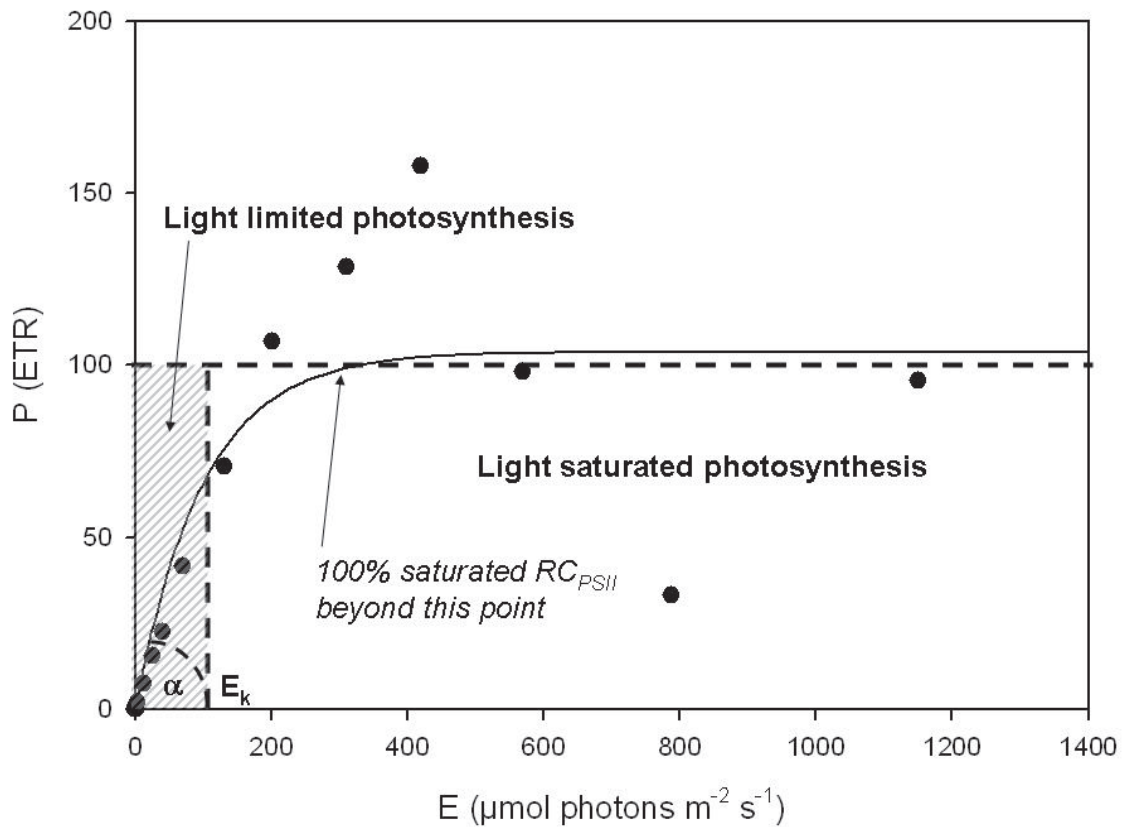


Figure 5. Low light acclimated chloroplasts in *Plakobranchnus ocellatus* from Lizard Island (Paper IV). The P vs. E plot gives an E_k of $90 \mu\text{mol photons m}^{-2} \text{s}^{-1}$, indicating low light acclimated chloroplasts. The shaded area denotes light limited and the region of linear photosynthesis (at irradiances lower than E_k). At P_{max} , most functional RC_{PSII} are saturated (light saturated photosynthesis). For a sacoglossan, retaining LL acclimated chloroplasts, exposing its retained functional chloroplasts to irradiances exceeding E_k may increase the decay rate of D1-protein of PSII, and RFC may be reduced.

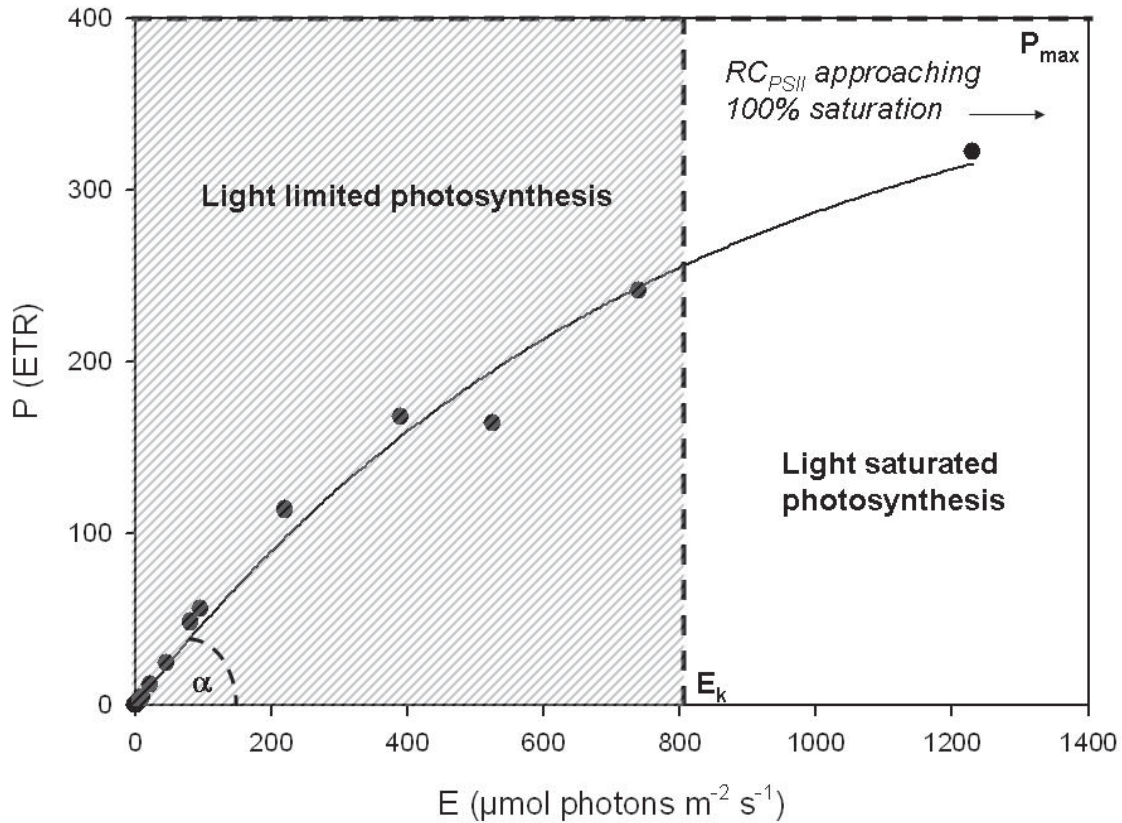


Figure 6. High light acclimated chloroplasts in *Thuridilla ratna* from Lizard Island (Paper IV). The P vs. E curves gives an E_k of $800 \mu\text{mol photons m}^{-2} \text{s}^{-1}$, indicating high light acclimated chloroplasts. The shaded area indicates that sacoglossans with HL acclimated chloroplasts are acclimated to a wider range of irradiances than LL acclimated chloroplasts. However, photosynthesis needs high irradiances to reach saturation and a high photosynthetic activity. The decay rate of functional D1-protein in PSII will also be higher at higher irradiances.

Summary *in vivo* methods

The Retention ability to retain functional chloroplasts in the digestive cells of sacoglossans can be expressed as RFC. RFC is Φ_{PSII} as a function of time in starved slugs indicating the lifespan of the chloroplasts in the digestive cells of the sacoglossans. RFC in sacoglossans can be classified into eight different levels (Table 1), ranging from non-functional chloroplasts being digested (Level 1-3) to functional chloroplasts being retained in a functional state from days to more than three months (Level 4-8). Results indicate that the majority of sacoglossans retaining functional chloroplasts are plakobranchoids.

The functionality of chloroplasts can not only be expressed as RFC, but also from the chloroplasts ability to photoacclimate. When chloroplasts are removed from their food algae and retained in a functional (photosynthetic) state in the digestive cells of sacoglossans, they can not divide nor synthesise any new pigments. But, the only indication of a partial photoacclimation in the chloroplasts is the increase in P_{max} and thus E_k , as a result of the chloroplasts speeding up the processing of e^- at higher irradiances.

When sacoglossans with functional chloroplasts are kept under starving conditions, the photosynthetic parameters Φ_{PSII} , P_{max} and α decline at similar rates as a function of time. This indicates that the chloroplasts are not able to repair themselves, but degrade as a function of age. How fast the photosynthetic chloroplasts degrade depends on the chloroplasts photoacclimation status (LL or HL), and the rate of photodamage they are exposed to by the slug.

3. In vitro methods – pigment composition, chemotaxonomy and histology

3.1 Pigment composition in the chloroplasts

As pointed out in chapter 2.3, chloroplasts in the digestive cells of sacoglossans do not divide, and their ability to photoacclimate is partially arrested because they can not synthesise any new pigments. Therefore, the pigment composition observed in the chloroplasts found in the sacoglossans should more or less reflect the pigment composition and photoacclimation status of the food algae (Paper III and IV).

Green algal chloroplasts from *Codium fragile* contain only chl *a*, chl *b*, siphonaxanthin, siphonein, violaxanthin, neoxanthin and β, ϵ -carotene (Paper III). The evidence that any new pigments are not synthesised by these chloroplasts when retained in the digestive cells of sacoglossans, is seen when they are retained by *Elysia viridis* in Paper III. Here the ratio of chl *a*: *b* is the same (2.2) for LL acclimated chloroplasts in *Codium* as for the same LL acclimated chloroplasts retained in the slug, and the ratio of the different carotenoids also remains the same. However, when slugs are allowed to feed, there is usually a higher turnover of chloroplasts by the slug (up to 75% during 9 days according to Gallop et al 1980), and there is less chl *a* and chl *b* in the slugs chloroplasts (μg pigment per gram slug) than in chloroplasts from *Codium*, because some of the chl *a* in the slugs is degraded (12%). This gives an indication that chloroplasts in sacoglossans are not able to synthesise any new pigments, even though E_k in the chloroplasts increased 2-6 fold as a result of branching of the digestive gland tubules (see Chapter 2.3 and 3.3). In sacoglossans not able to retain functional chloroplasts (e.g. the limapontoid *Placida dendritica*, Figure 1b in this study, Paper III), the pigment composition includes degraded chl *a* (40%), and the carotenoid neoxanthin is absent. However, the total μg pigment per gram slug in *P. dendritica* remains similar to the total μg pigment per gram *Codium*, indicating that pigments have only been altered. This may explain the bright green colour in these slugs even though they do not retain functional chloroplasts.

The pigment composition observed in sacoglossans collected *in situ* seems to be indicative of chloroplasts retained from different food algae (Paper IV). *Plakobranthus ocellatus* had a ratio of chl *a* : siphonaxanthin+siphonein that was up to 5 times higher than in *Elysia ornata* and *Thuridilla ratna*, but a chl *a*: violaxanthin ratio that was 2 times lower than in *E. ornata*

and *T. ratna*. These differences can be explained in the presence of lutein in *P. ocellatus*, which was not present in the other two sacoglossans (Paper IV). It has been suggested that in the chlorophyte *Caulerpa*, an interconversion between lutein and siphonaxanthin replaces the violaxanthin-antheraxanthin-zeaxanthin cycle. The presence of chloroplasts of *Caulerpa* in *P. ocellatus* is validated in Chapter 3.4.

One way to assess the photoacclimation status in the chloroplasts is to estimate E_k , but significant differences in ratios of pigment relative to chl *a*, can also be indicative of photoacclimation (Johnsen & Sakshaug 1993, Rodriguez et al. 2006). Coenocytic and siphonous chlorophytes have lower chl *a*: *b* ratios than other algae and higher plants (Yamazaki et al 2005). The chl *a*: *b* ratios in e.g. seagrasses lie between 3 to 5 (Keast & Grant 1976). All slugs in this study (Paper III and IV) retain coenocytic and siphonous chlorophytes (siphonaxanthin and siphonein is present in all, see Chapter 3.2), and the chl *a*: *b* ratios vary from 1.2-3.2. The lower chl *a*: *b* ratio is the result of marine green algae being more abundant in chl *b*, which absorbs blue-green light more efficiently (Anderson 1983), and further more that coenocytic and siphonous chlorophytes also have two unique carotenoids, siphonaxanthin and siphonein, which have main absorption bands between 500 and 550 nm making them efficient photosynthetic pigments specifically capturing blue-green light (Kageyama & Yokohama 1978). However, the chl *a*: *b* ratios did not correspond with the observed E_k values. *P. ocellatus* and *E. ornata* retaining LL acclimated chloroplasts had chl *a*: *b* ratios from 2.4-3.2 (w:w), whereas *Thuridilla ratna* and *E. ornata* retaining HL acclimated chloroplasts had chl *a*: *b* ratios varying from 1.2-2.3 (w:w) (Paper IV). LL acclimated chloroplasts should have a lower chl *a*: *b* ratios than HL acclimated chloroplasts (Anderson et al 1973). In fact, Keast & Grant (1976) observed little consistency in the ratio between chl *a* and chl *b* in a number of coenocytic and siphonous Ulvophyceae kept in LL and HL conditions, and suggested that the chl *a*: *b* ratios are characteristic of the algal species themselves. For the sacoglossans this implies that variations in chl *a*: *b* ratios indicate chloroplasts from different species of algae (see Chapter 3.3). It has been suggested this rigidity in pigment composition reflects the habit of green macroalgae to use chloroplast movement in response to daily changes in light intensity (Takagi 2003, Yamazaki et al 2005).

3.2. Chemotaxonomy

Pigments of marine macro- and microalgae comprise approximately 10 major chlorophylls (chl *a*, chl *b* and divinyl *a* and *b*, and the chl *c* group), >30 different major carotenoids (carotenes and xanthophylls), and three major phycobiliprotein groups (allophycocyanins, phycocyanins, and phycoerythrins) (Rowan 1989, Jeffrey et al 1997). In living cells, the pigments are bonded to apo-proteins, together forming a variety of pigment–protein complexes (Rowan 1989, Johnsen & Sakshaug 2007). Chl *a* and *b*, together with photosynthetic carotenoids, are the major light harvesting pigments in green algae and higher plants, chl *a* and phycobiliproteins in rhodophytes and chl *a*, *c* and phycobiliproteins in chromists (Table 2). This reflects the evolution of a green line (chl *b*) bringing about the green algae and higher plants, and the red line evolving into chromists (chl *c*) via rhodophytes and phycobilins (Falkowski et al 2004). In this study (Paper III and IV) all chloroplasts found to be functional in the digestive cells of the sacoglossans, belonged to the green algae (they contained chl *a* and chl *b* and β, ϵ -carotene), and they all also contained the photosynthetic carotenoids siphonaxanthin and siphonein. In the green algae, it is the coenocytic and siphonous algae of the Bryopsidales which are unique in having LHC containing siphonaxanthin and its esterified form siphonein (Yokohama et al 1992). In this regard, all sacoglossans observed retaining functional chloroplasts, except for three species, have obtained their chloroplasts from this algal group (Jensen 1993). The exception is *Elysia timida* from the Mediterranean retaining chloroplasts from the dasycladalean *Acetabularia*, *Elysia chlorotica* retaining chloroplasts from the chromist *Vaucheria* (cf. Table 1 for their respective RFC's and references), and the limapontoid *Hermaea bifida* which has been observed to retain chloroplasts from the rhodophyte *Griffithsia* (Kremer & Schmitz 1976). However, the latter association must be considered carefully since the ^{14}C assimilation rate in the algae was estimated to be a thousand times higher than in the slug. In this regard, observations on another limapontoid sacoglossan, *Ercolania kencolesi*, feeding on the dasycladalean *Boergesenia*, showed Φ_{PSII} values varying from 0.2-0.1 mol e^- mol photons $^{-1}$ for 10-15 days, with F_0 values subsequently measured at below 150 mV for all days (Grzymbovski et al 2007). However, such low F_0 values are in the range of what is considered too low for the signal to noise ratio of the diving-PAM (Schreiber 1986). Other food-sources exploited by the sacoglossan which are not known to be a source of functional chloroplasts, include other chlorophytes from Cladophorales and Ulvales, and the marine magnoliophyte *Zostera*, which has been suggested to be in the diet of *Elysia catulus* (Jensen 1980). Also, a chain-forming

diatom, the bacillariophyte diatom *Biddulphia*, has been observed to be the food of *Elysia evelinae* (Jensen 1981). Here the diatom cell walls are pierced and the cell contents sucked out, but we do not know if chloroplasts are retained in any functional state. The potential pigment information of all these algal food-sources are summed up in Table 2. The diet of some sacoglossans as *Calliopaea oophaga*, *C. bellula*, *Stiliger vesiculosus* and *Olea hansinensis* feeding on the eggmasses from other opisthobranchs (Jensen 1980, Coelho et al 2005) is not considered a potential for a chloroplast-sacoglossan association and are omitted from Table 2. It is also important to note that diadinoxanthin, diatoxanthin and fucoxanthin, noted as pigments from bacillariophytes (Table 2), has also been found in sacoglossans in this study (Paper IV), however as pigments from epiphytes on the food algae or imbedded in the slug mucus. These three pigments are therefore not specific for bacillariophyte algae only, but occur also in other chromists as phaeophytes and xanthophytes. Thus, Table 2 can be used as a chemotaxonomic pigment chart to quickly assess the algal food source.

We can therefore conclude that the most convenient chemotaxonomical pigment markers of potential donor algae of functional chloroplasts are siphonaxanthin, siphonein and vaucheriaxanthin, all from coenocytic and siphonous macroalgae.

Table 2. Chemotaxonomical pigment markers that may be found in Sacoglossa.

Distribution, functionality and relative magnitude of pigments (CHL: chlorophylls, PC: photosynthetic carotenoids and carotenes, PPC: photoprotective carotenoids) in marine algae and plants used as food sources by sacoglossan species, and that may represent potential donors of photosynthetic chloroplasts (Jensen 1993, Paper IV). Large circles represent major pigments, medium are common pigments and small circles are minor trace pigments. This table can be used to classify chloroplasts pigments from sacoglossans into major algal groups. Pigments marked in red are specific chemotaxonomic markers for functional chloroplasts.

PIGMENTS / TAXON		PLANTAE							CHROMISTA		
		Chlorophyta					Magnoliophyta		Rhodophyta	Bacillariophyta	Ochromophyta
		Ulvophyceae					Monocots		Florideophyceae	Coscinodiscophyceae	Xanthophyceae
		Bryopsidales	Dasycladales	Siphonocladales	Cladophorales	Ulvales	Hydrocharitales	Potamogetonales	Ceramiales	Biddulphiiales	Vaucheriales
C H L	a	●	●	●	●	●	●	●	●	●	●
	b	●	●	●	●	●	●				
	c ₁								●	●	
	c ₂								●	●	
	c ₃										
P C	violaxanthin	●	●	●	●	●	●				●
	neoxanthin	●	●	●	●	●	●				
	lutein	●	●	●	●	●	●				
	siphonaxanthin	●		●	●	●					
	siphonein	●									
	fucoxanthin								●		
	vaucheriaxanthin										●
	β,β-carotene						●	●	●	●	
β,ε-carotene	●	●	●	●	●			●			
P P C	diadinoxanthin								●		
	diatoxanthin								●		
	antheraxanthin	●	●	●	●	●	●				
	zeaxanthin	●	●	●	●	●	●	●			
	lutein	●					●	●	●		
Phycobili								●			

3.3 Branching of the digestive gland in sacoglossans

As pointed out in the introduction, the observation of intact chloroplasts in the digestive cells of the sacoglossans is not in itself a proof that the slug retains functional chloroplasts. As shown in Chapter 2, the presence of photosynthetic chloroplasts can be detected by measuring the chl *a* fluorescence kinetics in PSII measured by PAM. Histological investigations can give information about the conditions the chloroplasts experience inside the slugs, and to some degree differentiate between types of chloroplasts (Chapter 3.4).

An increase in P_{\max} and thus E_k by a magnitude from 2-6 observed in chloroplasts from *Codium fragile* being ingested and retained into the digestive cells of *Elysia viridis*, indicated that τ decreased; τ is low at high irradiances, indicating that a decrease in τ may be a cause of higher irradiances in the digestive system of the slugs than in the cell environment of the algae (Chapter 2.3, Paper III). The increase in irradiance in the digestive system of the slug relative to the algae can be attributed to the digestive gland tubules in plakobranchoid sacoglossans branching throughout the entire body (Figure 7). A high degree of ramification of the digestive gland tubules has been proposed as a necessity for the ability to retain functional chloroplasts in the Sacoglossa to evolve (Jensen 1997). In the simple cerata bearing limapontoids, one large digestive gland tubule extends into each ceratum (Figure 7a). However, only one species of limapontoid sacoglossan, *Costasiella lilianae*, has been suggested to retain functional chloroplasts (Table 1). Most other limapontoids seem to retain degraded chloroplasts (Chapter 3.4). The branching of the digestive gland into the cerata might be advantageous to the slug keeping it in the same colour as the food algae (camouflage), since pigments do remain even though retained chloroplasts are found to be non-functional (Paper III).

In the parapodiabearing plakobranchoids *Elysia ornata* and *Elysia viridis* (Figure 7b and c), the digestive gland tubules ramify throughout the entire body. In *Plakobranthus ocellatus* (Figure 7d), the digestive gland tubules are accumulated into tubules arranged as longitudinal furrows dorsally. These examples illustrate different morphological strategies to bring chloroplasts to the outer tissue to provide more light. When packing the digestive cells full of chloroplasts, the digestive cells and chloroplasts will quickly shade each other (self shading). By branching the digestive gland (as in *Elysia*) or arranging the digestive glands dorsally in the parapodia (as in *Plakobranthus*), this problem is avoided.

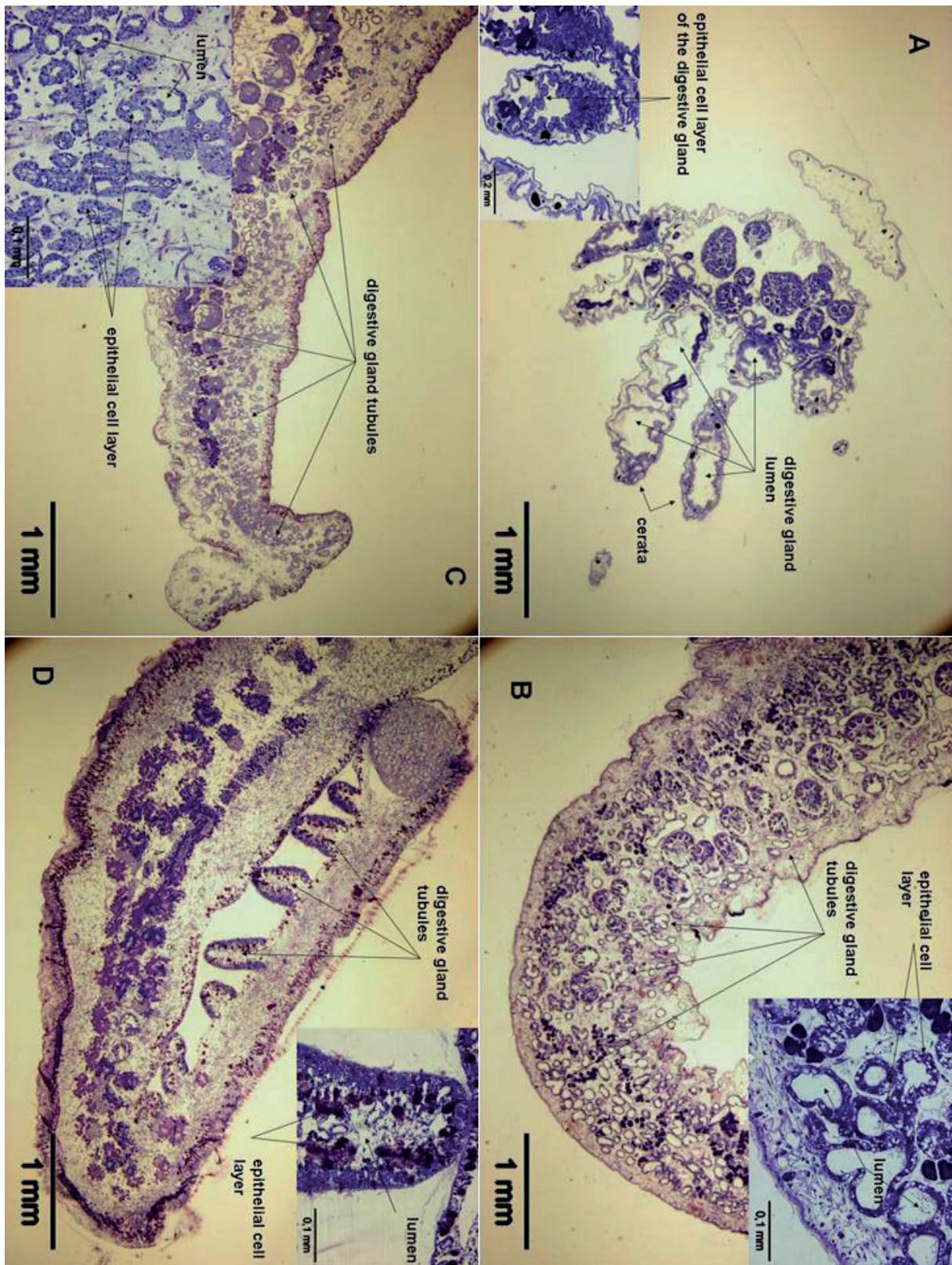


Figure 7. Light microscopy (LM) sections of *Placida dendritica* (A), *Elysia ornata* (B), *Elysia viridis* (C) and *Plakobranchus ocellatus* (D), showing the morphological distribution of digestive gland tubules.

3.4 Integrity of the chloroplasts in the digestive cells in sacoglossans

The structural integrity of the chloroplasts can also be studied to support observations on the functionality of the chloroplasts. Non-functional chloroplasts retained in the digestive cells of sacoglossans have a distinct morphology different from functional chloroplasts. The chloroplasts from *Codium* in the limapontoid *Placida dendritica* are degraded through digestion as soon as they are phagocytosed into the digestive cells of the slug and four different stages of chloroplast degradation is evident (Paper III). All chloroplasts are enclosed by a phagosome (a membrane surrounding the chloroplast as a result of phagocytosis, the cellular process of engulfing solid particles), and the thylakoid membranes quickly disintegrate. Further degradation involves the phagosome membrane breaking up, and the whole “digestive vacuole” containing the chloroplast swelling when the starch grain disintegrates (Figure 8a). This is in contrast to the same chloroplasts from *Codium* kept in the digestive cells of *Elysia viridis* (Paper III). Here all functional chloroplasts are surrounded by a phagosome. However, chloroplasts that are being degraded show breaches in the phagosome exposing the chloroplast to the cytoplasm. In these degraded chloroplasts the thylakoid membrane quickly disintegrates, but the chloroplast double membrane and the starch grain are the last to disintegrate. This indicates that chloroplasts in limapontoids and plakobranchoids are degraded differently, where the plakobranchoid digestion seems more ineffective on chloroplast membranes. Coenocytic and siphonous macroalgae have been described to have very robust chloroplasts (DeWreede 2006). When the cell wall is ruptured or in other ways damaged, these chloroplasts, or cytoplasts as they are described by Grant & Borowitzka (1984), are surrounded by a polysaccharide gelatinous membrane and are extremely resistant to changes in seawater osmolarity and pH (Wright & Grant 1978). However, our studies indicate that this “robustness” alone can not explain the different efficiency in degrading chloroplasts between limapontoids and plakobranchoids. Instead these differences may be adaptations in the digestive system of the sacoglossans.

Functional chloroplasts in the digestive cells of sacoglossans are also distinct from chloroplasts being degraded. The structural integrity of the chloroplasts surrounded by the phagosome is always intact, as seen in chloroplasts from *Codium* retained in a functional state by the digestive cells of *E. viridis* (Paper III, Figure 8b in this study). First of all, the thylakoid membranes are undamaged. This is very important, since it is here that the photosystems and light-harvesting complexes are located and where the light reactions drives the electron transport chain creating the chemo-osmotic potential to drive photophosphorylation and dark

reactions. Any sign of thylakoid membrane damage would be critical to photosynthetic activity. If chloroplasts are not able to synthesise any new galactolipids, as observations by Trench et al (1973a, b) imply, thylakoid membranes will gradually degrade. The presence of plastoglobuli in chloroplasts retained by sacoglossans, which function as collection sites for lipids when thylakoid membranes degrade, support the observation that chloroplasts are not able to repair themselves.

Chloroplast morphology varies greatly between algal groups, and different types of chloroplasts can be differentiated based on their outer membranes, thylakoid stacking, presence or absence of thylakoid organising bodies (TOB) and the presence or absence of pyrenoids (Wise 2006). Based on the observational differences of these structures (Table 3), potential donors of functional chloroplasts in sacoglossans can be identified further together with their pigment information (see Chapter 3.2). The green line in algal evolution that evolved to the higher plants has only one primary symbiotic lineage. This means that the chloroplasts in chlorophyte algae and higher plants have only one double membrane. The same goes for rhodophytes, the ancestor of the red lineage. However, the heterokont chromists are the result of a secondary symbiosis, and the chloroplasts are surrounded by four membranes. In the chlorophyte chloroplasts, the thylakoid membranes are stacked side by side to form adjoining lamellae traversing the length of the chloroplast (in higher plants the thylakoid membranes are organised into discrete granal stacks connected by paired membranes in the stroma). However, the number of thylakoid per stack (lamella) varies from two to six. In all rhodophytes there is an absence of stacked thylakoid regions, meaning that the thylakoids lie free in the chloroplast. However, the thylakoids in the ochrophyte *Vaucheria*, the thylakoids are again stacked side by side as in the chlorophytes. The presence or absence of a TOB and pyrenoid is here only used to further differentiate between the different groups of the Bryopsidales. The thylakoid organising body (TOB) is a concentric lamellar system at the base of chloroplasts only observed in Bryopsidales. The pyrenoid is present in many groups of algae, where it in some of them is known to contain Rubisco, or act as a site of starch formation.

The green macroalgal group Bryopsidales, which has in this study been shown to be the primary donors of functional plastids, can be further divided into Bryopsidinid chloroplasts which lack a TOB, and where a large central pyrenoid is common in the Bryopsidacea and the Derbeciaceae, but lacking in Codiaceae, and into Halimedinae which have a TOB, but where

pyrenoids are only sometimes found in the Caulerpacea (van den Hoek et al 1995). Based on TEM images of the digestive cells of sacoglossans from Australia, chloroplasts from Halimediane have been recognised in *Thuridilla ratna* (Figure 8c), chloroplasts from *Caulerpa* have been recognised in *Plakobranthus ocellatus* (Figure 8d), and chloroplasts from two different algal donors that belong to the Halimedinae and *Bryopsis* have been detected in *Elysia ornata* (Paper IV). These observations may explain the interspecific differences in ratios of pigments observed in sacoglossans in chapter 3.1. The sacoglossans in this study clearly exploit different species of bryopsidalean algae, from *Codium* in *Elysia viridis*, to *Caulerpa* in *Plakobranthus ocellatus*, to *Bryopsis* and *Halimeda* in *Elysia ornata* and *Thuridilla ratna*. This indicates that sacoglossans retain functional chloroplasts from a wide range of chlorophyte coenocytic and siphonous algae. As shown in chapter 2.2 and 2.3, this can be reflected in the RFC. Sacoglossans that prefer to explore a wide range of habitats and food algae, degrade their chloroplasts much quicker (retention of functional chloroplasts lasts only up to 20 days, Figure 4, corresponding to level 4-6 in Table 1). On the other hand, sacoglossans that adjust to similar light conditions as found in the same habitat as the food algae, or under LL and low temperature laboratory conditions, are able to retain their chloroplasts in a functional state up to several months (level 7 and 8 in Table 1).

Table 3. Chloroplast morphological characteristics in potential donor-algae of functional chloroplasts.

All functional chloroplasts found in sacoglossans originate from macroalgae with a coenocytic and siphonous thallus organisation. In green algae, the chloroplasts are only surrounded by a double chloroplast membrane, while in the ochrophyte algae are chromists surrounded by four chloroplast membranes. In the red algae, thylakoid membranes lie free in the chloroplast, in contrast to the green algae where the thylakoid membranes are banded (stacked into several layers, but into grana as in higher plants). The Thylakoid Organising Body (TOB) is only present in the halimedinid Bryopsidales. A large central pyrenoid is common in the bryopsidinid Bryopsidales (except *Codium*), and rudimentary pyrenoids are only present in a small number of *Caulerpa* in the halimedinid Bryopsidales. Table based on Hori (1974), Calvert et al (1976) and Hoek et al (1995).

		thallus organisation	chl p memb	thylakoid stacking	TOB	pyrenoid
CHLOROPHYTA	Bryopsidales	coenocyt. + siph.	2	stacked		
	Bryopsidinae	coenocyt. + siph.	2	stacked	no	likely
	Bryopsidacea	coenocyt. + siph.	2	stacked	no	yes
	Codiacea	coenocyt. + siph.	2	stacked	no	no
	Derbeciaceae	coenocyt. + siph.	2	stacked	no	yes
	Halimedinae	coenocyt. + siph.	2	stacked	yes	unlikely
	Caulerpaceae	coenocyt. + siph.	2	stacked	yes	in some
	Halimedacea	coenocyt. + siph.	2	stacked	yes	no
	Udotacea	coenocyt. + siph.	2	stacked	yes	no
	Dasycladales	large nucl. +siph.	2	stacked	no	no
	Siphonocladales	coenocyt. + siph.	2	stacked	no	yes
	Cladophorales	coenocyt. + siph.	2	stacked	no	yes
RHODOPHYTA	Ceramiales	coenocytic	2	free	no	in some
OCHROPHYTA	Vaucheriales	coenocyt. + siph.	4	stacked	no	no

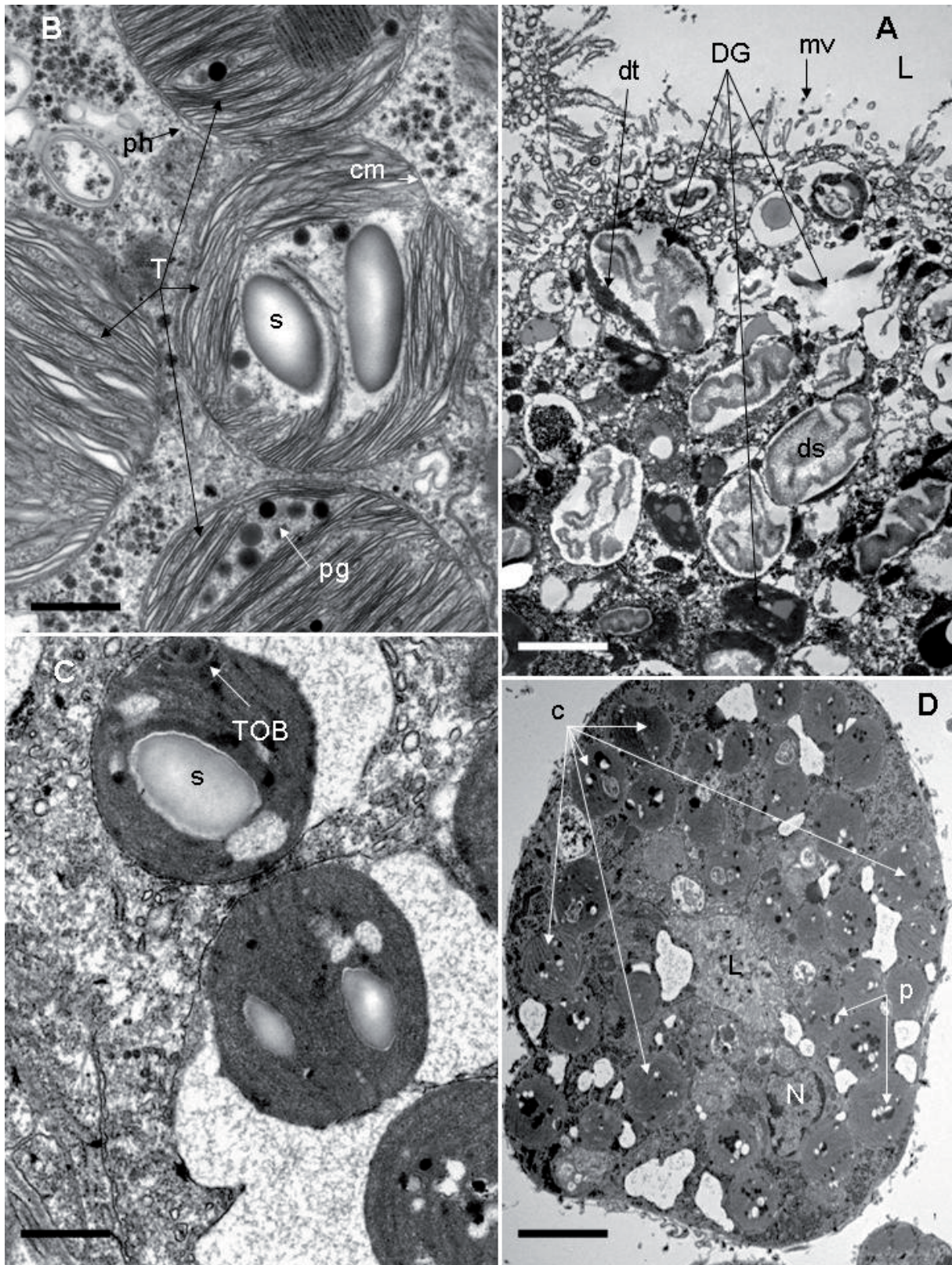


Figure 8. Transmission Electron Microscopy images of chloroplasts in the digestive cells of sacoglossans.

A) Digested chloroplasts (DG) from *Codium* in *Placida dendritica*, where the degraded thylakoid membranes (dt) are aggregated towards the border of the phagosome. Degraded

starch grains (ds) fill the remains of the digested chloroplast. Microvilli (mv) are lining the digestive cells into the lumen (L) of the digestive gland tubule. Scale bar 2 μm .

B) Functional chloroplasts from *Codium* in *Elysia viridis*. All chloroplasts are surrounded by the phagosome (ph), and the double chloroplast membrane (cm) is well defined. Starch grains (s) and plastoglobuli (pg) are common. The thylakoid membranes fill most of the chloroplast as long bands often stacked in several layers. Scale bar 1 μm .

C) Chloroplasts from a halimedinid algae in *Thuridilla ratna* have the characteristic TOB. Also in these chloroplasts starch grains (s) and plastoglobuli are common. Scale bar 1 μm .

D) Section through a digestive gland tubule in *Plakobranthus ocellatus*, which lumen (L) is surrounded by digestive gland cells. A large digestive cell nucleus (N) is present. Structurally intact chloroplasts (C) are packed into each digestive cell. Small pyrenoids (p) fused with starch are commonly found in chloroplasts belonging to the genus *Bryopsis*. Scale bar 5 μm .

Summary in vitro methods

Non-functional chloroplasts in the digestive cells of sacoglossans have up to 40% degraded chl *a* and lack some photosynthetic pigments (e.g. neoxanthin), indicating that chloroplasts are degrading.

Functional chloroplasts retained in the digestive cells of sacoglossans do not change the pigment composition compared to chloroplasts from the food algae.

Functional chloroplasts in the digestive cells of sacoglossans have the same pigment composition at the same percentage distribution as the food algae, indicating that they do not synthesise new pigments. However, the ratio of chl *a* to other pigments (chl *b*, violaxanthin, neoxanthin, siphonaxanthin and siphonein) decreases because some chl *a* is degraded as a result of a high turnover of chloroplasts in feeding sacoglossans.

The pigment composition in functional chlorophyte chloroplasts in the digestive cells of different sacoglossans collected *in situ*, reflect inter-specific differences in pigment ratios in coenocytic and siphonous chlorophytes, and indicate that sacoglossans retain chloroplasts from different food algae.

Siphonaxanthin and siphonein are specific carotenoid markers for the chlorophyte ulvophycean Bryopsidales. Vaucheriaxanthin is a specific carotenoid marker for the chromophyte xanthophycean *Vaucheria*. These carotenoids are specific chemotaxonomic markers for functional chloroplasts in sacoglossans obtained from coenocytic and siphonous macroalgae.

Branching of the digestive gland in sacoglossans has two different advantages: in limapontoids with non-functional chloroplasts, pigments give the sacoglossans similar colour as the food algae (camouflage). In plakobranchoids with functional chloroplasts, self-shading between chloroplasts is reduced and more light is available for photosynthesis.

The number of chloroplast membranes, stacking of thylakoid membranes, and the presence/absence of a TOB or pyrenoid, can be used to further identify algal donors of functional chloroplasts in sacoglossans.

Chloroplast integrity indicates that sacoglossans retaining non-functional chloroplasts disintegrate the whole chloroplast, while sacoglossans retaining functional chloroplasts do not effectively degrade the chloroplast membrane.

4. Conclusion

In this study, we can conclude that Φ_{PSII} as a function of time in starving slugs can be used to investigate phycozoan associations with emphasis on the algal component. In nudibranchs retaining zooxanthellae, two types of symbiotic relationship is evident (Paper I); the nudibranch *Pteraeolidia ianthina* which is able to maintain zooxanthellae numbers (Φ_{PSII} does not decrease significantly as a function of time during starvation), and species within the nudibranch genus *Phyllodesmium*, cryptic specialists on soft corals, which are not able to maintain zooxanthellae numbers (Φ_{PSII} decreases significantly as a function of time during starvation). This implies that nudibranchs which are highly adapted to their prey and which live most of their lifecycle on it may be independent of zooxanthellae, while more mobile nudibranchs that will benefit from tolerating long periods searching for food may adapt highly symbiotic associations with their zooxanthellae.

In sacoglossans, Φ_{PSII} as a function of time under starving conditions can be used to determine the ability to Retain Functional Chloroplasts in Sacoglossa (RFC, Paper II). The RFC is a measure on chloroplast functionality, and sacoglossans can be classified into eight different levels, depending on their RFC. Level 1-3 is used for sacoglossans retaining degraded chloroplasts for less than one day (mainly limapontoid sacoglossans), and level 4-8 denotes retention of functional chloroplasts from one day to more than three months (mainly plakobranchoid sacoglossans).

Chloroplast functionality can further be expressed by changes in the photosynthetic parameters Φ_{PSII} , α and P_{max} , and the pigment composition (Paper III). Chloroplasts retained by limapontoid sacoglossans are not functional, and chloroplast structure and pigment composition show different stages of degradation. Functional chloroplasts retained by plakobranchoid sacoglossans, degrade as a function of age, because they are not able to repair themselves, divide or synthesise any new pigments. They are only able to partially photoacclimate by adjusting the turnover time of electrons in PSII (τ).

The degradation of functional chloroplasts as a function of time is not only attributed to age, but also to the chloroplast photoacclimation status (E_k) and exposure to photodamage (Paper IV). Most sacoglossans in this study do not retain functional chloroplasts for more than two weeks (RFC level 4-6), they are found in a wide range of habitats, they feed on several

coenocytic and siphonous macroalgae, and they retain chloroplasts with photoacclimation statuses ranging from LL to HL. This indicates that sacoglossans moving in a wide range of habitats with varying light conditions will have a low RFC. Only sacoglossans inhabiting optimal light conditions are observed to have a long RFC.

The photosynthetic carotenoids siphonaxanthin and siphonein is present in all sacoglossans found with functional chloroplasts in this study. All functional chloroplasts retained by sacoglossans in this study thus originate from coenocytic and siphonous macroalgae. These two specific carotenoid markers, together with vaucheriaxanthin, can be used as chemotaxonomic pigment markers for functional chloroplasts.

Conclusively, to further investigate the association between green algal chloroplasts and sacoglossans, the chloroplast functionality in the digestive cells of sacoglossans must be investigated for more species of coenocytic and siphonous macroalgae. The results in this thesis is based on some groups of Bryopsidales, but as Table 3 in this thesis shows, many more macroalgal groups are potential donors of functional chloroplasts to sacoglossans.

Future studies should switch the emphasis from the algal component, to animal adaptations. Chloroplasts seem to be digested differently between limapontoid sacoglossans and plakobranchoid sacoglossans (Paper III); in *Placida dendritica* the entire chloroplast was degraded, but in *Elysia viridis*, the chloroplast membrane remains when the chloroplast was degraded. One possible sacoglossan adaptation may be that since coenocytic and siphonous chloroplasts are “robust”, the limapontoid digestion has adapted to a more “aggressive” digestion able to degrade the cytoplasts. The other possible sacoglossans adaptation is that the plakobranchoid digestion is “passive” in regard of lacking digestive enzymes that may degrade the cytoplasts.

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Paper I

Burghardt I, Evertsen J, Johnsen G, Wägele H (2005) Solar powered seaslugs – Mutualistic symbiosis of aeolid Nudibranchia (Mollusca, Gastropoda, Opisthobranchia) with *Symbiodinium*. *Symbiosis* 38:227-250

Solar Powered Seaslugs – Mutualistic Symbiosis of Aeolid Nudibranchia (Mollusca, Gastropoda, Opisthobranchia) with *Symbiodinium*

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Abstract

In this study, we investigate the mutualistic symbiosis of two aeolid nudibranchs, *Phyllodesmium briareum* Ehrenberg, 1831 and *Pteraeolidia ianthina* (Angas 1864), with zooxanthellae (Dinophyceae) of the genus *Symbiodinium* Freudenthal, 1962. These are the first long-term experiments with a Diving-PAM (submersible Pulse Amplitude Modulated Fluorometer) on a system, where an organism gets its symbiotic partners from a primary host, namely cnidarians. Long-term experiments with the nudibranchs kept under different environmental conditions indicate a highly evolved symbiotic relationship with zooxanthellae for both species, although the efficiency in photosynthesis is higher in *Pteraeolidia ianthina*. The relationship with *Symbiodinium* helps the adult nudibranch to overcome a period of food shortage by getting photosynthetic products. Juveniles of *Phyllodesmium briareum* are not able to survive without getting their primary food source, the soft coral *Briareum violacea* (Roule, 1908). In dark the number of photo-

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synthetically active zooxanthellae decreases in cultivated slugs. According to measured operational quantum yield of Chl a fluorescence (photosynthetic electron transfer rate), *Phyllodesmium briareum* and *Pteraeolidia ianthina* seem to house different types or even clades of *Symbiodinium*.

Keywords: Nudibranchia, symbiosis, mutualism, *Phyllodesmium briareum*, *Pteraeolidia ianthina*, *Symbiodinium*, zooxanthellae, photosynthesis, Pulse Amplitude Modulated fluorometry, irradiance

1. Introduction

A mutualistic symbiosis with unicellular dinoflagellates of the genus *Symbiodinium* Freudenthal, 1962, called zooxanthellae, is known from different marine invertebrates. Reef building hard corals (Hexacorallia, Madreporaria) are the most famous ones, using the metabolites of the zooxanthellae for their own nutrition. Some taxa of the Nudibranchia (Mollusca, Gastropoda, Opisthobranchia) are also known to house zooxanthellae inside of their body (namely inside the epithelial cells of the digestive gland). This was already described by Rousseau (1934, 1935). The nudibranchs get the dinoflagellates through their prey, mainly from cnidarians. Within the Nudibranchia, zooxanthellae have been found only in members of the taxon Cladobranchia but not in the Anthobranchia (Wägele and Johnsen, 2001). The importance of the relationship between intracellular symbiotic dinoflagellates and slugs is unknown in most cases. Zooxanthellae inside the nudibranch's digestive glandular cells were often detected only by histological and ultrastructural investigations (Kempf, 1984; Rudman, 1981a,b; 1982a,b; 1991; Marin and Ros, 1991; Wägele and Johnsen, 2001). These observations do not necessarily prove mutualism. In the investigated species, digestion of the zooxanthellae apart from their primary food (corals) cannot be excluded.

It has been assumed that camouflage offers a selective advantage which favours the retention of zooxanthellae in the digestive system of nudibranchs (Rudman, 1987). Additionally the zooxanthellae may enhance the ability of the seaslugs to survive periods of food shortage and allow them to search and test other food sources, e.g., other coral species. Finally in a mutualistic relationship the nudibranch would profit from the metabolites produced by the endosymbiotic zooxanthellae. An exchange of metabolites between host and endosymbiont has not been proved for nudibranchs' zooxanthellae yet, although studies of Hoegh-Guldberg and Hinde (1986) and Hoegh-Guldberg et al. (1986) suggest a transfer of organic carbon from *Symbiodinium* sp. to *Pteraeolidia ianthina* (Angas, 1864).

Morphological and behavioural adaptations in zooxanthellae-bearing nudibranchs were described by Rudman (1981b, 1982a, 1991) and Burghardt and Wägele (2004). Histological and ultrastructural investigations (Rudman, 1981a,b, 1982a,b, 1991; Wägele and Johnsen, 2001) show a correlation between morphological adaptations, e.g., branching structures of the digestive gland, and the housing of zooxanthellae. The branching of the digestive glandular system is much more extensive in Cladobranchia, which house zooxanthellae, than in those with no zooxanthellae. More extensive branching of the digestive gland enlarges the surface area for storage of zooxanthellae and allows higher photosynthetic light absorption and utilization. Rudman (1991) assumed a correlation between the branching patterns of the digestive gland, the evolution of the genus *Phyllodesmium* Ehrenberg, 1831 and the efficiency of photosynthetic performance inside the seaslug.

The aeolid genus *Phyllodesmium* known from the Indopacific with 15 described and approximately 11 undescribed species show a high diversity compared to other aeolid genera (Wägele, 2004). All known *Phyllodesmium* species exclusively feed on soft corals (Octocorallia). Some of these slug species are described to house zooxanthellae, but only by histological investigations (Rudman, 1991).

Here we investigate the symbiotic relationship of two nudibranch species for the first time with new methods in long-term experiments: *Phyllodesmium briareum* Ehrenberg, 1831 and *Pteraeolidia ianthina* (Angas, 1864). Additionally we compare the data of these nudibranchs with the primary food source of *P. briareum*, the octocoral *Briareum violacea* (Roule, 1908).

2. Material and Methods

Ten specimens (9 juveniles and 1 adult) of *Phyllodesmium briareum* and two adults of *Pteraeolidia ianthina* were collected in different sites around Lizard Island and investigated at Lizard Island Research Station (LIRS; Great Barrier Reef, Australia) between July and September 2002. For details of locality and the specimens see Table 1. Samples of the food of *P. briareum*, namely *Briareum violacea*, were also collected and studied. All investigated specimens were kept in aquaria with a flow-through water system at LIRS under moderate light conditions (up to a maximum of $\sim 350 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$ at solar noon). The water temperature was approximately 23–25°C. A Pulse Amplitude Modulated Fluorometer (Diving-PAM, Walz, Germany) was used to detect *in vivo* photosynthetic activity of zooxanthellae in *Phyllodesmium briareum*, *Briareum violacea* and *Pteraeolidia ianthina* by measuring the fluorescence emitted by Photosystem II (PSII) of chlorophyll a, the oxygen evolving side. This in turn allows distinguishing between active photosynthetic

Table 1. Synopsis of the investigated specimens of *Phylloidesmium briareum* and *Pteraeolidia ianthina* and the environmental conditions they were kept during experiments.

Species	Cultivation conditions	No.	Status	Collection date/ location	Cultivation (days)		Survival period/death	
					In light	In darkness		Back in light
<i>Phylloidesmium briareum</i>	Without soft corals as food source (in light)	Pb1	Adult	12.07.2002 Cobia Hole (~16 m, on rocks)	70	-	-	Artificial death after 70 days (20.09.2002)
		Pb7	Juvenile	23.07.2002 North Point (~18 m, on <i>Briareum</i> -colony)	32	-	-	Natural death after 32 days (24.08.2002)
		Pb8	Juvenile	24.07.2002 North Point (~18 m, on <i>Briareum</i> -colony)	58	-	-	Artificial death after 58 days (20.09.2002)
<i>Phylloidesmium briareum</i>	On <i>Briareum violacea</i> (food source) in light	Pb10	Juvenile	25.07.2002 North Point (~18 m, on <i>Briareum</i> -colony)	58	-	-	Artificial death after 58 days (21.09.2002)
		Pb2	Juvenile	22.07.2002 North Point (~18 m, on <i>Briareum</i> -colony)	3	21	-	Natural death after 24 days (15.08.2002)
		Pb6	Juvenile	23.07.2002 North Point (~18 m, on <i>Briareum</i> -colony)	3	28	-	Natural death after 31 days (23.08.2002)
<i>Phylloidesmium briareum</i>	Without food in darkness/after 33 days of starvation offer of soft corals	Pb5	Juvenile	22.07.2002 North Point (~18 m, on <i>Briareum</i> -colony)	2	31/3	starv./soft corals	Natural death after 36 days (27.08.2002)

Table 1. Continued.

Species	Cultivation conditions	No.	Status	Collection date/ location	Cultivation (days)		Survival period / death
					In light	In darkness	
<i>Phyllodesmium briareum</i>							
	Without food in darkness / after 34 days on <i>Briareum</i> in darkness	Pb4	Juvenile	22.07.2002 North Point (~18 m, on <i>Briareum</i> -colony)	2	32 / 2 starv. / <i>Briareum</i>	Natural death after 36 days (27.08.2002)
	Without food in darkness; back in light on <i>Briareum</i>	Pb3	Juvenile	22.07.2002 North Point (~18 m, on <i>Briareum</i> -colony)	2	30 5 (on <i>Briareum</i>)	Natural death after 37 days (29.08.2002)
	Without food in darkness; without food in light	Pb9	Juvenile	24.07.2002 North Point (~18 m, on <i>Briareum</i> -colony)	1	16	Natural death after 33 days (25.08.2002)
<i>Pteraeolidia ianthina</i>							
	Without food in light	Pte1	Adult	10.07.2002 Coconut Beach (intertidal, on coral rubble)	71	-	Artificial death after 71 days (19.09.2002)
	Without food in light; without food in darkness	Pte2	Adult	01.09.2002 Blue Lagoon between Lizard Is. and South Is.	9	11	Artificial death after 20 days (20.09.2002)

zooxanthellae inside the nudibranch and digested ones (Wägele and Johnsen, 2001).

The specimens of *Phyllodesmium briareum* were either kept in natural light conditions under starving conditions without any additional food (e.g., soft corals) or in natural light conditions with the primary food source (*Briareum violacea*). Natural light conditions means natural light climate conditions in which irradiance, spectral irradiance and photoperiod are driven by the sun's position (sun angle), clouds, the extinction coefficient of water and depth. Another group of specimens of *P. briareum* (juvenile animals) was kept under starving conditions, but after a few days in light they were put in total darkness in order to investigate behavioural and physiological changes of the slugs and changes of the photosynthetic performance. To some of these specimens different soft corals were offered as food source to investigate feeding preferences. Other specimens kept in darkness were brought back to the light after starvation in the darkness (Table 1). One specimen of *Pteraeolidia ianthina* (Pte1) was kept under natural sunlight conditions without any food in the aquarium for 71 days. The second specimen of *Pteraeolidia ianthina* (Pte2) was kept under the same conditions as Pte1 for the first 9 days, but afterwards brought in total darkness under continued starving conditions for 11 days (Table 1). For both nudibranch species the state of health was documented by taking digital photos of their external appearance.

The soft coral *Briareum violacea* was kept under natural light conditions, similar as specimens of *Phyllodesmium briareum*. Long-term experiments in the darkness were not performed because the polyps of the coral are retracted during night time. The zooxanthellae are not located in the coenenchyme, therefore no reliable measurements of the maximum quantum yield ($\Phi_{IIe-max}$) were possible.

All animals were exposed to different irradiances of natural sunlight between 0 and 1,500 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ for photosynthesis versus irradiance curves (P-E curves) with the Diving-PAM. Measurements were taken in the "open-air-lab" under a transparent roof at LIRS. For the attenuation of different irradiances spectrally neutral white cotton tissue and paper were used for shading. The fiber optics of the PAM was placed 0.5 cm from the part of the nudibranch with the highest concentration of zooxanthellae as detected by highest *in vivo* fluorescence – in aeolids mainly the cerata. Between the measurements breaks of at least 10 minutes were taken to allow the reaction centres of PS II to recover after light saturation.

There are sources of error when using PAM measurements. These need to be considered in order to interpret the results correctly. Usually the distance between the fiberoptics of the PAM and the measured animal was 0.5 cm, but this distance influences the F_0 values. The breaks between the measurements were generally 10 minutes, but after measurements in the full sunlight it was

often necessary to take longer breaks to allow the reaction centres of PS II to recover. This was the case for species with lowlight-adapted zooxanthellae (e.g. *Phyllodesmium briareum*). Another important factor was the "measuring spot" on the animal. We always tried to find the part of the slug with the highest F_0 signal, but because of the movements of the animals and the size of the fiberoptics it was not always possible to be absolutely accurate. Measurements, when not taken during darkness, were all taken in natural sunlight where clouds and changing humidity also influenced values to a certain degree. To reduce the effects of these errors, the number of measurements was high (e.g. 444 measurements for *Pteraeolidia ianthina*, Pte1).

Measurements of the maximum fluorescence yield ($\Phi_{IIe-max}$) were taken in darkness (during night time about 3 hours after sunset). $\Phi_{IIe-max}$ was plotted versus time in diagrams (Figs. 2 and 3) in order to show how long the zooxanthellae are able to stay photosynthetically active inside the nudibranch. For methodological details see Wägele and Johnsen (2001).

The photosynthesis versus irradiance curves (P-E-curves) and the resulting values (P_{max} , E_k , α) were analysed by the statistics software Kaleidagraph 3.6. For the calculations the following equation was used (for further details see Wägele and Johnsen, 2001):

$$P = P_{max} * (1 - \exp(-\alpha * E/P_{max}))$$

$$P = \text{Photosynthetic rate at a given actinic irradiance, } P = \Phi_{IIe} \cdot E$$

where:

$$P = \Phi_{IIe} (\text{mol charge separation} \cdot \text{mol quanta absorbed}^{-1}) \cdot E (\mu\text{mol quanta m}^{-2} \text{s}^{-1})$$

$$P_{max} = \text{Maximum photosynthetic rate (same units as P)}$$

$$\alpha = \text{Maximum light utilization coefficient } (\alpha = (\Phi_{IIe} \cdot E) \cdot E^{-1})$$

$$E = \text{Irradiance (PAR, 400–700 nm; } \mu\text{mol quanta m}^{-2} \text{s}^{-1})$$

$$E_k = P_{max} / \alpha = \text{light saturation index in } \mu\text{mol quanta m}^{-2} \text{s}^{-1}$$

After the long-term experiments all specimens were preserved in formalin/seawater for later histological investigations.

Additionally, some data (irradiance and yield measurements) were taken *in situ* under water by Scuba Diving. The irradiance in the intertidal zone (0–1 m depth) and at 15–20 m was typically ~1,500 and 50–100 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ respectively at solar noon.

3. Results

P-E (photosynthesis versus irradiance curves)

The E_k , α and P_{max} values of starved and fed specimens of *Phyllodesmium briareum*, *Briareum violacea* and *Pteraeolidia ianthina* are listed in Table 2.

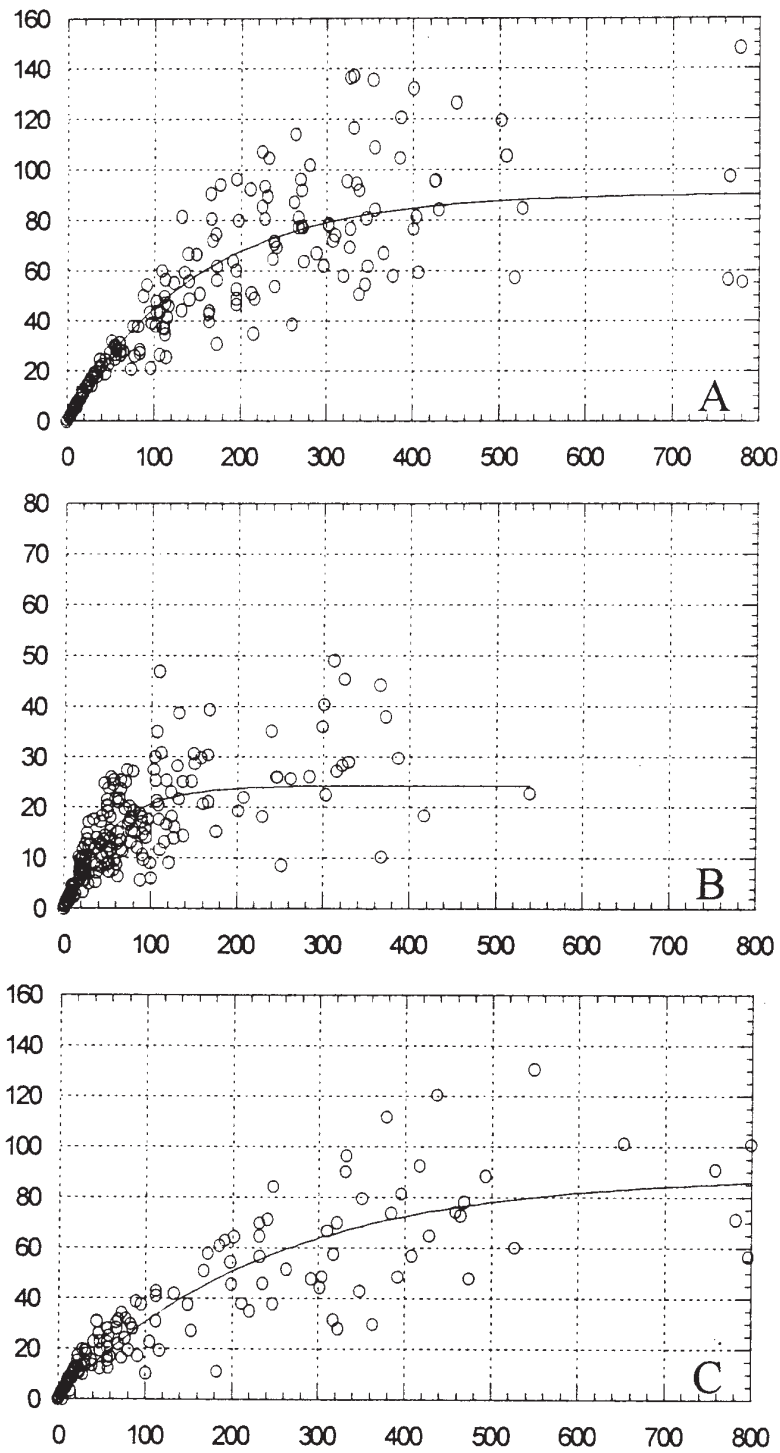


Figure 1. Photosynthetic rate ($P = \Phi_{IIe} * E$) versus irradiance (E ; [$\mu\text{mol quanta m}^{-2} \text{s}^{-1}$]) of *Briareum violacea*, *Phyllodesmium briareum* and *Pteraeolidia ianthina*. A: *Briareum violacea*. B: *Phyllodesmium briareum* Pb1. C: *P. briareum* Pb8.

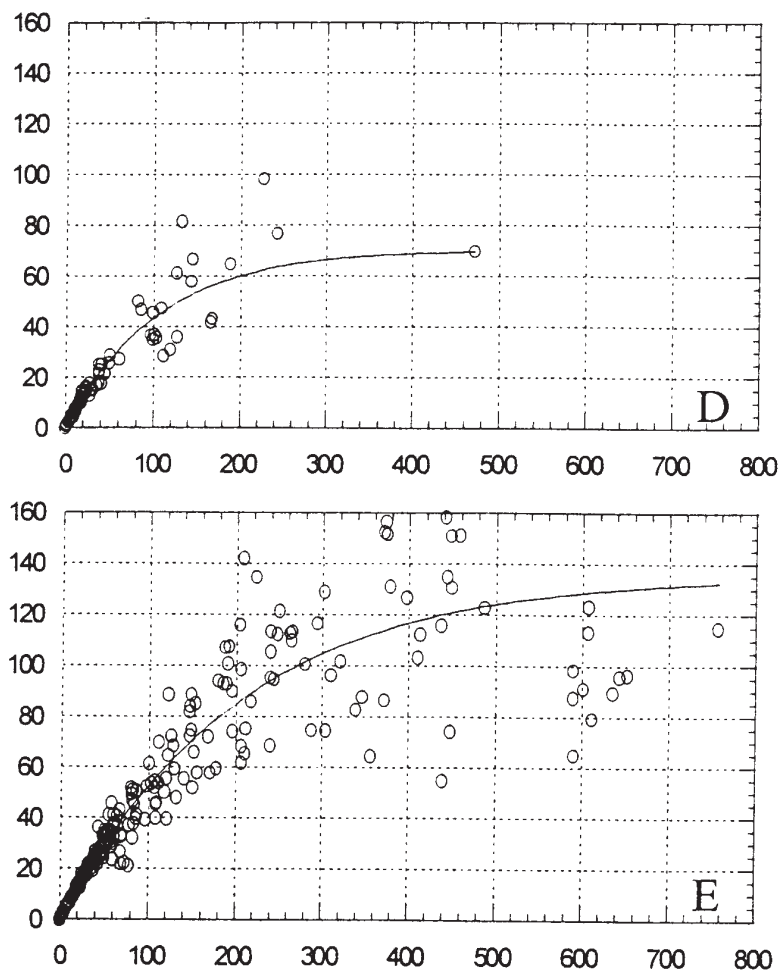


Figure 1. Photosynthetic rate ($P = \Phi_{IIe} * E$) versus irradiance (E ; [$\mu\text{mol quanta m}^{-2} \text{s}^{-1}$]) of *Briareum violacea*, *Phyllodesmium briareum* and *Pteraeolidia ianthina*. D: *P. briareum* Pb10. E: *Pteraeolidia ianthina* Pte1. x-axis: P, y-axis: E. Please note the different scales for P.

The E_k values of the starving specimens of *Phyllodesmium briareum* (here shown for Pb1) are generally lower than the ones of the fed specimens (Pb8 + Pb10). Similar results are observed for the P_{max} values (maximum photosynthetic rate) of the starving specimens in light: The mean values of these individuals are generally lower than the ones of the fed animals (Figs. 1C and 1D, Table 2). Compared to *Phyllodesmium briareum*, the P_{max} and α values of *Briareum violacea* are higher (Table 2). The E_k values are generally higher in *B. violacea* than in *P. briareum*. The P_{max} - and the E_k values of *Pteraeolidia ianthina* are significantly higher than the ones of *Phyllodesmium briareum* and *Briareum violacea* (Table 2).

Table 2. Photosynthetic characteristics of *Phyllodesmium briareum*, *Briareum violacea* and *Pteraeolidia ianthina* (calculated by Kaleidagraph 3.6, based on measurements with a Diving-PAM (Walz, Germany)).

Species	Conditions	No.	State	α	P_{\max}	E_k [$\mu\text{mol quanta}/\text{m}^{-2} \text{s}^{-2}$]
<i>Phyllodesmium briareum</i>						
	Without food in light	Pb1	Adult	0.46	24 (70 d)	52 (70 d)
		Pb7	Juvenile	0.57	21 (30 d)	37 (32 d)
	On food (<i>Briareum</i>) in light	Pb8	Juvenile	0.38	89 (58 d)	96 (58 d)
		Pb10	Juvenile	0.67	70 (58 d)	104 (58 d)
<i>Briareum violacea</i>						
	In light		Colony	0.63	90 (58 d)	144 (58 d)
<i>Pteraeolidia ianthina</i>						
	Without food in light	Pte1	Adult	0.67	136 (69 d)	203 (69 d)

$\Phi_{IIe} - T$ (PSII quantum yield vs. time-curves) and $F_0 - T$ (ground fluorescence vs. time-curves)

Comparisons of Φ_{IIe} and F_0 of different specimens of *Phyllodesmium briareum* indicate intraspecific differences (Fig. 2A). The starving individual kept in light (Pb1) obtained a gradual decrease in Φ_{IIe} versus time (from an average of 0.6 in the beginning to approximately 0.35 after 70 days). The curve of Pb7, the other individual kept under the same conditions as Pb1, is nearly identically, but this animal died much earlier. The curve is not shown here. By contrast, the Φ_{IIe} of the two specimens that were kept in light and fed with *Briareum violacea* (Pb8 + Pb10) stayed stable and decreased from 0.7 (start) to an average of 0.6 after 60 days in captivity (Fig. 2A).

There are also interspecific differences in the Φ_{IIe} versus time-curves between starving *Phyllodesmium briareum* (Pb1) and *Pteraeolidia ianthina* (Pte1; Fig. 2B). The Φ_{IIe} of Pte1 is close to the maximum (~0.6–0.7) during the experimental period.

Intraspecific differences between individuals of *Pteraeolidia ianthina* kept under different conditions are shown in Fig. 2C. Pte1 was kept under starvation without the coral in light for 71 days. Pte2, also kept without food, stayed in light for 8 days and was then brought into darkness for 11 days. In contrast to Pte1, the Φ_{IIe} of Pte2 decreased from 0.65 to 0.4 after being in darkness for 4 days (day 12 of cultivation).

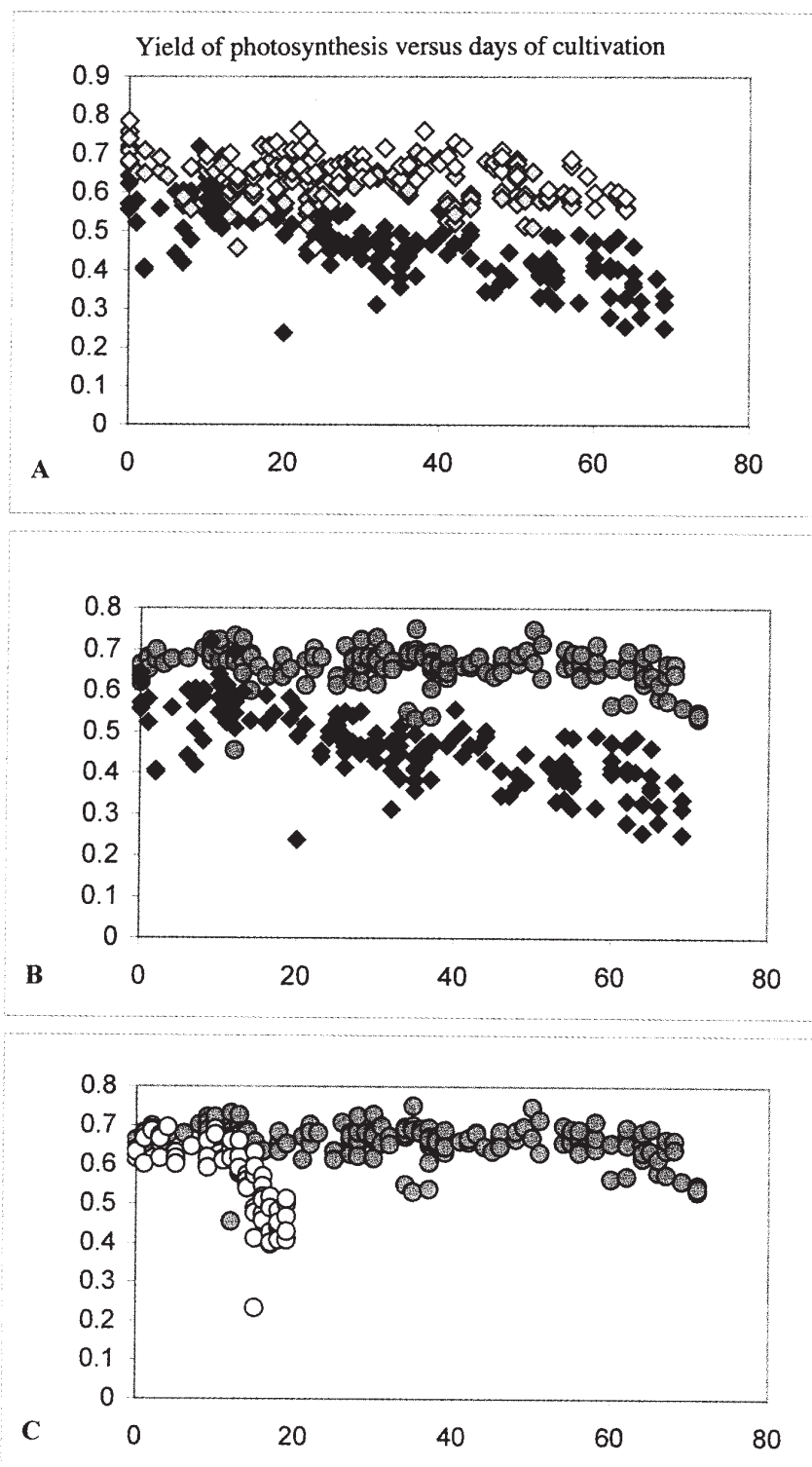


Figure 2. Yield of photosynthesis (Φ_{IIe}) versus time. A: *Phyllodesmium briareum* Pb1 starved (dark grey), Pb8 (white) and Pb10 (light grey) fed on *Briareum violacea*. B: *Phyllodesmium briareum* Pb1 (diamonds, dark grey) and *Pteraeolidia ianthina* Pte1 (circles, light grey), both under starving conditions in light. C: *Pteraeolidia ianthina* Pte1 kept in light (circles, light grey) and *P. ianthina* Pte2 kept in light for 9 days and then moved to darkness (circles, white).

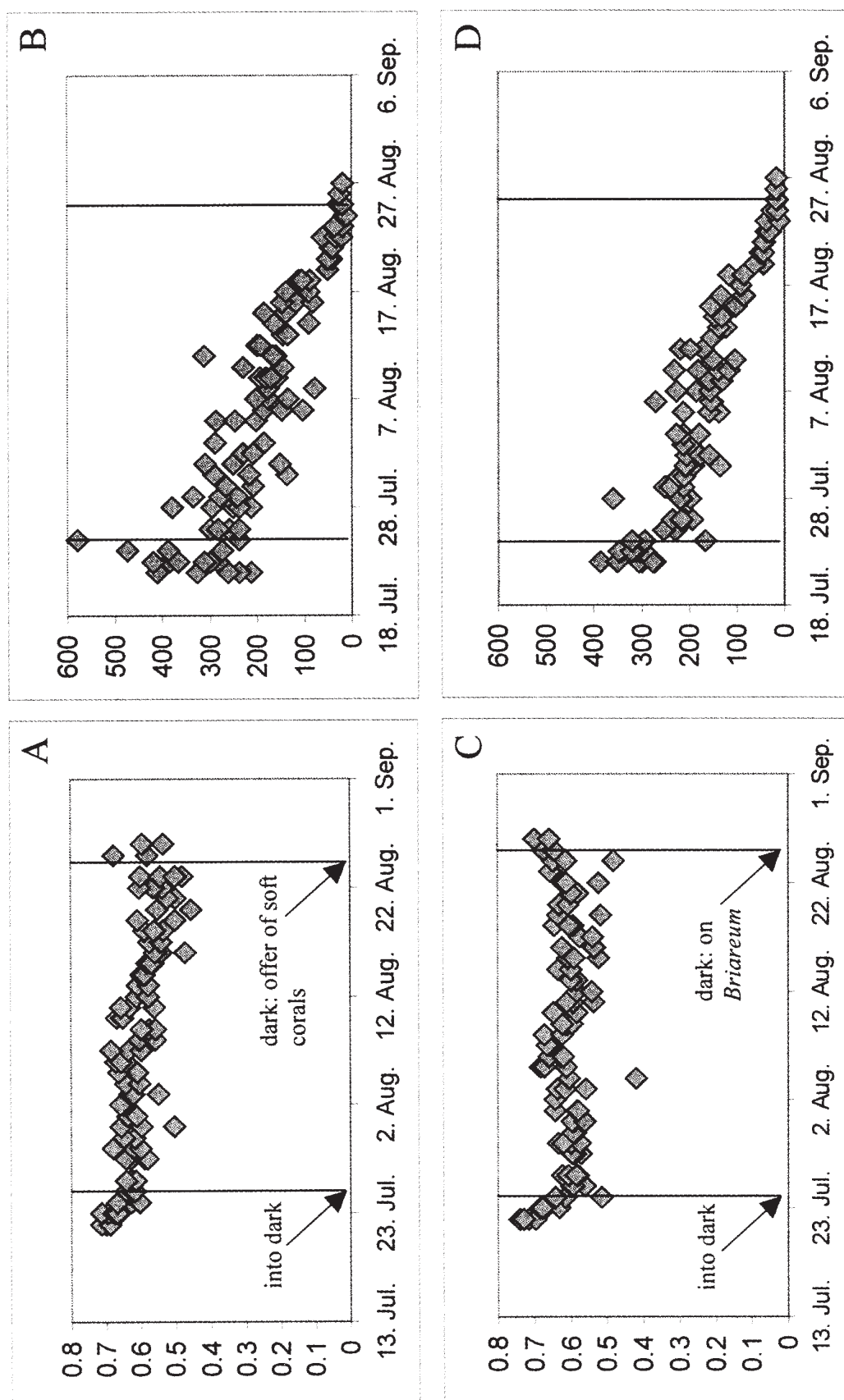


Figure 3. Φ_{IIe} versus time (A, C, E, G) and F_0 versus time (B, D, F, H) of different juvenile specimens of *Phylloidesmium briareum* kept in total darkness for some time (compare Table 1). First vertical line indicates change from light into darkness. A and B: *P. briareum* Pb5, second line indicates offer of different soft corals. C and D: Pb4, second line indicates offer of *Briareum violacea* in darkness.

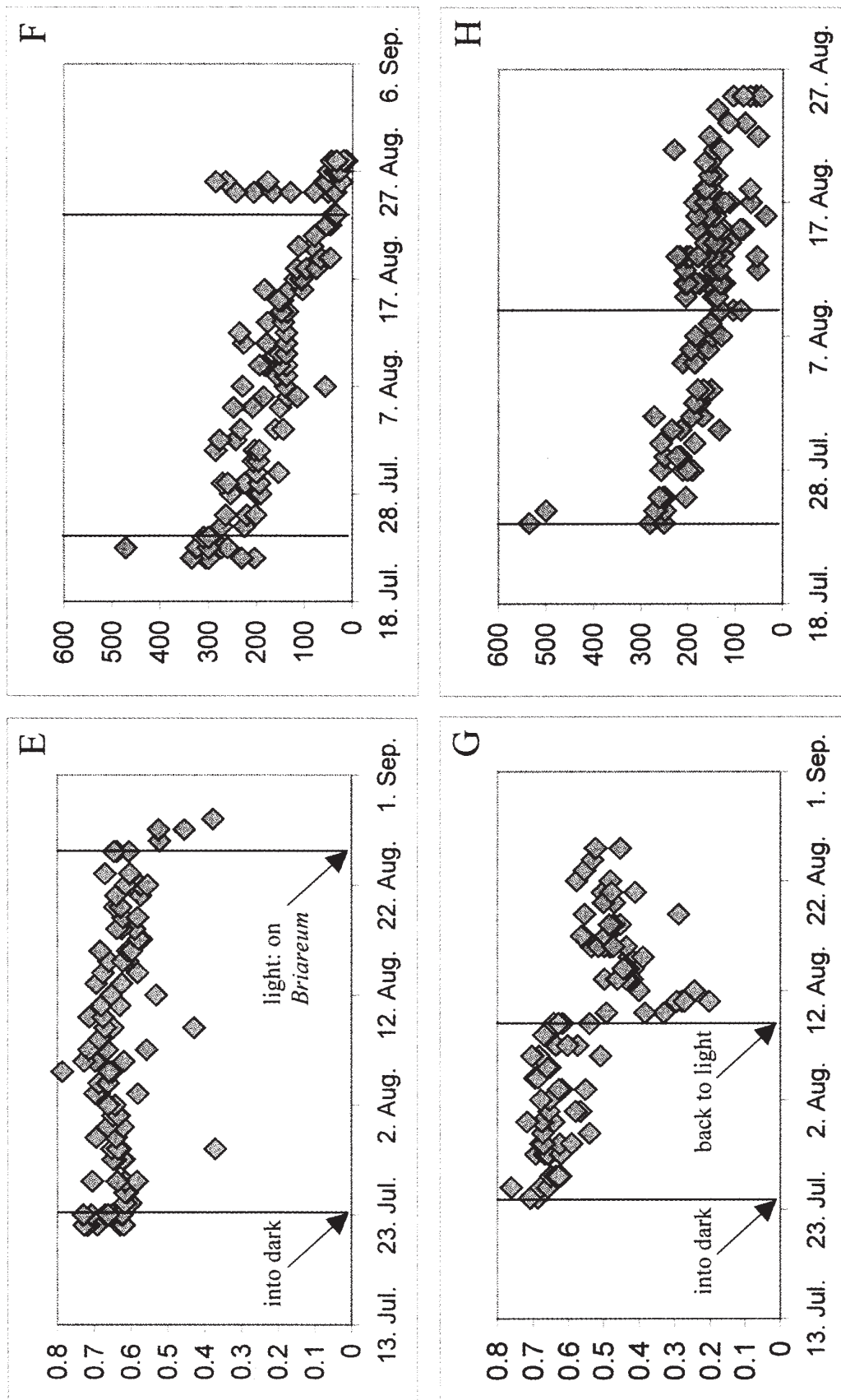


Figure 3. Φ_{IIe} versus time (A, C, E, G) and F_0 versus time (B, D, F, H) of different juvenile specimens of *Phyllodesmium briareum* kept in total darkness for some time (compare Table 1). First vertical line indicates change from light into darkness. E and F: *P. briareum* Pb3, second line indicates moving back into light and offering *Briareum*. G and H: *P. briareum* Pb9, second line indicates moving back into light without food offer.

The ground fluorescence (F_0) in the starving adult specimens of *Phyllodesmium briareum* and *Pteraeolidia ianthina* did not decrease considerably more than the yield values. Similar results can be observed for the fed specimens of *P. briareum* (Pb8 + Pb10). The F_0 values somewhat fluctuate (probably dependant on the distance between optical fiber and animal) but stayed in average on the same level for the whole time of the experiments (not shown here).

All starving specimens of *Phyllodesmium briareum* kept in darkness (Pb2, 3, 4, 5, 6, 9) show a slight decrease of Φ_{IIe} similar as the ones kept in light, but the F_0 of all specimens in the dark dropped to nearly zero (<10 mV) after approximately 30 days (Fig. 3).

The Φ_{IIe} and F_0 versus time curves of Pb5 show the typical course of the Φ_{IIe} – and F_0 values (Figs. 1A,B) of the animals kept in darkness. Additionally, different soft corals (*Sarcophyton* sp., *Sinularia* sp., *Xenia* sp.) were offered to Pb5 to check the food specificity of this species living under dark conditions. No feeding was observed and no increase of the F_0 values, that would indicate an uptake of fresh zooxanthellae, was measured.

Pb4 (Figs. 3C,D) was kept under similar conditions as Pb5, but instead of other soft corals, its proper food source (*Briareum violacea*) was offered. Again, there was no increase of F_0 values that would have indicated feeding and incorporation of zooxanthellae.

The Φ_{IIe} - and F_0 -data of specimen Pb3 (Figs. 3E,F) are similar to the ones of all dark cultivated individuals of *Phyllodesmium briareum*, but after putting the animal back to light into an aquarium with *Briareum violacea*, this specimen fed on the coral. The Φ_{IIe} (Fig. 3E) decreased quickly from 0.6 to 0.35 (5 days after the return to light conditions) and the F_0 (Fig. 3F) increased within one day up to 270 mV after introducing *B. violacea*. After one day on *Briareum violacea* the F_0 decreased again to nearly zero.

Pb9 was cultivated like the other starving and dark cultivated specimens but after 16 days, it was brought back into light. The Φ_{IIe} - and F_0 -data (Figs. 3G,H) show similar tendencies in the beginning, but after being back into light (still under starvation) a decrease of the Φ_{IIe} can be measured which then stabilises around yield values of 0.5 later on (Fig. 3G). The observed decrease of F_0 in the beginning of the experiment was stopped when the animal was brought back to the light. F_0 stabilized at ~150 mV (Fig. 3H).

Lifespans and behaviour of the investigated specimens of Phyllodesmium briareum and Pteraeolidia ianthina

Both (adult) specimens of *Pteraeolidia ianthina* survived the whole time of the experiments until they were preserved: Pte1 (71 days), Pte2 (20 days).

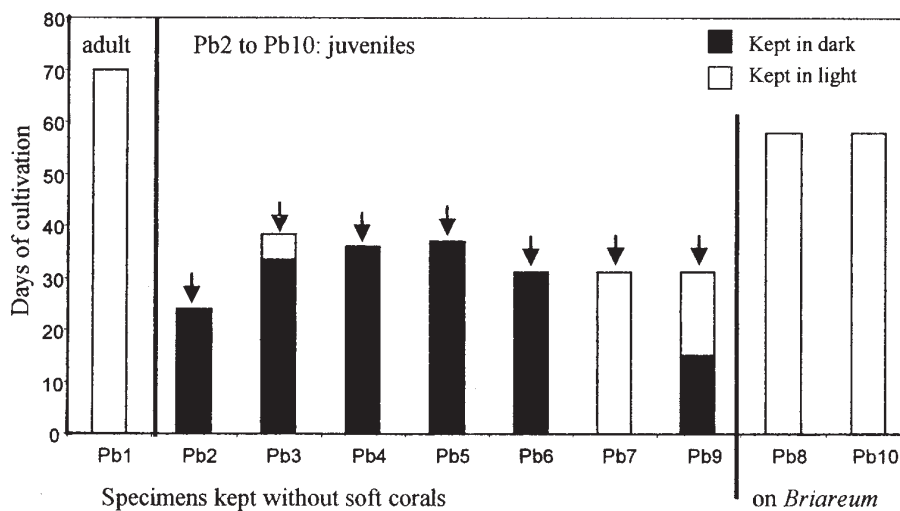


Figure 4. Life spans of juvenile and adult specimens of *Phyllodesmium briareum*, kept under different conditions (see Table 1). Specimens that died naturally are marked with an arrow. Pb1, Pb8 and Pb10 lived until experiments had to be finished.

Fig. 4 summarizes the life spans of the 10 investigated specimens of *Phyllodesmium briareum*. All juveniles that were kept under starving conditions (Table 1, Fig. 4) died after about 35 days, no matter whether they were kept in light or in darkness. Pb8 and Pb10, that were kept on colonies of *Briareum violacea* survived the whole experimental time. The specimen that was already adult in the beginning of our experiments (Pb1) was kept under starving conditions in the light and survived 70 days, until the end of experiments (Fig. 4). Although the starving adult specimen of *Phyllodesmium briareum* (Pb1) survived until the end of the experiments and although there was still photosynthetic activity, the animal lost the brownish colour of the zooxanthellae, became pale and did not grow (Fig. 5A). In the end of the experimental period, the slug became smaller and thinner. The starving juvenile in light (Pb7) went pale much quicker than Pb1 and died after only 32 days. It did not grow at all.

The two juvenile specimens that were cultivated on *Briareum violacea* (Pb8 and Pb10) grew fast in size. Although the starving individual Pb1 was a few millimetres longer than these two in the beginning of the experiments, in the end Pb8 was double the size of Pb1, dark brownish in colour and healthy looking, that means with a lot of thick cerata and no shrinking process visible (Fig. 5A). In general, the fed specimens were brown in colour and looked healthy during the whole time. They were mainly sitting between the polyps of *B. violacea*, were well camouflaged and did not move much. They were able to re-grow autotomized cerata and often laid egg clutches, mainly between the polyps.

The juvenile specimens of *Phyllodesmium*, that were kept in darkness (Pb2, 3, 4, 5, 6, 9), all went pale quickly. The cerata of these specimens went pale much faster than the rest of the body. Especially inside the foot there were still single brownish patches of zooxanthellae to see. During the first days of their captivity in darkness all of these juveniles produced a lot of brownish faeces that did not show any photosynthetic activity measured by the Diving-PAM. In the end of the cultivation all specimens in darkness were nearly transparent and the branched ducts of the mid-gut were clearly visible. The slugs lost size much faster than the starving specimens in light and developed local swellings over their body. Additionally they autotomized more and more cerata but were not able to re-grow them.

In general, the slugs kept in dark were less active than the ones kept in sunlight and showed unusual behaviour: They were often drifting near the water surface with spread-out cerata – a behaviour that was never observed for the specimens kept in light.

Fig. 5C presents the dark acclimated specimen Pb4 after 16 days in darkness. Fig. 5D shows the same animal after 34 days in darkness. In Fig. 5B two juveniles (Pb8 and Pb2) kept under different starving and light conditions after 2 weeks are presented. Pb8 which was cultivated in light on *Briareum violacea*, is double the size of Pb2 which was kept in darkness under starvation.

In general, *Phyllodesmium briareum* seemed to avoid high irradiances in full sunlight and was more active in moderate irradiances, e.g., when the sky was cloudy or during morning or evening hours. *Briareum violacea* was also sensitive to high irradiances. It retracted its polyps not only in night time but also in direct sunlight.

The two starving individuals of *Pteraeolidia ianthina*, Pte1 + Pte2 were much more active at higher irradiances than *Phyllodesmium briareum* and did not avoid direct sunlight. Pte1 stayed dark brown nearly until the end of the experiment but lost colour and size, from 40 mm length to 17 mm, suddenly a few days before preservation, i.e., after approximately 65 days of cultivation. Pte2 kept its brownish colour until it was preserved, even after cultivation in darkness for 11 days.

4. Discussion

Φ_{IIe} -E-curves

The data of the present study confirm the assumption of Hoegh-Guldberg and Hinde (1986), Rudman (1991) and Wägele and Johnsen (2001) that *Pteraeolidia ianthina* and *Phyllodesmium briareum* are nudibranchs with a highly evolved photosynthetic relationship with zooxanthellae. This study, based on PAM-

measurements also shows significant inter- and intraspecific differences of the photosynthetic parameters α , P_{\max} and E_k .

The P_{\max} - and E_k values of the starving adult *Phyllodesmium briareum* (Pb1) and in general the values of all specimens of *P. briareum* are much lower than the ones of *Pteraeolidia ianthina*. The other specimen of *Phyllodesmium briareum* (Pb7) that was kept under the same conditions (starvation in light), was a juvenile and showed similar results in the tests as Pb1, but the P_{\max} and E_k values were generally lower.

Comparing these data with the ones of the *Phyllodesmium briareum* specimens kept in light, but on their food source *Briareum violacea* (Pb8 + Pb10), there are significant differences to the starving animals. Both fed slugs showed high values of P_{\max} . The results for the E_k values are similar. The P_{\max} values of the fed specimens (Pb8 + Pb10) are more similar to the ones of *Briareum violacea* ($90 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$), whereas those of the starved specimens Pb1 and Pb7 are considerably lower. The E_k value of *Briareum violacea* was high compared to the data of the nudibranchs. Only *Pteraeolidia ianthina* showed higher values.

Low values of P_{\max} and E_k indicate low light adapted or acclimated photosynthetic active organisms, high values the opposite (Wägele and Johnsen, 2001). These data presented here match the locality where we found the investigated species. *Pteraeolidia ianthina* was collected in the intertidal zone in areas with exposure to high irradiances, whereas *Phyllodesmium briareum* was collected between 15 and 20 m, areas with lower light conditions ($50\text{--}100 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$ at noon on a bright day).

Pteraeolidia ianthina is known to forage on several hydrozoan species (Kempf, 1984; Willan, 1989; Hadfield, 1976; Gosliner, 1980), but we do not know what kind of prey our specimens were feeding on. Therefore, we do not know the source of the zooxanthellae. It is still unclear how many species of the genus *Symbiodinium* and zooxanthellae in general are involved in symbiotic relationships with nudibranchs. Hoegh-Guldberg and Hinde (1986) identified one species in *Pteraeolidia ianthina* as *Symbiodinium microadriaticum* Freudenthal, 1962 by morphological means. Marin and Ros (1991) assumed that the *Symbiodinium* they found in different nudibranchs also belong to this species. Contrarily to this assumption, Blank and Trench (1985) identified at least four different species in cnidarians on the basis of chromosome numbers and physiological behaviour. The current discussion in the literature distinguishes at least seven different phylogenetic clades or strains of *Symbiodinium*, based on data on ribosomal DNA (LaJeunesse, 2001; Rodriguez-Lanetty, 2003). Five of these clades are known to occur in corals. Unfortunately, there are no detailed investigations about *Symbiodinium* in octocorals. It is known that different clades of *Symbiodinium* can be associated with

individual coral colonies simultaneously (Rowan and Knowlton, 1995; van Oppen et al., 2001).

However, known examples of mixed zooxanthellae communities are limited (Rowan and Knowlton, 1995; Toller et al., 2001; van Oppen et al., 2001; Diekmann et al., 2002; LaJeunesse, 2002). Although some studies have found a lack of correlation between *Symbiodinium* phylotypes and photo-physiology (Savage et al., 2002), the distribution of zooxanthellae in some coral species correlates with light intensity and quality on different spatial scales. Light intensity and spectral composition are thus suggested to affect the composition of the zooxanthella community within individual colonies (Ulstrup and van Oppen, 2003). These data on corals are helpful for understanding the mutualism between the investigated nudibranchs and *Symbiodinium* because their only source of zooxanthellae are cnidarians.

Our results on the two different nudibranchs suggest that different ecotypes of *Symbiodinium* or even strains showing different photosynthetic characteristics are involved. *Pteraeolidia ianthina* houses a "highlight-adapted" type of *Symbiodinium*, *Phyllodesmium briareum* a "lowlight-adapted" one. *P. briareum* avoids high irradiances by escaping to shaded places and is more active in moderate irradiances. Interestingly, *Briareum violacea* also retracts its polyps in very high irradiances. This indicates that *P. briareum* and *B. violacea* may house similar light-sensitive zooxanthellae. In contrast to that, *P. ianthina* is also very active in direct sunlight irrespective of high irradiances.

When *Phyllodesmium briareum* feeds on *Briareum violacea*, a selective retention of different clades or types of *Symbiodinium* is possible, that would explain the differences of the E_k values between the soft coral and the fed slugs (see Table 2). One or more types of *Symbiodinium* that are more adapted to higher irradiances are probably not able to survive inside the slugs, but inside the coral. Thus the P_{max} - and E_k values of the fed specimens of *Phyllodesmium briareum* are more similar to the ones of *Briareum violacea* and higher than in the starving specimens, because they always had the opportunity to get "fresh" zooxanthellae from the coral.

Although the values in *Briareum violacea* are higher than in the starving animals of *Phyllodesmium briareum*, they are distinctly below the ones of *Pteraeolidia ianthina*. There seems to be no ecotype or strain of *Symbiodinium* adapted to extreme high irradiances in *Briareum violacea*, that was mainly found in depths of more than 13 meters around Lizard Island. Wägele and Johnsen (2001) indicate a much higher value of P_{max} and E_k in *Phyllodesmium briareum*. This might be due to the only few measurements and self-shading-effects in that study. High degree of self shading will give lower E_k values (Johnsen et al., 1997).

P-T-curves and F₀-T-curves

The results of the long-term experiments also show intra- and interspecific differences in Φ_{IIe} - and F_0 values over time. The Φ_{IIe} of the fed specimens of *Phyllodesmium briareum* (Pb8 + Pb10) was high (0.7–0.6) and did not decrease in comparison to the starved animals Pb1 and Pb7. This is certainly a consequence of permanent uptake of "fresh" zooxanthellae from *Briareum violacea*. These specimens can keep *Symbiodinium* cells photosynthetically active, but may replace them when new zooxanthellae are available. The decrease of the Φ_{IIe} in starving animals suggests that the portion of still photosynthetically active zooxanthellae is getting smaller and the animal is not able to keep all of them fully photosynthetic active over a long period. Nevertheless, the number of zooxanthellae seems to be stable over time indicated by nearly no changes in F_0 , even in the starving animals.

The interspecific differences between the starving specimens of *Phyllodesmium briareum* and *Pteraeolidia ianthina*, shown by the Φ_{IIe} plotted versus time, indicate that *P. ianthina* is able to keep the photosynthetic performance of its zooxanthellae stable for a long period (Pte1: at least 71 days). This confirms the results of Kempf (1984) that this species is able to survive at least some months exclusively on the photosynthetic products of its zooxanthellae. Their and our results suggest that *Pteraeolidia* has a stable, highly evolved and effective mutualistic symbiosis with *Symbiodinium*.

The rather slight decrease of Φ_{IIe} values in a specimen of *Phyllodesmium briareum* (Pb1) after more than 70 days of starvation from 0.7 to 0.6 also indicate the presence of a mutualistic symbiosis with zooxanthellae, but because of the decrease of the Φ_{IIe} the symbiotic relationship seems to be less stable and effective than in *Pteraeolidia ianthina*. The grade of branching of the digestive gland is high in *P. briareum* (Rudman, 1991) as well as in *P. ianthina*. The latter shows special adaptations of the digestive glands for housing zooxanthellae, namely the "tubules" described by Wägele and Johnsen (2001). This confirms the hypothesis of Rudman (1991) that a high grade of branching of the digestive gland indicates a symbiosis with *Symbiodinium*.

The fast decrease of the non-fed specimen of *Pteraeolidia ianthina* (Pte2) after 4 days in darkness (Fig. 2C) suggests that the probably high light-adapted zooxanthellae of *Pteraeolidia* are not able to survive several days in low-light or darkness. Comparing the PAM data between the different dark cultivated specimens of *Phyllodesmium briareum*, they show the same tendencies in Φ_{IIe} and F_0 versus time. Φ_{IIe} lies around 0.7 or decreases very slightly to 0.6, whereas the F_0 drops fast from around 300 mV to zero. This indicates that the number of photosynthetically active zooxanthellae decreases in darkness, but the remaining zooxanthellae are functional. Either the *Symbiodinium* cells are digested by the animal or they die because of the

lack of light. Under dark conditions the slugs shrink considerably. They lose their colour and get pale, an additional indication that the number of zooxanthellae decreases. In general, the animals obviously suffer more under starving conditions in darkness than in light, probably because of the missing nutrients produced by photosynthetic active zooxanthellae.

We assume that the zooxanthellae inside the dark cultivated slugs switch to a heterotrophic state and would then use metabolites of the nudibranch (Melkonian, pers. comm.). Since most of the zooxanthellae are situated in the midgut branches of the cerata, an autotomizing of the cerata would reduce the number of zooxanthellae in the slugs considerably. This assumption is supported by the observation that the starving dark cultivated specimen Pb4 did not accept the offered colony of *Briareum violacea*. Uptake of new zooxanthellae was avoided. Pb5, kept under the same conditions as Pb4, refused the offer of other soft corals. This may also indicate the avoidance of uptake of fresh zooxanthellae, but may also be interpreted as a narrow food spectrum of *Phyllodesmium briareum*.

The data of the dark cultivated specimen Pb9 after returning into light (Figs. 3G, H) show that there were still living zooxanthellae. The F_0 stabilized around 150 mV, indicating a stabilisation of the number of photosynthetically active zooxanthellae. The short drop of the Φ_{IIe} after returning into light may be a response of the zooxanthellae which were not able to cope with the quick switch to higher irradiances. The increase of the Φ_{IIe} two days later indicates chloroplast recovery and acclimation to higher irradiances. Additionally, there were more and more brownish patches to see in the body, especially in the foot. The Φ_{IIe} of the dark cultivated individual Pb3 show similar results as Pb9: the Φ_{IIe} decreases after returning the slug back to light. The sudden increase of the F_0 in Pb3 can be explained by the offer of *Briareum violacea* that allowed the uptake of fresh zooxanthellae by feeding. The dramatic decrease of F_0 only one day later suggests that the zooxanthellae were not able to survive as symbionts inside the slug – probably they were digested because of the bad state of the animal.

Fig. 4 shows that all juveniles of *Phyllodesmium briareum* that were kept under starving conditions died after about 35 days – no matter whether they were kept in light or darkness. Therefore we assume that the symbiotic relationship in juveniles is not developed enough to maintain metabolism or even growth without any other food. The two juveniles kept on *Briareum violacea* (Pb8 + Pb10) survived until they were preserved and showed a significant growth, so actually they were adults at the end of the experiments. We would most likely get different results for the non-fed experiments in darkness with adult animals, because of the probably underdeveloped symbiosis in juveniles. Therefore, the same experiments need to be repeated with adults.

According to the results presented here we assume that the energetic output of this mutualistic relationship with photosynthetic units (here zooxanthellae) helps the adult animals to overcome a period of food shortage (Marin and Ros, 1992). It also lengthens the period for search of adequate mating partners, whereas in juvenile specimens the mutualism is not established enough to nourish the animals over a longer period of time.

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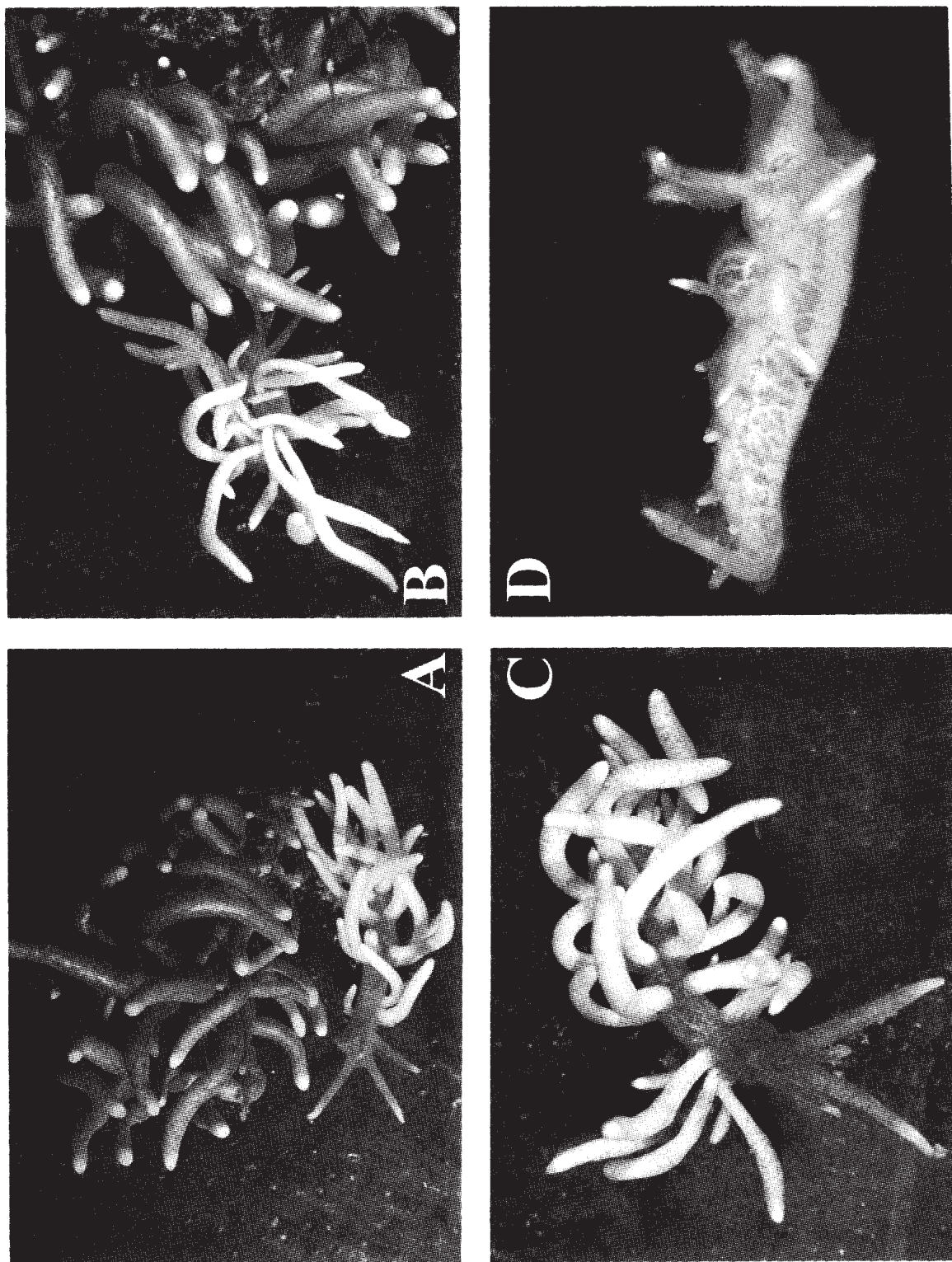


Figure 5. Living aspects of different specimens of *Phyllodesmium briareum* (see Table 1). A: *P. briareum* Pb1 (small specimen, 25th day without food in light) and *P. briareum* Pb8 (13th day in light on *Briareum violacea*). B: *P. briareum* Pb2 (small specimen, 15th day without food in darkness) and *P. briareum* Pb8 (16th day in light on *Briareum violacea*). C: *P. briareum* Pb4 (18th day without food, 16th day in darkness). D: *P. briareum* Pb4 (36th day without food, 34th day in darkness).

Paper II

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Paper III

Evertsen J, Johnsen G (2008) *In vivo* and *in vitro* differences in chloroplast functionality in the two North Atlantic sacoglossans (Gastropoda, Opisthobranchia) *Placida dendritica* and *Elysia viridis*. Mar Biol (submitted)

In vivo* and *in vitro* differences in chloroplast functionality in the two North Atlantic sacoglossans (Gastropoda, Opisthobranchia) *Placida dendritica* and *Elysia viridis

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Running head: *In vivo* and *in vitro* differences in chloroplasts functionality in sacoglossans

Abstract

The photosynthetic functionality in chloroplasts in the two sacoglossan molluscs *Placida dendritica* and *Elysia viridis* from the Trondheim fjord in Norway was studied. *P. dendritica* and *E. viridis* with no functional chloroplasts in their digestive system were introduced to the green macroalgae *Codium fragile*. Our results showed that *P. dendritica* was not able to retain functional (photosynthetic) chloroplasts. Transmission Electron Microscopy (TEM) showed that chloroplasts were directly digested when phagocytosed into the digestive cells. Four stages of chloroplast degradation were observed. A corresponding operational quantum yield of chl *a* fluorescence ($\Phi_{\text{PSII}} \sim 0$) indicated autofluorescence, and the presence of highly degraded chl *a*, supported these observations. In contrast, *E. viridis* was able to retain functional chloroplasts. For this species it took only one week for the chloroplasts inside the digestive cells to acquire the same Φ_{PSII} and light utilisation coefficient (α) as *C. fragile* kept under the same light conditions. Data for 8 days showed a 2-6 fold increase in the maximum photosynthetic rate (P_{max}) and light saturation index (E_k) relative to *C. fragile*. This increase in available light was probably caused by a reduced package effect in the digestive gland of *E. viridis* relative to *C. fragile*, resulting in a partial photoacclimation response by reducing the turnover time of electrons (τ). Isolated pigments from *C. fragile* compared to *E. viridis* showed the same levels of photosynthetic pigments (chl *a* and *b*, neoxanthin, violaxanthin, siphonaxanthin, siphonein and β, ϵ -carotene) relative to $\mu\text{g chl } a$ (w:w), indicating that the chloroplasts in *E. viridis* did not synthesise any new pigments. After 73 day starvation it was estimated that chloroplasts in *E. viridis* were able to stay photosynthetic 5 to 9 months relative to the size of the slugs, corresponding to a RFC of level 8 (a retention ability to retain functional chloroplasts (RFC) for more than 3 months). The reduction in Φ_{PSII} , P_{max} and α as a function of time was caused by a reduction in chloroplast health and number (chloroplast thylakoid membranes and PSII are degraded). These observations therefore conclude that chloroplasts from *C. fragile* can not divide or synthesise new pigments when retained by *E. viridis*, but are able to partially photoacclimate by decreasing τ as a response to more light. This study also points out the importance of siphonaxanthin and siphonein as chemotaxonomic markers for the identification of algal sources of functional chloroplasts.

Keywords: chloroplast functionality, kleptoplasty in Sacoglossa, photoacclimation status, chemotaxonomy, histology

Introduction

Placida dendritica (Alder & Hancock, 1843) and *Elysia viridis* (Montagu, 1804) are two sacoglossan molluscs (Gastropoda, Opisthobranchia) commonly found associated with the coenocytic green macroalgae *Codium fragile* (Suringar) Hariot, 1889 (Ulvothlyceae, Bryopsidales) in north Atlantic waters (Trowbridge 2002). Sacoglossans have a specific feeding habit feeding almost exclusively on the cytoplasm from coenocytic (multinucleate cells lacking transverse cell walls: e.g. chlorophyte macroalgae of the Bryopsidales, and xanthophyte macroalgae like *Vaucheria*) and siphonous (multinucleate cells with transverse cell walls: e.g. chlorophytes of the Siphonocladales, and rhodophytes like *Griffithsia*) macroalgae (Jensen 1980, Jensen 1997, DeWreede 2006). Some sacoglossans are also able to retain functional chloroplasts in the digestive system for several months, and eight different levels of retention have been described for sacoglossans retaining non-functional chloroplasts (level 1-3) to retaining functional chloroplasts from less than one day for up to 9 months (level 4-8: Clark et al. 1990, Evertsen et al. 2007). This ability to retain functional chloroplasts (RFC) refers to how well a given sacoglossan is able to retain chloroplasts in a functional state in their digestive cells as a function of time (Evertsen et al. 2007).

Chloroplasts have been observed in the digestive cells of *P. dendritica* (Taylor 1968, McLean 1976) and it has been suggested that these chloroplasts are functional for a short time (Taylor 1968, Greene & Muscatine 1972, Hinde 1980).

On the other hand, *E. viridis* has been observed to retain chloroplasts inside its digestive cells (Taylor 1968, Trench et al. 1973a, Hawes 1979, Hawes & Cobb 1980, Hinde 1980, Trench 1980, Williams & Cobb 1989) and the chloroplasts have been observed to be functional up to three months (Hinde & Smith 1972, Trench et al. 1973b). Observations of whether or not chloroplasts inside the digestive cells of *E. viridis* divide are inconclusive (Trench & Olhorst 1976). It has been observed that the chloroplasts in *E. viridis* are not synthesising any photosynthetic chlorophylls, galactolipids or proteins (Trench & Smith 1970, Trench et al. 1973b), indicating that chloroplast division is not taking place. As far as we know, no studies yet have pursued the pigment composition, chemotaxonomy (specific pigment tracers), functionality (RFC, Φ_{PSII} , photosynthetic and photoprotective pigments), degradation and photoacclimation status of the chloroplasts inside the sacoglossan digestive system, which is necessary if we want to understand which factors that govern the functionality of the chloroplasts when retained by sacoglossans. Photoacclimation is responsible for minimising variations in growth rate in fluctuating light, and can be attributed to three major physiological changes in chloroplasts (Falkowski 1980); active changes in the amount and ratios of photosynthetic and photoprotective pigments, changes in photosynthetic parameters, and changes in enzymatic activities involved in photosynthesis and respiration. Photoacclimation is thus the sum of compensation mechanisms which allow the chloroplasts to work under a wide range of irradiances at nearly the same optimal cell growth rate, in this regard chloroplast division rate.

This study investigates the functionality of chloroplasts being ingested by *P. dendritica* and *E. viridis* feeding on the same green macroalgae *C. fragile*. The *in vivo* method is based on Pulse Amplitude Modulated fluorometry measurements of living sacoglossans using a diving-PAM. Previous investigations by Wägele & Johnsen (2001), Burghardt et al. (2005), Evertsen et al. (2007) have shown that the diving-PAM can be used to investigate photosynthetic activity of chloroplasts and zooxanthellae in opisthobranchs. The diving-PAM measures the quantum yield of chlorophyll *a* (chl *a*) fluorescence from reaction centres (RC_{PSII}) in photosystem II (PSII) (Eq. 1 and 2), indicating the fraction of functional chloroplasts related to the quantum yield of electrons generated relative to the amount of photons absorbed. For green macroalgae, most *in vivo* fluorescence is emitted by chl *a* in PSII. When chloroplasts acclimated in darkness, the chl *a* fluorescence emitted from chloroplasts is minimal when all functional RC_{PSII} are open (F_0). However, under saturation light conditions, the chl *a* fluorescence increases until reaching a maximum, F_m , when all RC_{PSII} are closed (Eq. 1 and 2). The fraction of open RC_{PSII} is given as the operational (dark acclimated chloroplasts) and maximum (in actinic light) quantum yield of PSII estimated as defined by Butler (1978) and Dau (1994) with annotations as suggested by van Kooten & Snel (1990)

$$\Phi_{\text{PSII}} \text{ (mol e}^- \text{ mol photons}^{-1}\text{)} = F_m - F_0 / F_m \text{ (maximum quantum yield of chl } a \text{ fluorescence for dark acclimated chloroplasts)} \quad (1)$$

$$\Phi_{\text{PSII}}' \text{ (mol e}^- \text{ mol photons}^{-1}\text{)} = F_m' - F_0' / F_m' \text{ (operational quantum yield of chl } a \text{ fluorescence under actinic light)} \quad (2)$$

A decline in Φ_{PSII} may be a result of RC_{PSII} becoming photosynthetically incompetent due either to inefficient excitation transfer from light harvesting complexes (LHC) to RC_{PSII} ,

impairment of primary photochemistry, or disruption of electron transport between Q and PQ (Kroon et al. 1993). The initial Φ_{PSII} , measured on the day of collection, can be divided by the reduction of $\Phi_{PSII} \text{ day}^{-1}$ to estimate a time period in days in which the chloroplasts are functional inside the sacoglossans (Evertsen et al. 2007).

Φ_{PSII} as function of the incident irradiance (E , $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$) is defined as the relative electron transfer rate ($\text{ETR} = \Phi_{PSII} * E = (\text{mol e}^- \text{ mol photons}^{-1}) \mu\text{mol photons m}^{-2} \text{ s}^{-1}$). Since ETR here denotes how much electrons are generated relative to the amount of photons absorbed as a function of the incident irradiance, ETR can be used to create photosynthesis versus irradiance (P vs. E) curves to obtain information on the photoacclimational state of the chloroplasts. P vs. E curve fitting of ETR vs. E (Eq. 3) was done according to Webb et al. (1974)

$$P(\text{ETR}) = P_{\max}(1 - \exp(-\alpha * E / P_{\max})) \quad (3)$$

α , the light utilisation coefficient, is related to light absorbed by PSII, \bar{a}_{PSII} ($\text{m}^{-2} \cdot \text{mg chl } a^{-1}$) (Johnsen & Sakshaug 2007) and is spectrally dependent ($\alpha = \Phi_{PSII} * \bar{a}_{PSII}$). P_{\max} , the maximum photosynthetic rate, is dependent on the concentration of chl a in the photosynthetic unit (PSU) (q) and the minimum turnover time for processing photons (τ) (Dubinsky et al. 1986), thus obtaining no spectral dependency ($P_{\max} = \Phi_{PSII} / q \tau$). E_k is the ratio between P_{\max} / α and is denoted as the light saturation parameter ($\mu\text{mol photons m}^{-2} \text{ s}^{-1}$) (Kroon et al. 1993, Sakshaug et al. 1997).

The isolation of pigments using High Precision Liquid Chromatography (HPLC) can be used to check for taxon specific pigment markers (Jeffrey et al. 1997, Johnsen & Sakshaug 2007), the functionality of pigments in the sacoglossans food source (coenocytic macroalgae) and in the sacoglossan digestive cells in regard of photosynthetic and photoprotective pigments, and their corresponding degradation status. The amount of pigment relative to the amount of chl a (w:w) can be used to compare the photoacclimation status in the chloroplasts in their natural cell environment in the macroalgae relative to the chloroplasts in the digestive cells of the sacoglossans. Significant differences in ratios of pigment relative to chl a , can be indicative of photoacclimation (Rodriguez et al. 2006).

Light microscopy studies can be used to investigate the organisation of the digestive gland of the sacoglossans. A high degree of ramification of the digestive gland tubules has been proposed as a prior condition for the ability to retain functional chloroplasts in the Sacoglossa (Jensen 1997). TEM methodology can further be used to investigate the contents of the digestive cells, type of organelles and how many functional and degraded chloroplasts are present. This will tell us something about the conditions in the digestive cells that the chloroplasts are exposed to. It has been suggested that chloroplasts from *C. fragile* in the digestive cells of *E. viridis* are either directly exposed to the digestive cell cytoplasm, or contained within a phagosome (Trench 1980). The structural integrity of the chloroplasts in the digestive cells of the sacoglossans will therefore be investigated in regard of the phagosome, the chloroplast double membrane, the thylakoid membranes and starch grains.

Material and methods

Elysia viridis and *Placida dendritica* were collected by SCUBA-diving at Mausundvær, Frøya (63 52'30N, 08 38'36E) at the Trøndelag coast of Norway in April 2005. No *Codium fragile* was found at the sampling site at 5-7 meters depth, and was therefore later obtained

from Tautra (63°34'00N, 10°36'52E) in the Trondheimsfjord. The algae and slugs were brought back to the laboratory, and kept in running seawater aquaria similar to *in situ* conditions at 11 °C and under light conditions at 30 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ at the surface of the aquaria (no background light) under 6 hour day length (09:00-15:00) for all experiments.

Two experiments were conducted to investigate the functionality of *C. fragile* chloroplasts in the digestive cells of *P. dendritica* and *E. viridis*. Experiment 1 investigated functionality of chloroplasts from *C. fragile* as function of being retained in empty (containing no functional chloroplasts before the experiment) digestive cells of *P. dendritica* and *E. viridis*. *In vivo* measurements of the P vs. E parameters P_{max} , α and E_k were conducted as a function of 8 days using 8 different irradiance levels (0, 5, 10, 25, 40, 70, 145, and 520 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) for each curve. *In vitro* investigation of chloroplast functionality was conducted through HPLC pigment extraction and histology using LM and TEM.

Experiment 2 investigated photosynthetic activity of chloroplasts from *C. fragile* in starving *E. viridis*. *In vivo* measurements of P vs. E curves were conducted every second or third day for 73 days for *E. viridis* only. To achieve this 15 different irradiance levels (0, 3, 6, 13, 25, 40, 60, 90, 120, 190, 260, 350, 540, 740 and 1020 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) were used to create the P vs. E curves. Φ_{PSII} , P_{max} , α and E_k were then investigated as a function of time. The wet weight of each slug was measured five times and presented as a mean wet weight to the nearest 0.01 g for all experiments.

The fluorescence induction curve was measured with a diving-PAM (Heinz Waltz GmbH, Germany) using a weak non-actinic (non-photosynthetic) probe flash (at 0.15 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, obtained from a light emitting diode (LED at 650 nm) using a pulse modulated probe light sending pulses at a frequency of 0.6 KHz), measuring F_0 and F_0' (Eq. 1 and 2). A change from F_0 and F_0' to F_m and F_m' was induced by a saturating flash with a peak irradiance of $\sim 10\,000 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ of 0.8 seconds duration with the probe light (Halogen, white light) obtaining data at a frequency of 20 KHz, corresponding to the plateau level P of the Kautsky curve (see Govindjee 1995) before non-photochemical quenching processes start to reduce the chl *a* fluorescence (Kromkamp & Forster 2003).

P vs. E curves were obtained by placing individual sacoglossans and pieces of *C. fragile* into separate 24 ml glass vials with seawater put next to each other in a white styrofoam box for even light distribution. Pieces of black cardboard were put between the vials during PAM measurements to prevent the PAM flash from influencing the light acclimation of the adjacent slugs. A slide projector with slides with different layers of plankton-net as neutral density filters was used to create different irradiances levels. The spectral irradiance from each slide was checked with a RAMSES ACC underwater spectroradiometer at 350-800 nm (Trios GmbH, Germany), and showed similar spectra. $E(\text{PAR})$ during experiments was determined from inside the vials with the PAR 2π light collector (model Diving-LS, Heinz Waltz GmbH, Germany) attached to the diving-PAM. Prior to P vs. E curve experiments all organisms were dark acclimated for 15 minutes. Exposure to subsequent irradiance levels was 2 minutes incubation time. After each P vs. E experiment, all organisms were again dark acclimated for 15 minutes to check that Φ_{PSII} recovered, where the Φ_{PSII} after experiments was on average 13% lower than initial Φ_{PSII} values. The P vs. E curves were plotted in Sigmaplot version 10.0 to estimate the photosynthetic parameters P_{max} and α using equation 3.

For pigments analysis, all sample preparations were done under subdued light to avoid pigment degradation. The slugs were immediately dried with some paper cloth to avoid

excess water reducing extraction efficiency. Correspondingly, the *C. fragile* tissue was thoroughly squeezed without rupturing it to get as much water as possible out of the tissue. Pigment extraction was done in glass tubes with 2-4 ml pre-cooled methanol bubbled with N₂ to avoid oxidation. Tissue was grinded with a glass rod allowing 24 hours of extraction in darkness at 4 °C. After extraction, the samples were filtered through sterile syringes equipped with a 0.45 µm Millipore filter to remove debris, and 150 µl extract was then injected into the HPLC system (Hewlett-Packard HPLC Series 1100) equipped with a quaternary pump system and diode array detector, using the protocol of Rodriguez et al. (2006). Chlorophylls and carotenoids were detected by absorbance at 420, 440, 460 and 480 nm and identified by an absorbance detector (350–750 nm with 1.2 nm spectral resolution). HPLC calibration was performed using chl *a* and β,β-carotene standards from SIGMA (Aldrich, UK) and own chlorophyll and carotenoid standards (chl *b*, chl *c1*, violaxanthin, neoxanthin, siphonaxanthin and siphonin). The extinction coefficient of 250 L g⁻¹ cm⁻¹ at 445 nm in acetone for β,β-carotene (Jeffrey et al. 1997) was used to quantify siphonaxanthin and siphonin, adjusting for differences in molecular weight between pigments according to Johnsen & Sakshaug (1993).

For light microscopy (LM), the slugs were preserved in 4% final concentration of 39% formaldehyde (Merck, Germany) diluted in seawater. After 24 hours the slugs were dried in increasing concentrations of ethanol from 70% for 24 hours, followed by two 1 hour washes in 80%, and three 1 hour washes in 90% and 1 hour in 100% ethanol. They were then embedded whole in Technovit 7100 (Heraeus Kulzer GmbH, Germany), and cut into 2.5 µm thick sections with a powered microtome (Autocut model 1140) and stained with toluidine blue (Merck, Germany), before examined in a light microscope (Zeiss Axiovert 200).

For the transmission electron microscopy studies, tissue samples from the slugs were cut in 1 mm³ pieces and immersed in a final concentration of 2% glutaraldehyde buffered with 0.1 M phosphate buffer (pH 7.3) for 12 hours at 4°C. Samples were then washed in 0.1 M phosphate buffer once, and then postfixed in final concentration of 2% osmium tetroxide (OsO₄) in 0.1 M phosphate buffer for 1 hour. After fixation the samples were rinsed in 0.1 M phosphate buffer and dehydrated in increasing concentrations of ethanol at 30, 50, 70 and 96% twice each at 5 minute intervals and in 100% ethanol with molecular sieve three times in 5 minute intervals. Infiltration started with 1 hour embedding in 1:1 solution of Epon (Hexion, Amsterdam, Netherlands) and 100% ethanol, followed by two immersions for 1 hour in pure Epon at room temperature (20°C). Samples were then embedded in pure Epon and polymerised for 24 hours at 60°C, and stabilised for 24 hours at room temperature (20°C). Thin sections of 70 nm were cut with a diamond knife in a Leica EMU C6 ultramicrotome and collected on 200 mesh copper grids. Samples were then poststained for 25 minutes with a final concentration of 2% uranyl acetate (UO₂(OCOCH₃)₂·2H₂O) in 50% ethanol, and for 5 min in a final concentration of 0.1% aqueous lead citrate (Pb(C₆H₅O₇)₂·3H₂O) following protocols of Reynolds (1963). Samples were then observed and photographed in a Jeol NEM-1011 electron microscope.

Results

Experiment 1, *in vivo* results

1 week after *in situ* collection with no *Codium fragile* available, both *Elysia viridis* and *Placida dendritica* showed only autofluorescence ($\Phi_{PSII} = 0$). This indicated that no functional chloroplasts were present inside the digestive cells of the slugs. After this, 3 specimens of *P. dendritica* and *E. viridis* were put in an aquarium containing *C. fragile* and allowed feeding for 8 days.

The mean wet weight of *P. dendritica* at the start was 0.095g and 0.105g at the end of the experiment. Correspondingly, *E. viridis* weighed 0.17g at the start and 0.19g at the end of the experiment, giving an increase in wet weight of 9.5% for *P. dendritica* and 10.5% for *E. viridis*, relative to the initial wet weight.

P. dendritica did not show any photosynthetic responses throughout the whole experiment ($\Phi_{\text{PSII}} = 0$ for all days, indicating autofluorescence, Figure 1 and Table 1).

E. viridis displayed a gradual increase in Φ_{PSII} values over the first four days of the experiment (40% lower than *C. fragile*), and similar values as *C. fragile* on the last two days of the experiment (Fig. 1 and Table 1).

α in *E. viridis* was similar to *C. fragile* for the whole experiment period. However, P_{max} and E_k in *E. viridis* varied significantly compared to *C. fragile* (Table 1), with values ranging from two to six times higher than in *C. fragile* for the whole experiment.

Experiment 1, *in vitro* results

The extraction of pigments from *C. fragile*, *E. viridis* and *P. dendritica* (Table 2), concluding experiment 1, showed the presence of both photosynthetic chlorophylls and carotenoids in all samples. In *C. fragile*, only photosynthetic pigments were found: chl *a* and *b*, and the carotenoids siphonaxanthin and siphonein, violaxanthin, neoxanthin and β, ϵ -carotene.

The percent chl *b* to chl *a* (w:w) for both slugs was similar to *C. fragile*, ranging from 45-47%. However, there was 8% chl *a* like and 31% phaeophorbide *a* pigments in *P. dendritica*, and 12% phaeophytin *a* in *E. viridis*, indicating degradation of chlorophylls. The presence of chl *c* like pigment in both slugs was not found in *C. fragile*, indicating remains of phaeophytes (brown algae) in the slugs.

Of the photosynthetic carotenoids present in *C. fragile*, neoxanthin was absent in *P. dendritica*. However, the percent violaxanthin and β, ϵ -carotene content relative to chl *a* (w:w) was 4% and 15% higher in *P. dendritica* than in *C. fragile*. *E. viridis* contained all photosynthetic pigments found in *C. fragile*, only the percentage of neoxanthin and siphonein relative to chl *a* were 4% and 6% higher in *E. viridis* compared to *C. fragile*. The LM sections of *P. dendritica* showed that the digestive gland extends into the cerata (Fig. 2a-c). This seemed to be the only extension of the digestive gland from the gut, and no other ramifications of the digestive gland tubules were observed. The digestive gland tubule in a cerata occupied the whole volume with a very wide lumen.

The LM sections through the parapodium of *E. viridis* were perforated with narrow digestive gland tubules (Fig. 3a-c). This showed that the digestive gland was extended throughout the entire body of the slug and was highly ramified.

TEM sections of digestive cells in the cerata of *P. dendritica* showed degraded chloroplasts in several stages of digestion (chlp 1-4 in Fig. 4a and b). Some chloroplasts seemed structurally compact with a phagosome membrane surrounding the chloroplast (chlp1) indicating the first stage of degradation. Chlp1-chloroplasts are characterised by the dense stacking of the thylakoid membranes so that they can not be discerned from the chloroplast double membrane, and the intact starch grain. As degradation of the chloroplasts progressed (chlp2), the phagosome membrane, the chloroplast double membrane, and the thylakoid membranes are disintegrating. The starch grain began to break up. At level 3 (chlp3), the breaking up of the starch grain doubled the size of the “degrading chloroplast vacuole”. We

could still see fragments of the phagosome membrane, the chloroplast double membrane and the thylakoid membranes dispersed around the edges of the vacuole. The last stage of degradation appeared to be electron dense material occupying the vacuole with undifferentiated material dispersed around the edges (chlp4), leaving what seemed to be an empty vacuole. These observations showed that in *P. dendritica*, the phagosome membrane, the chloroplast double membrane, the thylakoid membranes and the starch grain degraded simultaneously leaving only empty vacuoles. No plastoglobuli were observed in any of the degraded chloroplasts in the digestive cells of *P. dendritica*.

The TEM images of the digestive glands in *E. viridis* consisted mostly of intact chloroplasts, but in some of the chloroplasts, the phagosome membrane and the chloroplast double membrane appeared to have burst open, exposing the thylakoid membranes to the digestive cell cytoplasm (Fig. 5a). We could see that the phagosome membrane and the thylakoid membranes were degraded, leaving only the chloroplast double membrane with a more or less intact starch grain inside it (dgc in Fig. 5a and b). All intact chloroplasts were surrounded by a phagosome and had a spherical shape with distinct thylakoid membranes, and in most the starch grains was visible, and plastoglobuli were present (Fig. 5a and b). The digestive cell contained apart from 46-57% chloroplasts, a digestive cell nucleus, mitochondria, digestive vacuoles and lots of ribosomes dispersed throughout the digestive cell cytoplasm (Fig. 5a).

Experiment 2, *in vivo* results

Three individuals of *E. viridis* were kept without the possibility to feed on *C. fragile* and ingest fresh chloroplasts. The starving conditions as a function of time resulted in a loss of 33-49% wet weight in the largest to the smallest slugs during 73 days of starvation (Fig. 6a). The photosynthetic activity also decreased as a function 73 days of starvation and size of the slugs. First of all, Φ_{PSII} decreased 28-29% for the two larger slugs, and by 47% for the smallest slug (Fig. 6b). In this regard, a RFC of 155 days could be estimated for the smallest slug, and a RFC of 261 and 273 days for the larger slugs.

Secondly, plotting the photosynthetic parameters as a function of time, gave a decrease in P_{max} of 49-74% from the smallest to the largest slug (Fig. 7a), and a decrease in α of 70% to 20-29% from the smallest to the largest slugs (Fig. 7b). However, E_k as a function of time varied differently (Fig. 7c), where the smallest slug showed E_k to increase by 68%, and decrease by 22-57% in the two larger slugs.

Discussion

Placida dendritica

In experiment 1, the *in vivo* results indicate that the chloroplasts in the digestive cells of *Placida dendritica* feeding on *Codium fragile* are not functional ($\Phi_{PSII} = 0$) (Fig.1, Table 1). This is in contrast to Taylor (1968), Greene & Muscatine (1972) and Hinde (1980) who observed photosynthetic ^{14}C incorporation in *P. dendritica*. In this regard, Greene and Muscatine explained a low uptake of ^{14}C in chloroplasts from *C. fragile* in *P. dendritica* as a result of passive uptake of ^{14}C in slugs (which they defined as “heterotrophic fixation”) and not by photosynthesis. In fact, structural damage to the chloroplasts in *P. dendritica* were observed within minutes after ingestion by McLean (1976), suggesting a quick loss of photosynthetic activity. McLeans’ observations still show structurally intact chloroplasts when they are in the process of being phagocytosed into the digestive cell. The thylakoid membranes are still clearly visible and it is easy to discern their organisation into layers. However, as soon as the chloroplasts are truly incorporated into the digestive cell, the

thylakoid membrane structure becomes indistinct. The two first degradation stages described in this study (chlp 1 and 2 in Fig. 4b) are therefore also found in McLean's study, as well as in Hinde (1980) and Taylor (1968).

The pigment composition in *P. dendritica* compared to the food source suggested a mix of photosynthetic and degraded chloroplasts (Table 2). However, the presence of highly degraded chlorophylls in *P. dendritica*, with 31% of phaeophorbide *a* and 8% of chl *a* like pigment relative to chl *a*, indicates that chloroplasts are being digested as soon as they enter the digestive cells. The digestive gland branches into each ceratum, where it appears wrinkled but seems to occupy the ceratum as a single tubule (Fig. 2b). In this regard long main ducts and rather wide lumina of the digestive gland tubules are associated with reduced functional kleptoplasty (Clark et al. 1990). The presence of functional chloroplasts have been observed by means of by ^{14}C uptake in related limapontid species like *Hermaea bifida* and *Costasiella lilianae* (Kremer & Schmitz 1976, Clark et al. 1981) and by Φ_{PSII} measurements in *Ercolania kencelesi* (Grzybowski et al. 2007). The observation of ^{14}C uptake in *H. bifida* and *C. lilianae* needs further study, since Green & Muscatine (1972) suggested that the presence of ^{14}C in *P. dendritica* was caused by passive uptake of ^{14}C ; in *H. bifida*, ^{14}C assimilation rate in the algae was estimated to be a thousand times higher than in the slug, and in *E. kencelesi*, Φ_{PSII} values were lower than $0.2 \text{ mol e}^- \text{ mol photons}^{-1}$ in all measurements (Grzybowski et al. 2007), indicating that low Φ_{PSII} values are attributed to degraded chloroplasts. This implies that most limapontid sacoglossans can be designated to RFC level 1-3, retention of non-functional chloroplasts (Clark et al. 1990, Evertsen et al. 2007).

Elysia viridis

During the 8 days that *Elysia viridis* was allowed to feed on *C. fragile*, photosynthetic activity appeared as a gradual increase and stabilisation of Φ_{PSII} and α values (Table 1). This is probably a result of *E. viridis* filling the digestive cells with functional chloroplasts (Fig. 5a and b). The similarity of Φ_{PSII} and α between *E. viridis* and *C. fragile* indicates that the photoacclimation status of the chloroplasts in the digestive cells of *E. viridis* is the same as for chloroplasts in *C. fragile*. However, P_{max} and E_k varied greatly in *E. viridis*, and were 2-6 times higher than in *C. fragile* (Table 1). Since α was constant as a function of time in *E. viridis* from the first day, E_k varies as a function of P_{max} . The variations indicate a high turnover of chloroplasts when *E. viridis* is feeding on *C. fragile*, and reflects the photoacclimation status of *C. fragile*. Gallop et al. (1980) observed that feeding animals replaced 75% of their chloroplasts during a 9 day period, while starving animals only lost 15% in the same period. This implies that with the constant replacement of chloroplasts throughout the whole digestive gland system, the threshold where all RC_{PSII} are saturated with photons will also vary as a function of [functional chloroplasts] in the digestive system. The 2-6 times higher values of P_{max} and E_k also indicate that the chloroplasts in the digestive cells of *E. viridis* receive more light than chloroplasts in *C. fragile*, e.g. as high light acclimation. We find this to be unlikely since chloroplasts in the slugs need to divide to actively photoacclimate (Cran & Possingham 1974). Instead, the higher E_k values probably reflects the morphological adaptations in plakobranchiid (= Elysiidae) sacoglossans, where an increased branching of the digestive gland provides larger surface areas for retention of photosynthetic chloroplasts (Jensen 1997). This implies that the package effect, which is an effect of intracellular self-shading dependent on cell size and shape, cellular pigment composition, chloroplast size, shape, number and morphology, and thylakoid stacking (Johnsen et al. 1994), is less for chloroplasts in *E. viridis* than for chloroplasts in *C. fragile*, inducing higher E_k values in the former. Since both α and P_{max} comprise Φ_{PSII} , the former

parameter can not explain the increase in P_{\max} and E_k . Since the pigment composition remains unchanged, α and q should not either change. The increase in E_k may therefore be attributed to an increase of τ , leading to a decreased turnover time of e^- , indicating that chloroplasts in the digestive cells of sacoglossans experience higher irradiances than in the food algae. The LM images of the parapodium of an *E. viridis* support these results, where we observed that the digestive gland tubules are evenly distributed throughout the slug tissue (Fig. 3b).

The amount of photosynthetic pigments relative to chl *a* (w:w) of the chloroplasts in *E. viridis* were overall the same as for *C. fragile*, which support the observations for Φ_{PSII} that all light harvesting pigments are present compared to the food source, and that the photosynthetic chloroplasts in *E. viridis* do not photoacclimate in regard of synthesising new pigments, because of arrested chloroplast division in the digestive cells (Table 2). The presence of 12% pheophytin *a* relative to chl *a* in *E. viridis* indicates that some degradation of chloroplasts is present, reflecting the turnover of up to 75% of the chloroplast as observed by Gallop et al. (1980).

The degradation of the *C. fragile* chloroplasts in *E. viridis* seems different from *P. dendritica*. In *P. dendritica*, the phagosome membrane, the chloroplast double membrane, the thylakoid membranes and the starch grain disintegrate at the same rates (Fig. 4b). In *E. viridis*, only the phagosome and the thylakoid membranes seem to be degraded, leaving only the chloroplast double membrane containing an intact starch grain (Fig. 5b). These observations may explain the confusion whether the phagosome membrane envelops the chloroplast throughout the existence of the chloroplast or not (Trench et al. 1973b, Hawes 1979, Hawes & Cobb 1980), where the former suggested that the phagosome membrane is re-absorbed so that functional chloroplasts lie free in the cytoplasm of the digestive cell. What we see, is in fact only damaged chloroplasts exposed to the cytoplasm of the digestive cell, leading to degradation. The functional chloroplasts are still enveloped in the phagosome in this study. In this regard these cytoplasm-exposed and degraded chloroplasts are related to the turnover of chloroplasts in feeding *E. viridis* explained by Gallop et al. (1980). It is also important to note that a large number of ribosomes were dispersed in clusters all over the digestive cell cytoplasm. It is possible that they are linked to the leakage and utilisation of photosynthetic products from the chloroplasts (Gallop 1974). Plastoglobuli were present inside most of the chloroplasts (Fig. 5c). They function as lipid storage sites outside the thylakoid membranes and contain the lipophilic quinones functioning as the oxidation-reduction catalysts in the photochemically active thylakoid membranes (Tevini & Steinmuller 1985), indicating that the thylakoid membranes of the chloroplasts are not being repaired, but showing a sign of beginning senescence. This indicates that the synthesis of thylakoid membranes in the chloroplasts is arrested, which is supported by Hawes & Cobb (1980) who observed an increase in the number of plastoglobuli per chloroplast in the digestive cells of *E. viridis* after 28 days of starvation.

In experiment 2, the starving experiment with *E. viridis* removed from *C. fragile* for 73 days, the observations indicate an arrested chloroplast division and no active photoacclimation in the chloroplasts. The decline in Φ_{PSII} (Fig. 6a) can be related to chloroplast health status (Björkmann & Demmig 1987), as a result of a reduction in the number of functional RC_{PSII} of the PSU. A lack of functional D1, one of the reaction centre proteins of PSII, is related to a decrease in Φ_{PSII} when the PSU is not repaired (Vasilikiotis & Melis 1994). This is also supported by the decrease of α in our results (Fig. 7b). Since the chloroplasts are not able to rebuild their membranes, repair damaged PSU's or synthesise new pigments, the

chloroplasts will degrade as function of time. This is also correlated with the decrease in P_{\max} in our study (Fig. 7a), which is related to the chl *a* content in the PSU and the enzymatic processes related to the electron transport chain (Dubinsky et al. 1986). The varying responses in E_k (Fig. 7c) may be attributed to P_{\max} and α whose decrease is related to a reduction in PSII functionality (non-functional D1, arrested pigment synthesis and PSU repair) and declining enzyme activity caused by arrested chloroplast division. Related studies have observed that chloroplasts from *C. fragile* in the digestive cells of *E. viridis* can not synthesise chl *a*, galactolipids or membrane proteins (Trench & Smith 1970, Trench et al. 1973b, Trench & Olhorst 1976). This implies that chloroplasts from *C. fragile* when they are sucked out of the algal cell environment and phagocytosed into the digestive cells of *E. viridis*, are not able to neither renew thylakoid membranes nor replace pigments. Hawes & Cobb (1980) observed in their experiments on the effects on starvation of the chloroplasts from *C. fragile* in *E. viridis*, still intact chloroplasts present in the digestive cells of *E. viridis* after 40 days of starvation, displaying increased swelling and disintegration of the thylakoid membranes indicating a progressive degradation of the chloroplasts.

The gradual degradation and overall decrease of 28-47% in Φ_{PSII} of the chloroplasts in *E. viridis* can also be linked to the loss of 33-49% wet weight in this study (Fig. 6a and b). The two larger slugs with relatively more chloroplasts per animal lost only 33% of their wet weight, and had an estimated RFC up to 9 months. The smaller slug lost 49% of its wet weight, and had an estimated RFC of up to 5 months. Our observations on the changes in weight in starving *E. viridis* are similar to Hinde & Smith's (1975) observations where *E. viridis* fed on *C. fragile* lost 40% of initial weight after 10 weeks starvation.

The estimated RFC values of 5-9 months for *E. viridis* in this study, corresponds to level 8, characterised by retention of functional chloroplasts for more than 3 months according to Evertsen et al. (2007). In this regard, our results far exceed previous studies on RFC for *E. viridis*. Hinde & Smith (1972 and 1975) observed continuous CO₂ fixation for up to three months in *E. viridis* using ¹⁴C assimilation methods. But, there are some important differences in the experimental setup that might explain the discrepancies: in this study the slugs were kept under irradiances at 30 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ at 11° C. In Hinde & Smith (1972 and 1975) the sacoglossans were kept at 18° C and 440 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, inducing high light conditions that cause a relatively higher respiration to photosynthetic rates compared to our cool and low light conditions. High light conditions result in a rapid turnover of the D1 protein in RC_{PSII} which plays a major role in maintaining PSII integrity in high light (Franklin & Larkum 1997).

The presence of siphonaxanthin and siphonein in green macroalgae is variable, however, among the Ulvophyceae, siphonaxanthin and siphonein is present in the Bryopsidales (=Caulerpales) no matter their depth or habitat distribution (Yokohama 1981). For the other ulvophyceae taxa, siphonaxanthin, but not siphonein, is present in the Ulvales, Siphonocladales and Cladophorales in deep water or shaded habitats. Siphonaxanthin and siphonein is lacking in the Dasycladales. For details considering the phylogeny of the ulvophyceae taxa, confer Lam & Zechman (2006), Hayden & Waaland (2002), Leliaert et al (2007) and Zechman (2003). The presence of siphonaxanthin and siphonein together in a sacoglossan with photosynthetic chloroplasts can therefore be used as chemotaxonomic markers to indicate Bryopsidales as a food source. This is important since sacoglossans are not only reported to feed on coenocytic and siphonous green algae, but also red algae, brown algae and seagrasses (Jensen 1980), and only a handful of sacoglossans species with

photosynthetic chloroplasts have been associated with a chloroplast donor algae (Clark et al 1990, Evertsen et al 2007).

Conclusion

The chloroplasts from *Codium fragile* seem to be retained differently in *Placida dendritica* compared to *Elysia viridis*. In *P. dendritica* the membranes of the phagosomes, the chloroplast, the thylakoides, and the starch grain, is degraded as soon as the chloroplasts are phagocytosed into the digestive cells. In *E. viridis*, chloroplasts exposed to the digestive cell cytoplasm are all in the process of being degraded, but the chloroplast double membrane and the starch grain remain throughout degradation. These differences reflect that the “robustness” that has been described for coenocytic chlorophytes (Grant & Borowitzka 1984) may not be enough to explain retention of functional chloroplasts in the Sacoglossa. Instead it appears to be adaptations in the digestive system of the sacoglossans that render the slugs able to keep the chloroplasts in a functional state. Functional chloroplasts from *C. fragile* in the digestive cells of *E. viridis* are always kept within the phagosome, and have a capacity to maintain photosynthesis for 5-9 months. Even though the chloroplasts are able to maintain photosynthesis for long time periods, this study indicates that chloroplast division is arrested and that the chloroplasts are not able to photoacclimate regarding pigment synthesis, but may partially be able to adjust their turnover time of electrons (τ) to higher irradiance levels. This implies that the functionality of retained chloroplasts in various sacoglossans must also be investigated in regard of synthesis of pigments, lipids, proteins, nucleic acids, and starch formation. In this regard, it has been observed that the sacoglossan *Elysia chlorotica* from the East coast of North America which feeds on the siphonous xanthophyte *Vaucheria litorea*, retains chloroplasts that not only have a photosynthetic capacity up to 10 months, but which are also capable of synthesising several photosynthetic proteins like the carbon fixating enzyme RuBisCO, the D1, D2, and CP43 core complexes of PSII, and electron transport chain proteins like cyt f and others (Pierce et al. 1996, Mujer et al. 1996, Green et al. 2000). This demonstrates that chloroplasts have different functional capabilities depending on which algae the sacoglossan has collected chloroplasts from, and on the type of sacoglossan. It is therefore very important to use chemotaxonomical markers, as siphonaxanthin and siphonoin in this study, to identify groups of potential coenocytic algae that they harvest functional chloroplasts from.

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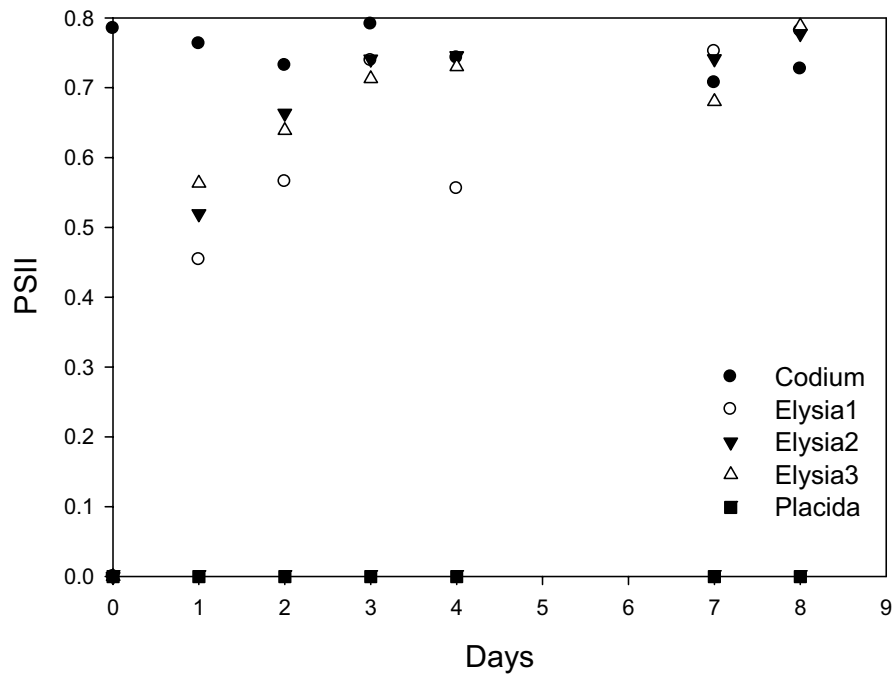


Figure 1. Experiment 1, Φ_{PSII} measured in *Codium fragile*, *Elysia viridis* and *Placida dendritica* for 8 days after being introduced to *Codium fragile*. Note that all three specimens of *P. dendritica* only showed $\Phi_{\text{PSII}} = 0$ for all days.

Table 1. Experiment 1, the P vs. E parameters measured in *Codium fragile* and in *Elysia viridis* and *Placida dendritica* introduced to *C. fragile* for a period of 8 days.

Day 1					
	Pmax	α	Ek	R2	Φ PSII
Codium	25	0.82	30.4	0.99	0.763
Elysia1	45	0.55	82.0	0.98	0.454
Elysia2	77	0.65	118.3	0.99	0.520
Elysia3	69	0.66	103.9	0.99	0.563
Placida	0	0	0.0	0	0.000
Day2					
	Pmax	α	Ek	R2	Φ PSII
Codium	19	0.82	23.2	0.99	0.732
Elysia1	49	0.74	66.2	0.95	0.566
Elysia2	93	0.82	113.4	0.99	0.663
Elysia3	66	0.84	78.6	0.98	0.639
Placida	0	0	0.0	0	0.000
Day 3					
	Pmax	α	Ek	R2	Φ PSII
Codium	45	0.91	49.5	0.99	0.791
Elysia1	92	0.95	96.8	0.99	0.739
Elysia2	93	0.8	116.3	0.99	0.741
Elysia3	73	0.96	76.0	0.97	0.713
Placida	0	0	0.0	0	0.000
Day 4					
	Pmax	α	Ek	R2	Φ PSII
Codium	24	1.04	23.1	0.97	0.743
Elysia1	22	0.78	28.2	0.85	0.746
Elysia2	62	0.99	62.6	0.99	0.730
Elysia3	37	0.98	37.8	0.92	0.752
Placida	0	0	0.0	0	0.000
Day 7					
	Pmax	α	Ek	R2	Φ PSII
Codium	22	1.02	21.6	0.91	0.707
Elysia1	78	0.95	82.1	0.99	0.741
Elysia2	55	0.89	61.8	0.98	0.680
Elysia3	45	0.79	57.0	0.98	0.781
Placida	0	0	0.0	0	0.000
Day 8					
	Pmax	α	Ek	R2	Φ PSII
Codium	15.8	0.96	16.5	0.9	0.727
Elysia1	102.8	0.94	109.4	0.99	0.735
Elysia2	113.2	0.82	138.0	0.99	0.778
Elysia3	141.5	0.91	155.5	0.99	0.788
Placida	0	0	0.0	0	0.000

Table 2. Experiment 1, photosynthetic pigments (chl *a* and *b*, siphonaxanthin, siphonoin, violaxanthin, neoxanthin and β , ϵ -carotene) and degraded pigments (chl *a* and *c* like pigments, pheophytin *a* and pheophorbide *a*) in *Elysia viridis* and *Placida dendritica* compared to *Codium fragile*, isolated by HPLC.

Pigments	Peaks	pigm:chl a		pigm:chl a		pigm:chl a	
		<i>C. fragile</i>	<i>C. fragile</i>	<i>P. dendritica</i>	<i>P. dendritica</i>	<i>E. viridis</i>	<i>E. viridis</i>
chl <i>a</i>	411,430,536,580,616,662 [1.22]	112.39	1.00	8.31	1.00	6.53	1.00
chl <i>a</i> like	431,534,575,617,662 [1.66]			0.63	0.08		
pheophytin <i>a</i>	410,475,506,535,560,609,665 [2.39]					0.82	0.12
pheophytin <i>a</i> like	410,503,536,608,666 [2.71]						
pheophorbide <i>a</i>	(401),412,478,510,541,610,667 [2.20]			2.59	0.31		
chl <i>b</i>	(425),457,596,645 [2.79]	51.09	0.45	3.89	0.47	3.00	0.46
chl <i>c</i> like	442,580,632			1.49	0.18	0.11	0.02
neoxanthin	415,439,467[0.91]	6.23	0.06			0.66	0.10
violaxanthin	418,441,471 [0.92]	0.80	0.01	0.38	0.05	0.16	0.02
viola like	418,443,472, [0.89]	1.06	0.01			0.10	0.02
siphonaxanthin	454,471	8.30	0.07	0.49	0.06	0.51	0.08
siphonoin	456,471	5.09	0.05	0.53	0.06	0.69	0.11
β , ϵ -carotene	(424),448,476 [0.5]	6.42	0.06	1.54	0.19	0.44	0.07

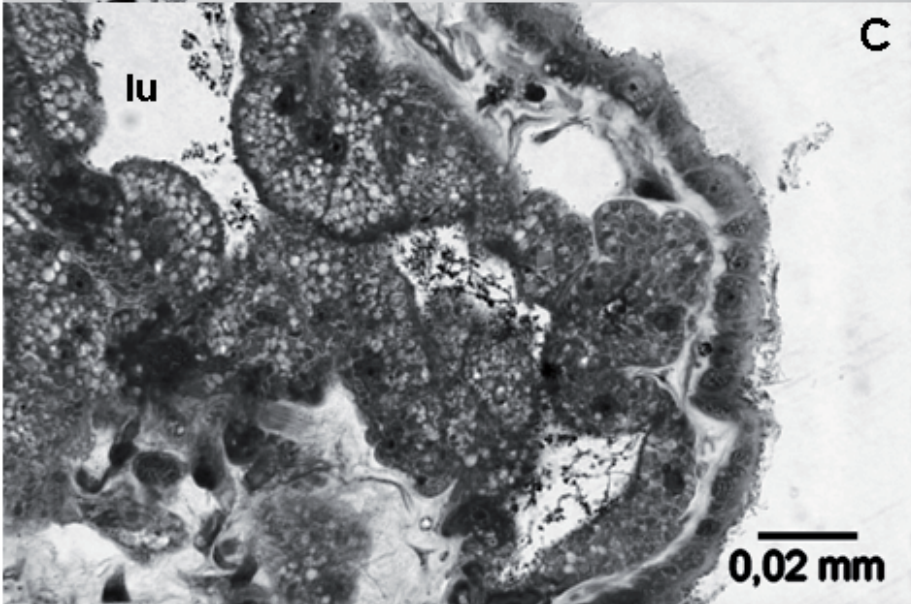
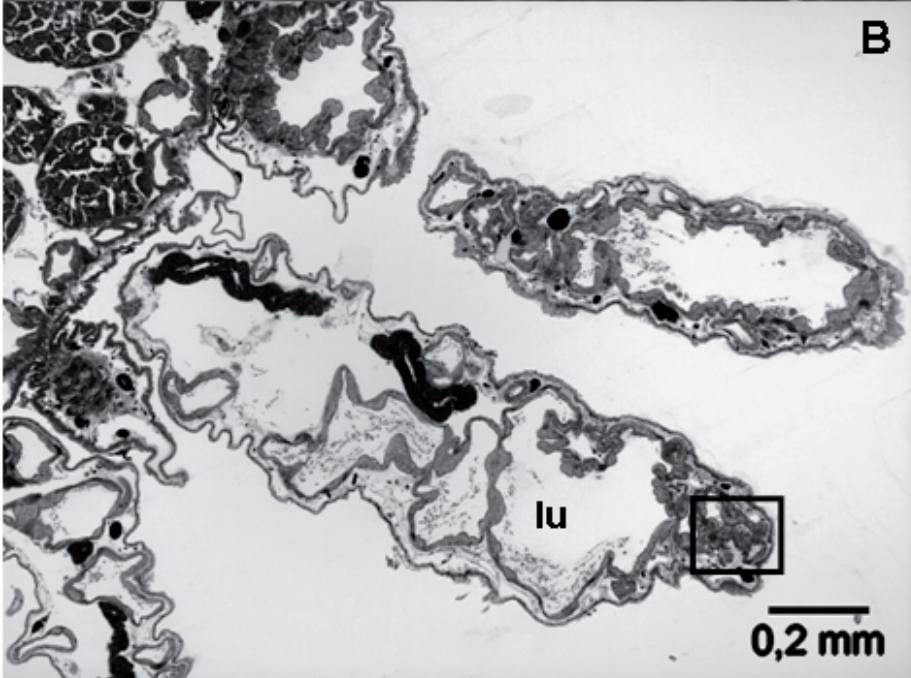
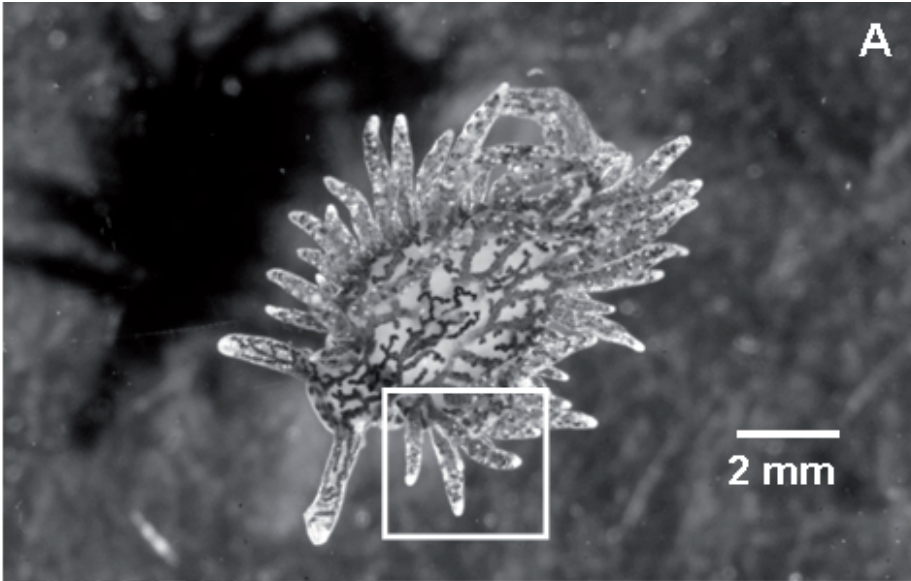


Figure 2.

a) The cerata-bearing sacoglossan *Placida dendritica*. The square denotes where the light microscope sections were taken.

b) Light microscope sections showing the wide lumina of the digestive gland extending into the cerata (lu = lumen).

c) Close up of the tip of the ceratum (indicated by the square in b) showing the digestive cells lining the inside of the lumen.

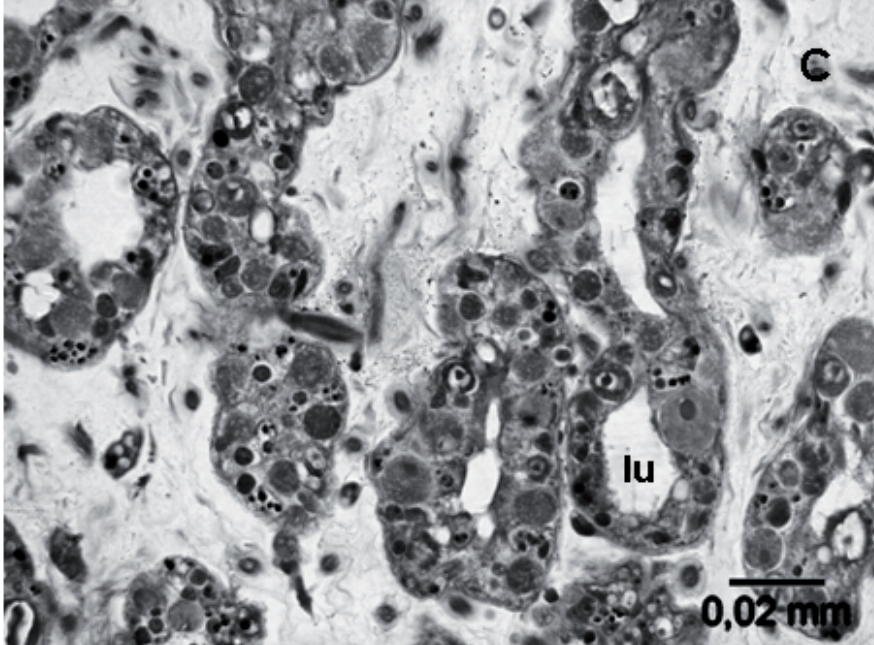
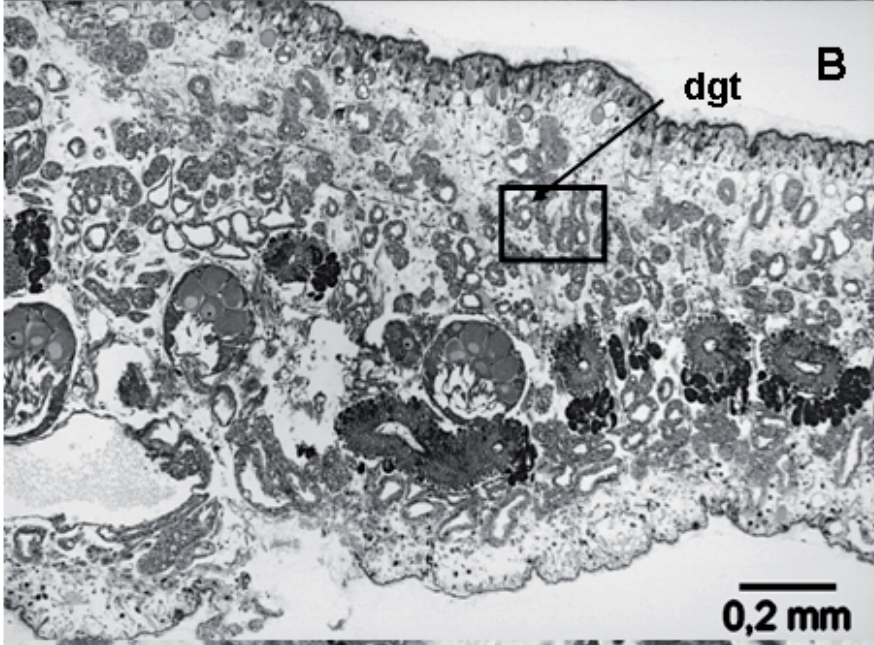
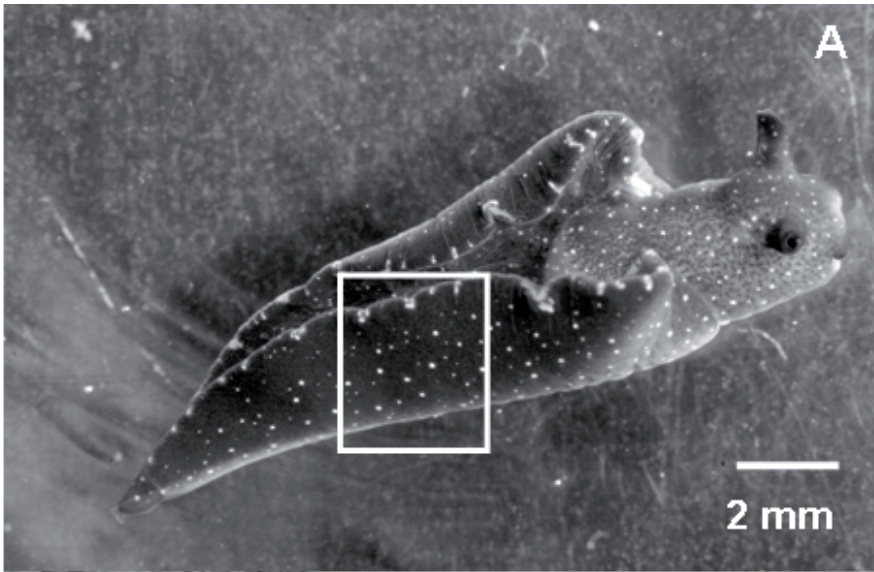


Figure 3.

- a) The parapodia-bearing plakobranchid sacoglossan *Elysia viridis*. The square indicates the light microscopy sections cut from the parapodium.
- b) A light microscopy section through the parapodium of *E. viridis* which clearly shows the digestive gland tubules (dgt) perforating the most of the tissue.
- c) A close up of the square indicated in b, showing the digestive gland tubules as doughnut shaped bodies surrounding a lumen.

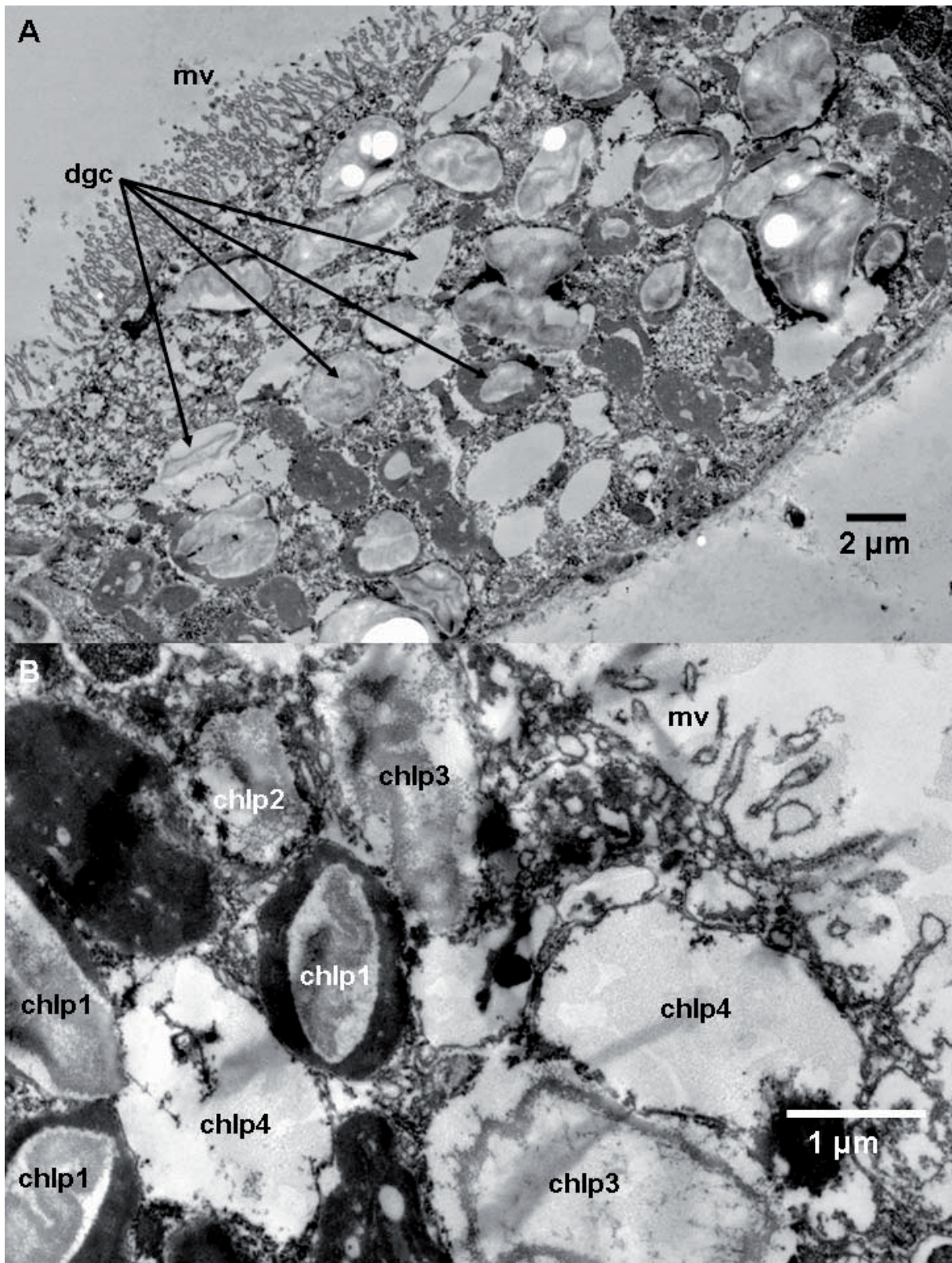


Figure 4.

a) The digestive cells in *Placida dendritica* with microvilli (mv) showing retained degraded chloroplasts (dgc).

b) A close up of the degraded chloroplasts reveals four stages of degradation (chlp1-4): chlp1 are chloroplasts surrounded by an intact phagosome membrane, thylakoid membranes are not distinct surrounding a starch grain, chlp2 also have a phagosome membrane but thylakoide

membranes are beginning to disintegrate and the starch grain is still visible, chlp3 are only the remnants of the starch surrounded by fragments of the thylakoide membranes, chlp4 are totally disintegrated chloroplasts only appearing as electron dense (empty) vacuoles.

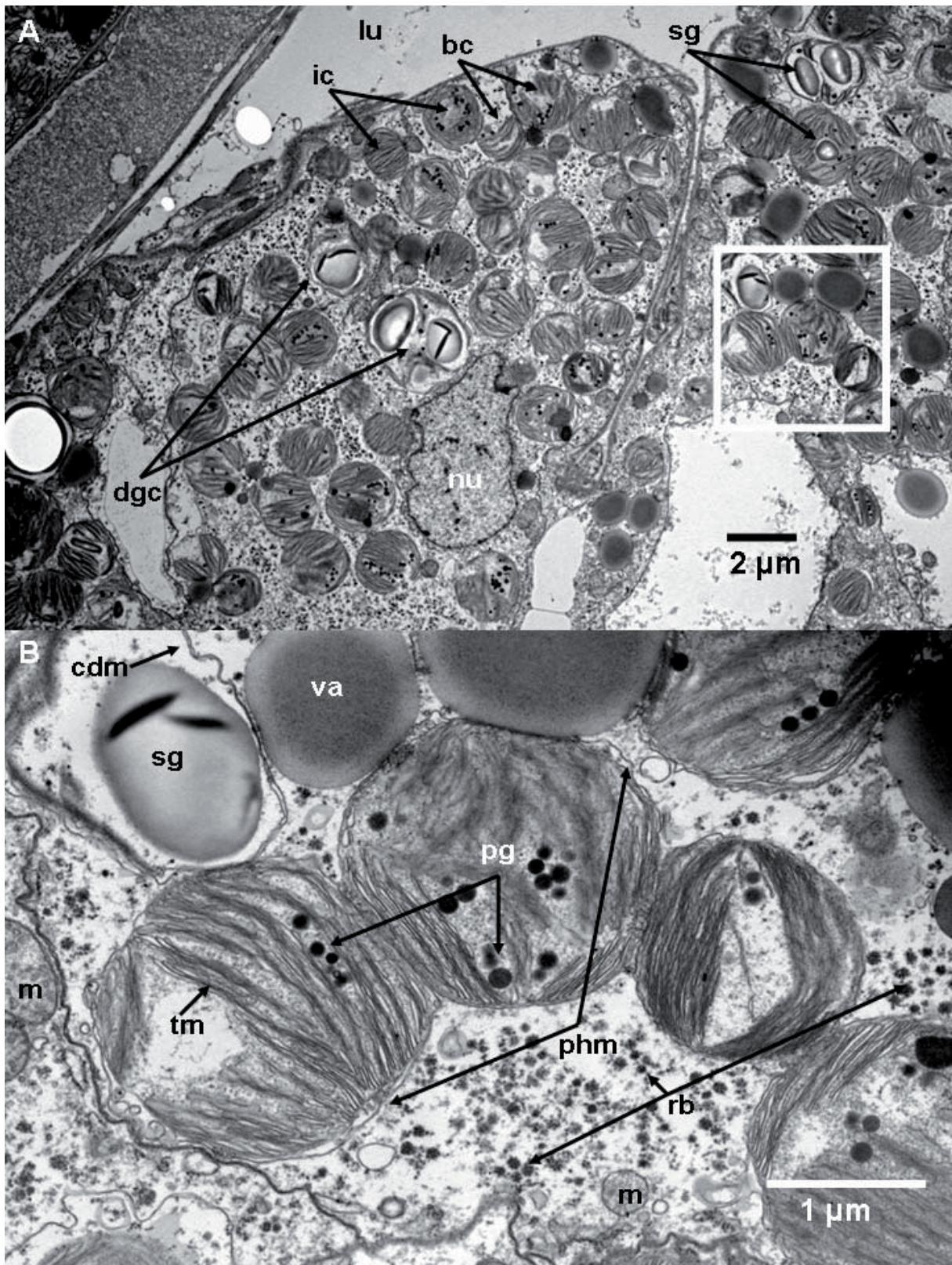


Figure 5.

a) Digestive cell in *Elysia viridis* with intact chloroplasts (it), broken chloroplasts exposed to the digestive cell cytoplasm (bc), digested chloroplasts where only the chloroplast membrane and starch grain (sg) are left (dgc), a nucleus (nu), lumen (lu). The square indicates the close up of chloroplasts shown in b. The estimated area covered by chloroplast ranging from 2-2.5 μm in diameter is 110-137 μm^2 covered by the 35 chloroplasts counted in this digestive cell.

The area of this digestive cell is estimated to $240 \mu\text{m}^2$. The area covered by the chloroplasts amounts to 46-57% of the digestive cell.

b) Close up of intact chloroplasts surrounded by the phagosome membrane (phm) and digested chloroplasts with a starch grain (sg) and a double chloroplast membrane (dcm). Note the presence of distinct thylakoid membranes (tm) and plastoglobuli (pg) in the intact chloroplasts. The cytoplasm surrounding the chloroplasts is filled with ribosomes (rb), some mitochondria (m) and vacuoles (va).

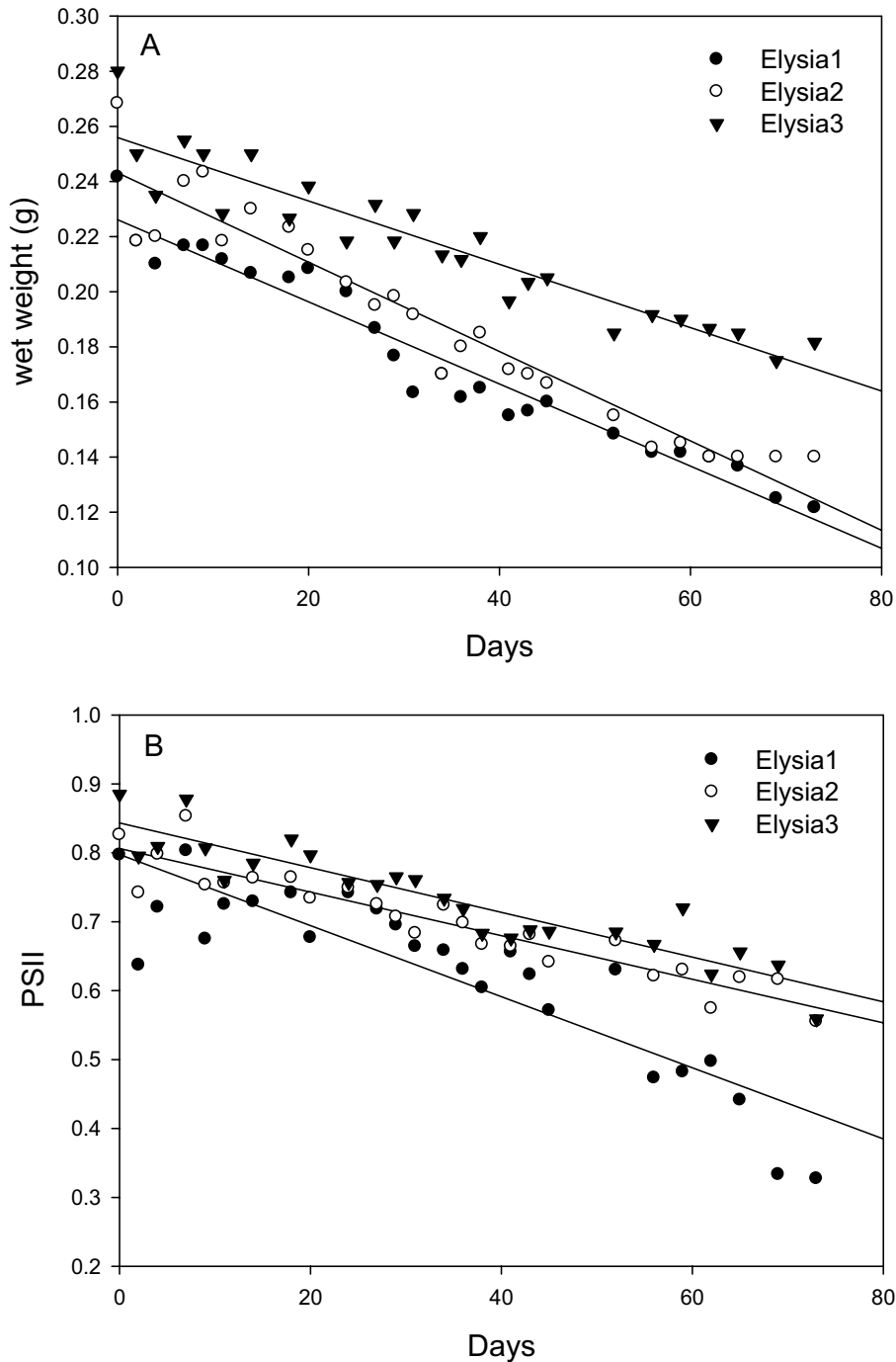
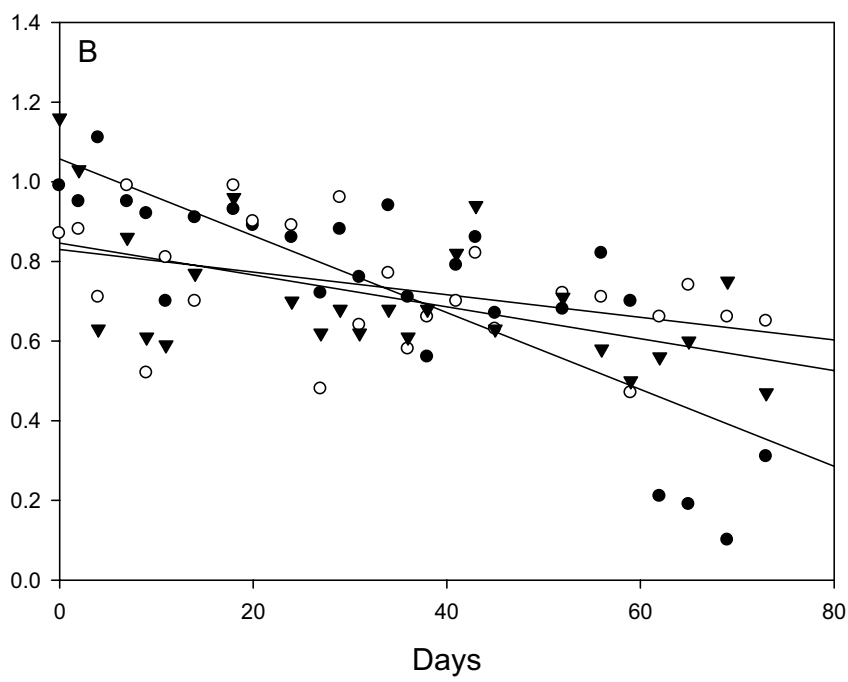
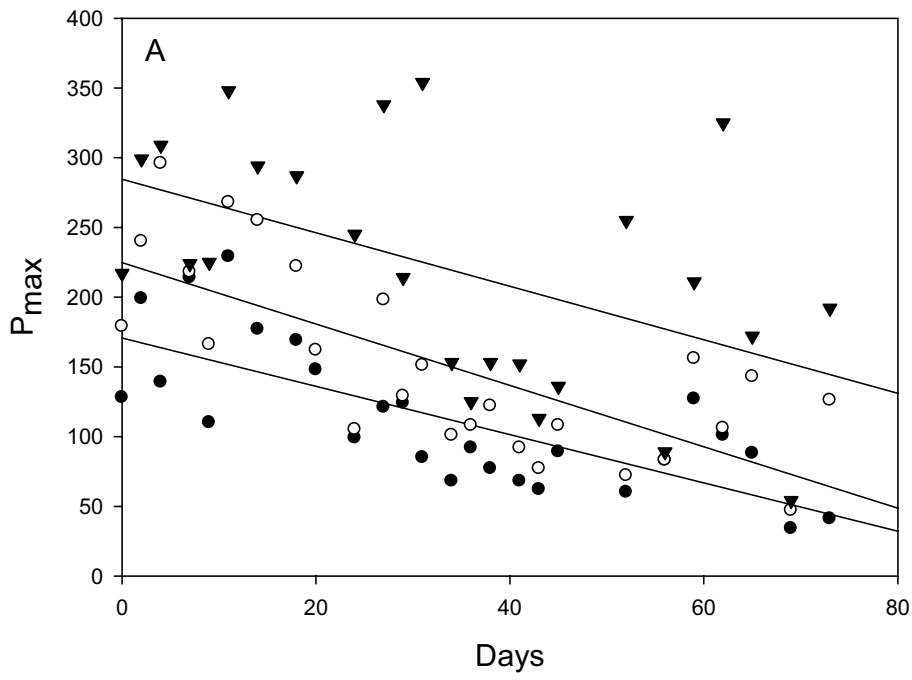


Figure 6. Experiment 2, starving experiment with *Elysia viridis* removed from *Codium fragile*, showing Φ_{PSII} and change in wet weight as a function of days.

a) The initial wet weight in the smallest slug (Elysia1) was 0.242 g, and 0.268 and 0.280 g in the two larger slugs (Elysia2 and 3), giving a daily decrease in wet weight of 0.0015, 0.0016 and 0.0012 g day⁻¹ respectively from the smallest to the largest slug.

b) The daily decrease in Φ_{PSII} in the three specimens of *E. viridis* is calculated to 0.0032 Φ_{PSII} day⁻¹ for the smallest slug (Elysia1) and 0.0052 Φ_{PSII} day⁻¹ for the two larger slugs (Elysia2 and 3). The respective RFC values for each specimen are then estimated to 155 days estimated for the smallest slug based on an initial Φ_{PSII} of 0.797 mol e⁻ mol photons⁻¹, 261 and 273 days for the two larger slugs estimated from initial Φ_{PSII} of 0.826 and 0.855 mol e⁻ mol photons⁻¹ respectively.



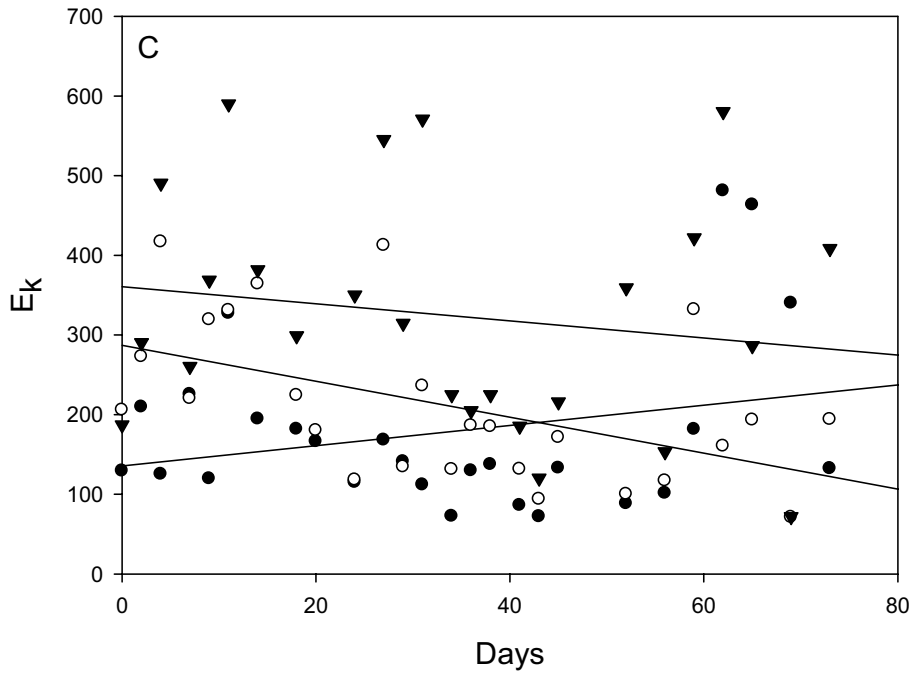


Figure 7. Experiment 2, starving experiment with *Elysia viridis* removed from *Codium fragile*, showing the P vs. E parameters P_{\max} , α and E_k as a function of days.

a) The daily decrease in P_{\max} is calculated to 1.73, 2.20 and 1.92 ((mol e^- mol photons $^{-1}$)(μ mol photons m^{-2} s^{-1})) day^{-1} from the smallest (Elysia1) to the smallest slug (Elysia3).

b) The daily decrease in α is calculated to 0.00960 to 0.0028 and 0.0040 ((mol e^- mol photons $^{-1}$)(μ mol photons m^{-2} s^{-1}) 2) day^{-1} from the smallest (Elysia1) to the two larger slugs (Elysia2 and 3).

c) In the smallest of the three specimens (Elysia1), the E_k increased by 1.27 μ mol photons m^{-2} s^{-1} day^{-1} , whilst for the two larger specimens E_k decreased by 2.25 and 1.07 μ mol photons m^{-2} s^{-1} day^{-1} (Elysia2 and 3).

Paper IV

Evertsen J, Johnsen G, Tvedt K (2008) *In vivo* and *in vitro* characteristics of photosynthetic chloroplasts retained in some sacoglossans (Gastropoda, Opisthobranchia) from Lizard Island, Great Barrier Reef. (manuscript)

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Doctoral theses in Biology
Norwegian University of Science and Technology
Department of Biology

Year	Name	Degree	Title
1974	Tor-Henning Iversen	Dr. philos. Botany	The roles of statholiths, auxin transport, and auxin metabolism in root gravitropism
1978	Tore Slagsvold	Dr. philos. Zoology	Breeding events of birds in relation to spring temperature and environmental phenology.
1978	Egil Sakshaug	Dr. philos. Botany	"The influence of environmental factors on the chemical composition of cultivated and natural populations of marine phytoplankton"
1980	Arnfinn Langeland	Dr. philos. Zoology	Interaction between fish and zooplankton populations and their effects on the material utilization in a freshwater lake.
1980	Helge Reinertsen	Dr. philos. Botany	The effect of lake fertilization on the dynamics and stability of a limnetic ecosystem with special reference to the phytoplankton
1982	Gunn Mari Olsen	Dr. scient. Botany	Gravitropism in roots of <i>Pisum sativum</i> and <i>Arabidopsis thaliana</i>
1982	Dag Dolmen	Dr. philos. Zoology	Life aspects of two sympatric species of newts (<i>Triturus</i> , <i>Amphibia</i>) in Norway, with special emphasis on their ecological niche segregation.
1984	Eivin Røskaft	Dr. philos. Zoology	Sociobiological studies of the rook <i>Corvus frugilegus</i> .
1984	Anne Margrethe Cameron	Dr. scient. Botany	Effects of alcohol inhalation on levels of circulating testosterone, follicle stimulating hormone and luteinizing hormone in male mature rats
1984	Asbjørn Magne Nilsen	Dr. scient. Botany	Alveolar macrophages from expectorates – Biological monitoring of workers exposed to occupational air pollution. An evaluation of the AM-test
1985	Jarle Mork	Dr. philos. Zoology	Biochemical genetic studies in fish.
1985	John Solem	Dr. philos. Zoology	Taxonomy, distribution and ecology of caddisflies (<i>Trichoptera</i>) in the Dovrefjell mountains.
1985	Randi E. Reinertsen	Dr. philos. Zoology	Energy strategies in the cold: Metabolic and thermoregulatory adaptations in small northern birds.
1986	Bernt-Erik Sæther	Dr. philos. Zoology	Ecological and evolutionary basis for variation in reproductive traits of some vertebrates: A comparative approach.
1986	Torleif Holthe	Dr. philos. Zoology	Evolution, systematics, nomenclature, and zoogeography in the polychaete orders <i>Oweniimorpha</i> and <i>Terebellomorpha</i> , with special reference to the Arctic and Scandinavian fauna.
1987	Helene Lampe	Dr. scient. Zoology	The function of bird song in mate attraction and territorial defence, and the importance of song repertoires.
1987	Olav Hogstad	Dr. philos. Zoology	Winter survival strategies of the Willow tit <i>Parus montanus</i> .

1987 Jarle Inge Holten	Dr. philos Botany	Autecological investigations along a coast-inland transect at Nord-Møre, Central Norway
1987 Rita Kumar	Dr. scient Botany	Somaclonal variation in plants regenerated from cell cultures of <i>Nicotiana sanderae</i> and <i>Chrysanthemum morifolium</i>
1987 Bjørn Åge Tømmerås	Dr. scient. Zoology	Olfaction in bark beetle communities: Interspecific interactions in regulation of colonization density, predator - prey relationship and host attraction.
1988 Hans Christian Pedersen	Dr. philos. Zoology	Reproductive behaviour in willow ptarmigan with special emphasis on territoriality and parental care.
1988 Tor G. Heggberget	Dr. philos. Zoology	Reproduction in Atlantic Salmon (<i>Salmo salar</i>): Aspects of spawning, incubation, early life history and population structure.
1988 Marianne V. Nielsen	Dr. scient. Zoology	The effects of selected environmental factors on carbon allocation/growth of larval and juvenile mussels (<i>Mytilus edulis</i>).
1988 Ole Kristian Berg	Dr. scient. Zoology	The formation of landlocked Atlantic salmon (<i>Salmo salar</i> L.).
1989 John W. Jensen	Dr. philos. Zoology	Crustacean plankton and fish during the first decade of the manmade Nesjø reservoir, with special emphasis on the effects of gill nets and salmonid growth.
1989 Helga J. Vivås	Dr. scient. Zoology	Theoretical models of activity pattern and optimal foraging: Predictions for the Moose <i>Alces alces</i> .
1989 Reidar Andersen	Dr. scient. Zoology	Interactions between a generalist herbivore, the moose <i>Alces alces</i> , and its winter food resources: a study of behavioural variation.
1989 Kurt Ingar Draget	Dr. scient Botany	Alginate gel media for plant tissue culture,
1990 Bengt Finstad	Dr. scient. Zoology	Osmotic and ionic regulation in Atlantic salmon, rainbow trout and Arctic charr: Effect of temperature, salinity and season.
1990 Hege Johannesen	Dr. scient. Zoology	Respiration and temperature regulation in birds with special emphasis on the oxygen extraction by the lung.
1990 Åse Krøkje	Dr. scient Botany	The mutagenic load from air pollution at two work-places with PAH-exposure measured with Ames Salmonella/microsome test
1990 Arne Johan Jensen	Dr. philos. Zoology	Effects of water temperature on early life history, juvenile growth and prespawning migrations of Atlantic salmon (<i>Salmo salar</i>) and brown trout (<i>Salmo trutta</i>): A summary of studies in Norwegian streams.
1990 Tor Jørgen Almaas	Dr. scient. Zoology	Pheromone reception in moths: Response characteristics of olfactory receptor neurons to intra- and interspecific chemical cues.
1990 Magne Husby	Dr. scient. Zoology	Breeding strategies in birds: Experiments with the Magpie <i>Pica pica</i> .
1991 Tor Kvam	Dr. scient. Zoology	Population biology of the European lynx (<i>Lynx lynx</i>) in Norway.
1991 Jan Henning L'Abée Lund	Dr. philos. Zoology	Reproductive biology in freshwater fish, brown trout <i>Salmo trutta</i> and roach <i>Rutilus rutilus</i> in particular.
1991 Asbjørn Moen	Dr. philos Botany	The plant cover of the boreal uplands of Central Norway. I. Vegetation ecology of Sølendet nature reserve; haymaking fens and birch woodlands
1991 Else Marie Løbersli	Dr. scient Botany	Soil acidification and metal uptake in plants

1991 Trond Nordtug	Dr. scient. Zoology	Reflectometric studies of photomechanical adaptation in superposition eyes of arthropods.
1991 Thyra Solem	Dr. scient. Botany	Age, origin and development of blanket mires in Central Norway
1991 Odd Terje Sandlund	Dr. philos. Zoology	The dynamics of habitat use in the salmonid genera <i>Coregonus</i> and <i>Salvelinus</i> : Ontogenic niche shifts and polymorphism.
1991 Nina Jonsson	Dr. philos.	Aspects of migration and spawning in salmonids.
1991 Atle Bones	Dr. scient. Botany	Compartmentation and molecular properties of thioglucoside glucohydrolase (myrosinase)
1992 Torggrim Breiehagen	Dr. scient. Zoology	Mating behaviour and evolutionary aspects of the breeding system of two bird species: the Temminck's stint and the Pied flycatcher.
1992 Anne Kjersti Bakken	Dr. scient. Botany	The influence of photoperiod on nitrate assimilation and nitrogen status in timothy (<i>Phleum pratense</i> L.)
1992 Tycho Anker-Nilssen	Dr. scient. Zoology	Food supply as a determinant of reproduction and population development in Norwegian Puffins <i>Fratercula arctica</i>
1992 Bjørn Munro Jenssen	Dr. philos. Zoology	Thermoregulation in aquatic birds in air and water: With special emphasis on the effects of crude oil, chemically treated oil and cleaning on the thermal balance of ducks.
1992 Arne Vollan Aarset	Dr. philos. Zoology	The ecophysiology of under-ice fauna: Osmotic regulation, low temperature tolerance and metabolism in polar crustaceans.
1993 Geir Slupphaug	Dr. scient. Botany	Regulation and expression of uracil-DNA glycosylase and O ⁶ -methylguanine-DNA methyltransferase in mammalian cells
1993 Tor Fredrik Næsje	Dr. scient. Zoology	Habitat shifts in coregonids.
1993 Yngvar Asbjørn Olsen	Dr. scient. Zoology	Cortisol dynamics in Atlantic salmon, <i>Salmo salar</i> L.: Basal and stressor-induced variations in plasma levels and some secondary effects.
1993 Bård Pedersen	Dr. scient. Botany	Theoretical studies of life history evolution in modular and clonal organisms
1993 Ole Petter Thangstad	Dr. scient. Botany	Molecular studies of myrosinase in Brassicaceae
1993 Thrine L. M. Heggberget	Dr. scient. Zoology	Reproductive strategy and feeding ecology of the Eurasian otter <i>Lutra lutra</i> .
1993 Kjetil Bevanger	Dr. scient. Zoology	Avian interactions with utility structures, a biological approach.
1993 Kåre Haugan	Dr. scient. Bothany	Mutations in the replication control gene trfA of the broad host-range plasmid RK2
1994 Peder Fiske	Dr. scient. Zoology	Sexual selection in the lekking great snipe (<i>Gallinago media</i>): Male mating success and female behaviour at the lek.
1994 Kjell Inge Reitan	Dr. scient. Botany	Nutritional effects of algae in first-feeding of marine fish larvae
1994 Nils Røv	Dr. scient. Zoology	Breeding distribution, population status and regulation of breeding numbers in the northeast-Atlantic Great Cormorant <i>Phalacrocorax carbo carbo</i> .
1994 Annette-Susanne Hoepfner	Dr. scient. Botany	Tissue culture techniques in propagation and breeding of Red Raspberry (<i>Rubus idaeus</i> L.)
1994 Inga Elise Bruteig	Dr. scient. Bothany	Distribution, ecology and biomonitoring studies of epiphytic lichens on conifers

1994 Geir Johnsen	Dr. scient Botany	Light harvesting and utilization in marine phytoplankton: Species-specific and photoadaptive responses
1994 Morten Bakken	Dr. scient. Zoology	Infanticidal behaviour and reproductive performance in relation to competition capacity among farmed silver fox vixens, <i>Vulpes vulpes</i> .
1994 Arne Moksnes	Dr. philos. Zoology	Host adaptations towards brood parasitism by the Cuckoo.
1994 Solveig Bakken	Dr. scient Bothany	Growth and nitrogen status in the moss <i>Dicranum majus</i> Sm. as influenced by nitrogen supply
1995 Olav Vadstein	Dr. philos Botany	The role of heterotrophic planktonic bacteria in the cycling of phosphorus in lakes: Phosphorus requirement, competitive ability and food web interactions.
1995 Hanne Christensen	Dr. scient. Zoology	Determinants of Otter <i>Lutra lutra</i> distribution in Norway: Effects of harvest, polychlorinated biphenyls (PCBs), human population density and competition with mink <i>Mustela vison</i> .
1995 Svein Håkon Lorentsen	Dr. scient. Zoology	Reproductive effort in the Antarctic Petrel <i>Thalassoica antarctica</i> ; the effect of parental body size and condition.
1995 Chris Jørgen Jensen	Dr. scient. Zoology	The surface electromyographic (EMG) amplitude as an estimate of upper trapezius muscle activity
1995 Martha Kold Bakkevig	Dr. scient. Zoology	The impact of clothing textiles and construction in a clothing system on thermoregulatory responses, sweat accumulation and heat transport.
1995 Vidar Moen	Dr. scient. Zoology	Distribution patterns and adaptations to light in newly introduced populations of <i>Mysis relicta</i> and constraints on Cladoceran and Char populations.
1995 Hans Haavardsholm Blom	Dr. philos Bothany	A revision of the <i>Schistidium apocarpum</i> complex in Norway and Sweden.
1996 Jorun Skjærmo	Dr. scient Botany	Microbial ecology of early stages of cultivated marine fish; impact fish-bacterial interactions on growth and survival of larvae.
1996 Ola Ugedal	Dr. scient. Zoology	Radiocesium turnover in freshwater fishes
1996 Ingibjörg Einarsdottir	Dr. scient. Zoology	Production of Atlantic salmon (<i>Salmo salar</i>) and Arctic char (<i>Salvelinus alpinus</i>): A study of some physiological and immunological responses to rearing routines.
1996 Christina M. S. Pereira	Dr. scient. Zoology	Glucose metabolism in salmonids: Dietary effects and hormonal regulation.
1996 Jan Fredrik Børseth	Dr. scient. Zoology	The sodium energy gradients in muscle cells of <i>Mytilus edulis</i> and the effects of organic xenobiotics.
1996 Gunnar Henriksen	Dr. scient. Zoology	Status of Grey seal <i>Halichoerus grypus</i> and Harbour seal <i>Phoca vitulina</i> in the Barents sea region.
1997 Gunvor Øie	Dr. scient Bothany	Eevaluation of rotifer <i>Brachionus plicatilis</i> quality in early first feeding of turbot <i>Scophthalmus maximus</i> L. larvae.
1997 Håkon Holien	Dr. scient Botany	Studies of lichens in spruce forest of Central Norway. Diversity, old growth species and the relationship to site and stand parameters.
1997 Ole Reitan	Dr. scient. Zoology	Responses of birds to habitat disturbance due to damming.
1997 Jon Arne Grøttum	Dr. scient. Zoology	Physiological effects of reduced water quality on fish in aquaculture.

1997 Per Gustav Thingstad	Dr. scient. Zoology	Birds as indicators for studying natural and human-induced variations in the environment, with special emphasis on the suitability of the Pied Flycatcher.
1997 Torgeir Nygård	Dr. scient. Zoology	Temporal and spatial trends of pollutants in birds in Norway: Birds of prey and Willow Grouse used as Biomonitor.
1997 Signe Nybø	Dr. scient. Zoology	Impacts of long-range transported air pollution on birds with particular reference to the dipper <i>Cinclus cinclus</i> in southern Norway.
1997 Atle Wibe	Dr. scient. Zoology	Identification of conifer volatiles detected by receptor neurons in the pine weevil (<i>Hylobius abietis</i>), analysed by gas chromatography linked to electrophysiology and to mass spectrometry.
1997 Rolv Lundheim	Dr. scient. Zoology	Adaptive and incidental biological ice nucleators.
1997 Arild Magne Landa	Dr. scient. Zoology	Wolverines in Scandinavia: ecology, sheep depredation and conservation.
1997 Kåre Magne Nielsen	Dr. scient. Botany	An evolution of possible horizontal gene transfer from plants to soil bacteria by studies of natural transformation in <i>Acinetobacter calcoaceticus</i> .
1997 Jarle Tufto	Dr. scient. Zoology	Gene flow and genetic drift in geographically structured populations: Ecological, population genetic, and statistical models
1997 Trygve Hesthagen	Dr. philos. Zoology	Population responses of Arctic charr (<i>Salvelinus alpinus</i> (L.)) and brown trout (<i>Salmo trutta</i> L.) to acidification in Norwegian inland waters
1997 Trygve Sigholt	Dr. philos. Zoology	Control of Parr-smolt transformation and seawater tolerance in farmed Atlantic Salmon (<i>Salmo salar</i>) Effects of photoperiod, temperature, gradual seawater acclimation, NaCl and betaine in the diet
1997 Jan Østnes	Dr. scient. Zoology	Cold sensation in adult and neonate birds
1998 Seethaledsumy Visvalingam	Dr. scient. Botany	Influence of environmental factors on myrosinases and myrosinase-binding proteins.
1998 Thor Harald Ringsby	Dr. scient. Zoology	Variation in space and time: The biology of a House sparrow metapopulation
1998 Erling Johan Solberg	Dr. scient. Zoology	Variation in population dynamics and life history in a Norwegian moose (<i>Alces alces</i>) population: consequences of harvesting in a variable environment
1998 Sigurd Mjøen Saastad	Dr. scient. Botany	Species delimitation and phylogenetic relationships between the Sphagnum recurvum complex (Bryophyta): genetic variation and phenotypic plasticity.
1998 Bjarte Mortensen	Dr. scient. Botany	Metabolism of volatile organic chemicals (VOCs) in a head liver S9 vial equilibration system in vitro.
1998 Gunnar Austrheim	Dr. scient. Botany	Plant biodiversity and land use in subalpine grasslands. – A conservation biological approach.
1998 Bente Gunnveig Berg	Dr. scient. Zoology	Encoding of pheromone information in two related moth species
1999 Kristian Overskaug	Dr. scient. Zoology	Behavioural and morphological characteristics in Northern Tawny Owls <i>Strix aluco</i> : An intra- and interspecific comparative approach
1999 Hans Kristen Stenøien	Dr. scient. Botany	Genetic studies of evolutionary processes in various populations of nonvascular plants (mosses, liverworts and hornworts)

1999 Trond Arnesen	Dr. scient. Botany	Vegetation dynamics following trampling and burning in the outlying haylands at Sølendet, Central Norway.
1999 Ingvar Stenberg	Dr. scient. Zoology	Habitat selection, reproduction and survival in the White-backed Woodpecker <i>Dendrocopos leucotos</i>
1999 Stein Olle Johansen	Dr. scient. Botany	A study of driftwood dispersal to the Nordic Seas by dendrochronology and wood anatomical analysis.
1999 Trina Falck Galloway	Dr. scient. Zoology	Muscle development and growth in early life stages of the Atlantic cod (<i>Gadus morhua</i> L.) and Halibut (<i>Hippoglossus hippoglossus</i> L.)
1999 Torbjørn Forseth	Dr. scient. Zoology	Bioenergetics in ecological and life history studies of fishes.
1999 Marianne Giæver	Dr. scient. Zoology	Population genetic studies in three gadoid species: blue whiting (<i>Micromisistius poutassou</i>), haddock (<i>Melanogrammus aeglefinus</i>) and cod (<i>Gradus morhua</i>) in the North-East Atlantic
1999 Hans Martin Hanslin	Dr. scient. Botany	The impact of environmental conditions of density dependent performance in the boreal forest bryophytes <i>Dicranum majus</i> , <i>Hylocomium splendens</i> , <i>Plagiochila asplenigides</i> , <i>Ptilium crista-castrensis</i> and <i>Rhytidiadelphus lokeus</i> .
1999 Ingrid Bysveen Mjølnærød	Dr. scient. Zoology	Aspects of population genetics, behaviour and performance of wild and farmed Atlantic salmon (<i>Salmo salar</i>) revealed by molecular genetic techniques
1999 Else Berit Skagen	Dr. scient. Botany	The early regeneration process in protoplasts from <i>Brassica napus</i> hypocotyls cultivated under various g-forces
1999 Stein-Are Sæther	Dr. philos. Zoology	Mate choice, competition for mates, and conflicts of interest in the Lekking Great Snipe
1999 Katrine Wangen Rustad	Dr. scient. Zoology	Modulation of glutamatergic neurotransmission related to cognitive dysfunctions and Alzheimer's disease
1999 Per Terje Smiseth	Dr. scient. Zoology	Social evolution in monogamous families: mate choice and conflicts over parental care in the Bluethroat (<i>Luscinia s. svecica</i>)
1999 Gunnbjørn Bremset	Dr. scient. Zoology	Young Atlantic salmon (<i>Salmo salar</i> L.) and Brown trout (<i>Salmo trutta</i> L.) inhabiting the deep pool habitat, with special reference to their habitat use, habitat preferences and competitive interactions
1999 Frode Ødegaard	Dr. scient. Zoology	Host specificity as parameter in estimates of arthropod species richness
1999 Sonja Andersen	Dr. scient. Bothany	Expressional and functional analyses of human, secretory phospholipase A2
2000 Ingrid Salvesen, I	Dr. scient. Botany	Microbial ecology in early stages of marine fish: Development and evaluation of methods for microbial management in intensive larviculture
2000 Ingar Jostein Øien	Dr. scient. Zoology	The Cuckoo (<i>Cuculus canorus</i>) and its host: adaptations and counteradaptations in a coevolutionary arms race
2000 Pavlos Makridis	Dr. scient. Botany	Methods for the microbial econtrol of live food used for the rearing of marine fish larvae
2000 Sigbjørn Stokke	Dr. scient. Zoology	Sexual segregation in the African elephant (<i>Loxodonta africana</i>)
2000 Odd A. Gulseth	Dr. philos. Zoology	Seawater tolerance, migratory behaviour and growth of Charr, (<i>Salvelinus alpinus</i>), with emphasis on the high Arctic Dieset charr on Spitsbergen, Svalbard

2000 Pål A. Olsvik	Dr. scient. Zoology	Biochemical impacts of Cd, Cu and Zn on brown trout (<i>Salmo trutta</i>) in two mining-contaminated rivers in Central Norway
2000 Sigurd Einum	Dr. scient. Zoology	Maternal effects in fish: Implications for the evolution of breeding time and egg size
2001 Jan Ove Evjemo	Dr. scient. Zoology	Production and nutritional adaptation of the brine shrimp <i>Artemia</i> sp. as live food organism for larvae of marine cold water fish species
2001 Olga Hilmo	Dr. scient. Botany	Lichen response to environmental changes in the managed boreal forest systems
2001 Ingebrigt Uglem	Dr. scient. Zoology	Male dimorphism and reproductive biology in corkwing wrasse (<i>Symphodus melops</i> L.)
2001 Bård Gunnar Stokke	Dr. scient. Zoology	Coevolutionary adaptations in avian brood parasites and their hosts
2002 Ronny Aanes	Dr. scient.	Spatio-temporal dynamics in Svalbard reindeer (<i>Rangifer tarandus platyrhynchus</i>)
2002 Mariann Sandsund	Dr. scient. Zoology	Exercise- and cold-induced asthma. Respiratory and thermoregulatory responses
2002 Dag-Inge Øien	Dr. scient. Botany	Dynamics of plant communities and populations in boreal vegetation influenced by scything at Sølendet, Central Norway
2002 Frank Rosell	Dr. scient. Zoology	The function of scent marking in beaver (<i>Castor fiber</i>)
2002 Janne Østvang	Dr. scient. Botany	The Role and Regulation of Phospholipase A ₂ in Monocytes During Atherosclerosis Development
2002 Terje Thun	Dr. philos. Biology	Dendrochronological constructions of Norwegian conifer chronologies providing dating of historical material
2002 Birgit Hafjeld Borgen	Dr. scient. Biology	Functional analysis of plant idioblasts (Myrosin cells) and their role in defense, development and growth
2002 Bård Øyvind Solberg	Dr. scient. Biology	Effects of climatic change on the growth of dominating tree species along major environmental gradients
2002 Per Winge	Dr. scient. Biology	The evolution of small GTP binding proteins in cellular organisms. Studies of RAC GTPases in <i>Arabidopsis thaliana</i> and
2002 Henrik Jensen	Dr. scient. Biology	Causes and consequences of individual variation in fitness-related traits in house sparrows
2003 Jens Rohloff	Dr. philos. Biology	Cultivation of herbs and medicinal plants in Norway – Essential oil production and quality control
2003 Åsa Maria O. Espmark Wibe	Dr. scient. Biology	Behavioural effects of environmental pollution in threespine stickleback <i>Gasterosteus aculeatus</i> L.
2003 Dagmar Hagen	Dr. scient. Biology	Assisted recovery of disturbed arctic and alpine vegetation – an integrated approach
2003 Bjørn Dahle	Dr. scient. Biology	Reproductive strategies in Scandinavian brown bears
2003 Cyril Lebogang Taolo	Dr. scient. Biology	Population ecology, seasonal movement and habitat use of the African buffalo (<i>Syncerus caffer</i>) in Chobe National Park, Botswana
2003 Marit Stranden	Dr. scient. Biology	Olfactory receptor neurones specified for the same odorants in three related Heliothine species (<i>Helicoverpa armigera</i> , <i>Helicoverpa assulta</i> and <i>Heliothis virescens</i>)
2003 Kristian Hassel	Dr. scient. Biology	Life history characteristics and genetic variation in an expanding species, <i>Pogonatum dentatum</i>

2003	David Alexander Rae	Dr.scient Biology	Plant- and invertebrate-community responses to species interaction and microclimatic gradients in alpine and Arctic environments
2003	Åsa A Borg	Dr.scient Biology	Sex roles and reproductive behaviour in gobies and guppies: a female perspective
2003	Eldar Åsgard Bendiksen	Dr.scient Biology	Environmental effects on lipid nutrition of farmed Atlantic salmon (<i>Salmo Salar</i> L.) parr and smolt
2004	Torkild Bakken	Dr.scient Biology	A revision of Nereidinae (Polychaeta, Nereididae)
2004	Ingar Pareliussen	Dr.scient Biology	Natural and Experimental Tree Establishment in a Fragmented Forest, Ambohitantely Forest Reserve, Madagascar
2004	Tore Brembu	Dr.scient Biology	Genetic, molecular and functional studies of RAC GTPases and the WAVE-like regulatory protein complex in <i>Arabidopsis thaliana</i>
2004	Liv S. Nilsen	Dr.scient Biology	Coastal heath vegetation on central Norway; recent past, present state and future possibilities
2004	Hanne T. Skiri	Dr.scient Biology	Olfactory coding and olfactory learning of plant odours in heliothine moths. An anatomical, physiological and behavioural study of three related species (<i>Heliothis virescens</i> , <i>Helicoverpa armigera</i> and <i>Helicoverpa assulta</i>).
2004	Lene Østby	Dr.scient Biology	Cytochrome P4501A (CYP1A) induction and DNA adducts as biomarkers for organic pollution in the natural environment
2004	Emmanuel J. Gerreta	Dr. philos Biology	The Importance of Water Quality and Quantity in the Tropical Ecosystems, Tanzania
2004	Linda Dalen	Dr.scient Biology	Dynamics of Mountain Birch Treelines in the Scandes Mountain Chain, and Effects of Climate Warming
2004	Lisbeth Mehli	Dr.scient Biology	Polygalacturonase-inhibiting protein (PGIP) in cultivated strawberry (<i>Fragaria x ananassa</i>): characterisation and induction of the gene following fruit infection by <i>Botrytis cinerea</i>
2004	Børge Moe	Dr.scient Biology	Energy-Allocation in Avian Nestlings Facing Short-Term Food Shortage
2005	Matilde Skogen Chauton	Dr.scient Biology	Metabolic profiling and species discrimination from High-Resolution Magic Angle Spinning NMR analysis of whole-cell samples
2005	Sten Karlsson	Dr.scient Biology	Dynamics of Genetic Polymorphisms
2005	Terje Bongard	Dr.scient Biology	Life History strategies, mate choice, and parental investment among Norwegians over a 300-year period
2005	Tonette Røstelien	PhD Biology	Functional characterisation of olfactory receptor neurone types in heliothine moths
2005	Erlend Kristiansen	Dr.scient Biology	Studies on antifreeze proteins
2005	Eugen G. Sørmo	Dr.scient Biology	Organochlorine pollutants in grey seal (<i>Halichoerus grypus</i>) pups and their impact on plasma thyrid hormone and vitamin A concentrations.
2005	Christian Westad	Dr.scient Biology	Motor control of the upper trapezius

2005 Lasse Mork Olsen	PhD Biology	Interactions between marine osmo- and phagotrophs in different physicochemical environments
2005 Åslaug Viken	PhD Biology	Implications of mate choice for the management of small populations
2005 Ariaya Hymete Sahle Dingle	PhD Biology	Investigation of the biological activities and chemical constituents of selected <i>Echinops</i> spp. growing in Ethiopia
2005 Anders Gravbrøt Finstad	PhD Biology	Salmonid fishes in a changing climate: The winter challenge
2005 Shimane Washington Makabu	PhD Biology	Interactions between woody plants, elephants and other browsers in the Chobe Riverfront, Botswana
2005 Kjartan Østbye	Dr.scient Biology	The European whitefish <i>Coregonus lavaretus</i> (L.) species complex: historical contingency and adaptive radiation
2006 Kari Mette Murvoll	PhD Biology	Levels and effects of persistent organic pollutants (POPs) in seabirds Retinoids and α -tocopherol – potential biomarkers of POPs in birds?
2006 Ivar Herfindal	Dr.scient Biology	Life history consequences of environmental variation along ecological gradients in northern ungulates
2006 Nils Egil Tokle	Phd Biology	Are the ubiquitous marine copepods limited by food or predation? Experimental and field-based studies with main focus on <i>Calanus finmarchicus</i>
2006 Jan Ove Gjershaug	Dr.philos Biology	Taxonomy and conservation status of some booted eagles in south-east Asia
2006 Jon Kristian Skei	Dr.scient Biology	Conservation biology and acidification problems in the breeding habitat of amphibians in Norway
2006 Johanna Järnegren	PhD Biology	Acesta Oophaga and Acesta Excavata – a study of hidden biodiversity
2006 Bjørn Henrik Hansen	PhD Biology	Metal-mediated oxidative stress responses in brown trout (<i>Salmo trutta</i>) from mining contaminated rivers in Central Norway
2006 Vidar Grøtan	PhD Biology	Temporal and spatial effects of climate fluctuations on population dynamics of vertebrates
2006 Jafari R Kideghesho	phD Biology	Wildlife conservation and local land use conflicts in western Serengeti, Corridor Tanzania
2006 Anna Maria Billing	PhD Biology	Reproductive decisions in the sex role reversed pipefish <i>Syngnathus typhle</i> : when and how to invest in reproduction
2006 Henrik Pärn	PhD Biology	Female ornaments and reproductive biology in the bluethroat
2006 Anders J. Fjellheim	PhD Biology	Selection and administration of probiotic bacteria to marine fish larvae
2006 P. Andreas Svensson	phD Biology	Female coloration, egg carotenoids and reproductive success: gobies as a model system
2007 Sindre A. Pedersen	PhD Biology	Metal binding proteins and antifreeze proteins in the beetle <i>Tenebrio molitor</i> - a study on possible competition for the semi-essential amino acid cysteine
2007 Kasper Hancke	PhD Biology	Photosynthetic responses as a function of light and temperature: Field and laboratory studies on marine microalgae
2007 Tomas Holmern	PhD Biology	Bushmeat hunting in the western Serengeti: Implications for community-based conservation

2007 Kari Jørgensen	PhD Biology	Functional tracing of gustatory receptor neurons in the CNS and chemosensory learning in the moth <i>Heliothis virescens</i>
2007 Stig Ulland	PhD Biology	Functional Characterisation of Olfactory Receptor Neurons in the Cabbage Moth, <i>Mamestra Brassicae</i> / L. (Lepidoptera, Noctuidae). Gas Chromatography Linked to Single Cell Recordings and Mass Spectrometry
2007 Snorre Henriksen	PhD Biology	Spatial and temporal variation in herbivore resources at northern latitudes
2007 Roelof Frans May	PhD Biology	Spatial Ecology of Wolverines in Scandinavia
2007 Vedasto Gabriel Ndibalema	PhD Biology	Demographic variation, distribution and habitat use between wildebeest sub-populations in the Serengeti National Park, Tanzania
2007 Julius William Nyahongo	PhD Biology	Depredation of Livestock by wild Carnivores and Illegal Utilization of Natural Resources by Humans in the Western Serengeti, Tanzania
2007 Shombe Ntaraluka Hassan	PhD Biology	Effects of fire on large herbivores and their forage resources in Serengeti, Tanzania
2007 Per-Arvid Wold	PhD Biology	Functional development and response to dietary treatment in larval Atlantic cod (<i>Gadus morhua</i> L.)
2007 Anne Skjetne Mortensen	PhD Biology	Focus on formulated diets and early weaning Toxicogenomics of Aryl Hydrocarbon- and Estrogen Receptor Interactions in Fish: Mechanisms and Profiling of Gene Expression Patterns in Chemical Mixture Exposure Scenarios
2008 Brage Bremset Hansen	PhD Biology	The Svalbard reindeer (<i>Rangifer tarandus platyrhynchus</i>) and its food base: plant-herbivore interactions in a high-arctic ecosystem
2008 Jiska van Dijk	PhD Biology	Wolverine foraging strategies in a multiple-use landscape
2008 Flora John Magige	PhD Biology	The ecology and behaviour of the Masai Ostrich (<i>Struthio camelus massaicus</i>) in the Serengeti Ecosystem, Tanzania
2008 Bernt Rønning	PhD Biology	Sources of inter- and intra-individual variation in basal metabolic rate in the zebra finch, <i>Taeniopygia guttata</i>
2008 Sølvi Wehn	PhD Biology	Biodiversity dynamics in semi-natural mountain landscapes. - A study of consequences of changed agricultural practices in Eastern Jotunheimen
2008 Trond Moxness Kortner	PhD Biology	"The Role of Androgens on previtellogenic oocyte growth in Atlantic cod (<i>Gadus morhua</i>): Identification and patterns of differentially expressed genes in relation to Stereological Evaluations"
2008 Katarina Mariann Jørgensen	Dr.Scient Biology	The role of platelet activating factor in activation of growth arrested keratinocytes and re-epithelialisation
2008 Tommy Jørstad	PhD Biology	Statistical Modelling of Gene Expression Data

2008 Anna Kusnierczyk

PhD
Biology

Arabidopsis thaliana Responses to Aphid
Infestation