Diversity and composition of algal epiphytes on the Mediterranean seagrass *Cymodocea nodosa*: a scale-based study

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Table 1. Epiphytes on *Cymodocea nodosa* were identified from sequencing partial 18 S rDNA followed by nucleotide BLAST searches in NCBI and verified by searching the same sequences in the SILVA platform (Identity: alignment identity score; BPS: base pairs aligned; Quality: alignment quality; cf. Quast et al. 2013).

Sample / site	**Sequence bp**	**Accession Numbers**	BLAST/NCBI				SILVA					
			Taxon with closest match	**Query Cover**	**E.Value**	**PerIdentity**	**Taxon ID**	**Bp Score**	**Identity**	**BPS**	**Quality**	**Taxonomy**
KA1 Nea Karvali	1466	MW578779	*Posidonia oceanica* (AY491942.1)	69%	0.0	95.62%	Unclassified	66	70.972	1466	58	Unclassified
KA2 Nea Karvali	549	MW578780	*Prorocentrum micans* (MK405477.1)	98%	0.0	99.08%	Dinophyceae	96	981.378	549	97	Eukaryota; SAR; Alveolata; Dinoflagellata; Dinophyceae
KA3 Nea Karvali	556	MW578781	*Prorocentrum micans* (MK405477.1)	97%	0.0	98.16%	Dinophyceae	98	981.343	556	96	Eukaryota; SAR; Alveolata; Dinoflagellata; Dinophyceae
KB2 Nea Karvali	1457	MW578782	*Posidonia oceanica* (AY491942.1)	71%	0.0	97.07%	Unclassified	66	713.627	1456	63	Unclassified
KB4 Nea Karvali	527	MW578783	*Halurus flosculosus* (AF488381.1)	97%	0.0	95.76%	*Halurus*	92	851.711	526	90	Eukaryota; Archaeplastida; Rhodophyceae; Florideophycidae; Rhodymeniophycidae; *Halurus*
KC2 Nea Karvali	1376	MW578784	*Cymodocea rotundata* (JN034102.1)	96%	0.0	90.59%	Unclassified	78	893.267	1376	86	Unclassified
KC4 Nea Karvali	1175	MW578785	*Prorocentrum lima* (MK541784.1)	89%	0.0	96.04%	Unclassified	92	911.318	1175	91	Unclassified
KD1 Nea Karvali	1144	MW578786	*Prorocentrum lima* (MK541784.1)	89%	0.0	96.99%	Unclassified	89	916.162	1144	91	Unclassified

KD2 Nea Karvali	1122	MW578787	*Prorocentrum micans* (MK405477.1)	86%	0.0	98.38%	*Prorocentrum*	90	917.832	1122	89	Eukaryota; SAR; Alveolata; Dinoflagellata; Dinophyceae; Prorocentrales; *Prorocentrum*
KD3 Nea Karvali	1178	MW578788	*Prorocentrum micans* (AY833514.1)	84%	0.0	94.98%	Unclassified	90	893.155	1178	86	Unclassified
KD4 Nea Karvali	1135	MW578789	*Bostrychia radicans* (AY617138.1)	86%	0.0	98.53%	Unclassified	84	805.106	1135	82	Unclassified
THA1 Thasos	1179	MW578766	*Halurus flosculosus* (FR865645.1)	98%	0.0	97.40%	Rhodymenio-phycidae	93	925.676	1157	93	Eukaryota; Archaeplastida; Rhodophyceae; Florideophycidae; Rhodymeniophycidae
THA3 Thasos	1479	MW578767	*Posidonia oceanica* (LC027443.1)	74%	0.0	94.21%	Unclassified	70	787.076	898	73	Unclassified
THB1 Thasos	381	MW578768	*Hedychium coronarium*	97%	0.0	99.2%	Unclassified	33	457.627	381	46	Unclassified
THB2 Thasos	1097	MW578769	*Prorocentum lima* (MK541784.1)	98%	0.0	95.99%	*Halurus*	93	925.676	1157	93	Eukaryota; Archaeplastida; Rhodophyceae; Florideophycidae; Rhodymeniophycidae; *Halurus*
THB3 Thasos	1093	MW578770	*Prorocentrum lima* (MK541784.1)	98%	0.0	96.45%	*Prorocentrum*	94	946.703	1093	93	Eukaryota; SAR; Alveolata; Dinoflagellata; Dinophyceae; Prorocentrales; *Prorocentrum*
THB4 Thasos	1080	MW578771	*Prorocentrum micans* (MK405477.1)	97%	0.0	97.13%	Alveolata	98	958.371	1080	94	Eukaryota; SAR; Alveolata
THC1 Thasos	1101	MW578772	*Prorocentrum texanum*	92%	0.0	97.76%	Alveolata	98	952.657	1101	94	Eukaryota; SAR; Alveolata

			(MK995624.1)								
THC2 Thasos	1084	MW578773	*Bostrychia radicans* (AY617138.1)	94%	0.0	96.62%	*Halurus*	96	870.118	1069	86	Eukaryota; Archaeplastida; Rhodophyceae; Florideophycidae; Rhodymeniophycidae; *Halurus*
THC3 Thasos	1069	MW578774	*Halurus flosculosus* (FR865645.1)	95%	0.0	94.94%	*Halurus*	96	870.118	1069	86	Eukaryota; Archaeplastida; Rhodophyceae; Florideophycidae; Rhodymeniophycidae; *Halurus*
THC4 Thasos	1427	MW578775	*Posidonia oceanica* (LC027443.1)	72%	0.0	96.04%	Unclassified	80	844.595	1427	80	Unclassified
THD1 Thasos	381	MW578776	*Hedychium coronarium*	96%	0.0	99.46%	Unclassified	31	472.727	381	44	Unclassified
THD3 Thasos	629	MW578777	*Prorocentrum lima* (MK541780.1)	99%	0.0	98.74%	*Prorocentrum*	91	974.522	629	97	Eukaryota; SAR; Alveolata; Dinoflagellata; Dinophyceae; Prorocentrales; *Prorocentrum*
THD4 Thasos	635	MW578778	*Prorocentrum texanum* (JQ390504.1)	99%	0.0	99.53%	Dinophyceae	98	992.038	635	98	Eukaryota; SAR; Alveolata; Dinoflagellata

Table 2. Epiphytic taxa identified on the leaves of *Cymodocea nodosa* on the coast of Kavala Gulf, the Ecological Status Group (ESG) to which each taxon belongs is shown (+ indicates presence at the preliminary study, % cover ± standard error is given at the main study).

	Preliminary study (01/03/2008-21/10/2009)					**Main study (22/10/2009)**						
	Vrasidas (30-1-08)	Vrasidas (01-2-08)	Vrasidas (20-1-08)	Nea Karvali (18-3-09)	Vrasidas (23-3-09)	Thasos 2, TH2	Thasos 1, TH1	Vrasidas 2, B2	Vrasidas 1, B1	Nea Karvali 1, K1	Nea Karvali 2, K2	ESG
RHODOPHYTA												
Acrochaetium sp. Nägeli in Nägeli *et* Cramer			+				0.03±0.02	0.06±0.04		0.61±0.12	0.07±0.03	II
Callithamnion sp. Lyngbye			+									II
Ceramium ciliatum (J. Ellis) Ducluzeau		+										II
Ceramium comptum Børgesen	+											II
Ceramium tenerrimum (G. Martens) Okamura			+				0.02±0.02	0.06±0.03	0.01±0.01	0.1±0.06	0.06±0.03	II
Champia parvula (C. Agarth) Harvey	+	+										II
Chroodactylon ornatum (C. Agarth) Basson		+	+									II
Chondria capillaris (Hudson) M. Wynne					+		0.02±0.01	0.01±0.01				II
Gayliella transversalis (F.S. Collins *et* Hervey) T.O. Cho *et* Fredericq	+	+										II
Hydrolithon cruciatum (Bressan) Chamberlain				+		56.08±2.11	48.42±2.15	79.6±2.09	82.54±2.93	45.23±1.64	27.47±2.26	I
Carradoriella elongata (Hudson) Savoie *et* G.W.Saunders							0.01±0.01	0.32±0.15		0.07±0.04		II
HETEROKONTOPHYTA												
Feldmannia mitchelliae (Harvey) H.-S.Kim	+	+	+	+			1.83±0.46	5.59±1.23		40±4.11	19.91±2.03	II
Punctaria latifolia Greville				+								II
Scytosiphon lomentaria (Lyngbye) Link		+										II
Sphacelaria cirrosa (Roth) Agarth		+										II
CHLOROPHYTA												
Bryopsis hypnoides J.V. Lamouroux		+										II
Cladophora sp. Kützing	+						0.12±0.08	0.09±0.06	0.01±0.01			II
Ulva polyclada Kraft		+										II
Ulva prolifera O.F. Müller	+	+	+	+			+			+		II
Cyanobacteria						1.92±0.26	1.58±0.35	0.34±0.18	1.29±0.34		0.19±0.13	II

Table 3. Permutational Analysis of Variance of species/taxa cover (%) between meadows (fixed), sites (random, nested within meadows), leaves (random, nested within sites and meadows) and leaf side (fixed, orthogonal to meadows) (a) and pairwise analysis between meadows (b). MC indicates that Monte Carlo test was used to acquire the p statistic because permutations were too low.

a) Source	**df**	**MS**	**Pseudo-F**	**P(perm)**
Meadow	2	75441	10.673	0.0041 (MC)
Leaf side	1	1204.6	0.807	0.463
Site(meadow)	3	7068.3	3.67	0.006
Meadow X leaf side	2	1272.5	0.852	0.536
Leaf(site(meadow))	24	1926	5.242	<0.001
Site(meadow) X leaf side	3	1493.5	0.696	0.631
Leaf(site(meadow)) X leaf side	24	2145.5	5.839	<0.001
Residuals	360	367.45		
Total	419			

b) Groups	**t**	**P(perm)**
Nea Karvali, Vrasidas	3.449	0.019 (MC)
Nea Karvali, Thasos	2.859	0.032 (MC)
Vrasidas, Thasos	4.161	0.009 (MC)

Table 4. Nested ANOVA of Ecological Status Group (ESG) I and ESG II cover (%) between meadows (fixed), sites (random, nested within meadows), leaves (random, nested within sites and meadows).

		Df	Mean Sq	F value	Pr(>F)
ESGI	Meadow	2	71936	269.267	<0.001
	Meadow:site	3	4465	16.715	<0.001
	Meadow:site:leaf	24	1668	6.242	<0.001
	Residuals	390	267		
ESGII	Meadow	2	82.58	224.721	<0.001
	Meadow:site	3	11.42	31.072	<0.001
	Meadow:site:leaf	24	1.62	4.401	<0.001
	Residuals	390	0.37		

Table 5. Permutational Analysis of Variance of Ecological Status Group cover (%) between meadows (fixed), sites (random, nested within meadows), leaves (random, nested within sites and meadows) and leaf side (fixed, orthogonal to meadows) (b). MC indicates that Monte Carlo test was used to acquire the p statistic because permutations were too low.

a) Source	**df**	**MS**	**Pseudo-F**	**P(perm)**
Meadow	2	68029	9.689	0.006 (MC)
Leaf side	1	1259.2	0.852	0.455
Site(meadow)	3	7021.1	3.98	0.006
Meadow x leaf side	2	1040.9	0.705	0.615
Leaf(site(meadow))	24	1764.3	4.906	<0.001
Site(meadow) x leaf side	3	1477.4	0.721	0.617
Leaf(site(meadow)) x leaf side	24	2050.4	5.702	<0.001
Residuals	360	359.59		
Total	419			

b) Groups	**t**	**P(perm)**
Nea Karvali, Vrasidas	3.398	0.018 (MC)
Nea Karvali, Thasos	2.561	0.044 (MC)
Vrasidas, Thasos	4.129	0.009 (MC)

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Abstract

Cymodocea nodosa, a typical marine angiosperm species in the Mediterranean Sea, hosts a range of epiphytic algae. Epiphyte abundance varies at different spatial scales, yet epiphyte diversity and community composition are poorly understood. This study explores the epiphytes on *C. nodosa* from two reference meadows (Thasos, Vrasidas) and one anthropogenically stressed meadow (Nea Karvali) in the northern Aegean Sea (Kavala Gulf, Greece). A nested destructive sampling design at three spatial scales (metres, hundreds of metres, kilometres) and stereoscopic/microscopic observations were used. Light microscopy revealed a total of 19 taxa of macroalgae populating the leaves of *C. nodosa*. The most commonly encountered taxa with highest cover (%) were *Hydrolithon cruciatum* and *Feldmannia mitchelliae*. DNA sequencing (18S rDNA) confirms the presence of a number of dinoflagellate and red algal epiphytes, and this represents the first application of DNA metabarcoding to study the diversity of seagrass epiphytes. Epiphytic communities studied at species/taxon and functional (Ecological Status Groups) levels separated the reference low-stressed meadows from the degraded one, with the functional approach having higher success. The Ecological Evaluation Index classified the studied meadows into different Ecological Status Classes according to anthropogenic stress.

Key words

Macroalgae, abundance, image analysis, functional groups, metabarcoding

Introduction

Seagrasses often form highly productive (Holmer 2019, Pergent et al. 2014) and extensive meadows in world-wide shallow ecosystems (Larkum et al. 2006), holding a major role for benthic carbon sequestration (Trevathan-Tackett et al. 2015, Stankovic et al. 2018, Küpper and Kamenos 2018) and contributing to the cycling and storage of a plethora of nutrients. Furthermore, they provide a wide range of ecosystem services, such as cover, protection, substrate, and food for a wide range of organisms (Ruiz-Frau et al. 2017). At the same time, they enhance water quality by trapping suspended particles within their leaves and roots (Moore 2004), and they protect the coasts from erosion (Ondiviela et al. 2014). Furthermore, seagrasses can be used as bioindicators (Orfanidis et al. 2001) since they can respond quickly to any biotic and abiotic changes in their habitat (Orth et al. 2006).

Cymodocea nodosa is a common seagrass species in the Mediterranean Sea (Hemminga and Duarte 2000) that grows relatively fast in comparison to other seagrass genera (Tsioli et al. 2019, Marbà et al. 2004). Most of the leaf surface is photosynthetically active (Cancemi et al. 2002), contributing to a significant proportion of the total productivity (Hemminga and Duarte 2000), and is often populated by epiphytes (Borowitzka et al. 2006).

Seagrass epiphytes are mainly photosynthetic organisms, especially filamentous algae of various lineages and red coralline algae. Epiphytic algae found in seagrass meadows seem to cover the whole spectrum of algal lineages, although Rhodophyta are particularly prevalent (Browne et al. 2013, Ben Brahim et al. 2020), and diatoms can be found on almost any seagrass population (Chung and Lee 2008, Mazzella and Spinoccia 1992). Leaves of *C. nodosa* and, to a lesser degree, *Posidonia oceanica* have also been found to harbour toxic dinoflagellates as epiphytes (Turki 2005). Epiphytic cyanobacteria contribute to N_2 assimilation, while bacterial epiphytes on the rhizomes and on the leaves have a role in recycling nutrients (Hamisi et al. 2013). Finally, seagrass epiphytes include non-photosynthetic organisms like fungi, heterotrophic protozoa, sponges, bryozoa, hydrozoa, and ascidians (Borowitzka et al. 2006). Leaves and especially the upper parts of the oldest leaves often host the greatest variety and biomass of epiphytic algae (Mazzella et al. 1994, Uku and Björk 2001).

The colonization of seagrass leaves by epiphytes can be easily observed via the comparison of leaves that originate from the same bundle but differ in age. There are several factors that can affect epiphyte diversity and abundance. Both vary over the seasonal cycle, which seems to be a consequence of substratum availability and stability, the availability of epiphyte propagules (Jacobs et al. 1983, Trautman and Borowitzka 1999), as well as the environmental conditions conducive to epiphyte development. In some cases, epiphytes are mostly located on the outward side of seagrass leaves, which seems to be driven by light distribution (Trautman and Borowitzka 1999). In some seagrass species, epiphytic load is equally distributed between the two sides of the leaf, in particular when leaves are not curved, like *Posidonia australis* and *Cymodocea nodosa* (Trautman and Borowitzka 1999). Hydrodynamics significantly influence epiphyte allocation and abundance, as it affects trapping of the spores and the nutrient supply for epiphytic organisms (Fonseca et al. 1982, Kendrick and Burt 1997, Borowitzka et al. 2006). Nutrient supply is of great importance, as nutrient increase can lead to a significant boost of epiphytic biomass that may even lead to seagrass die-off (Duarte 1995). Epiphytes can affect the photosynthetic efficiency of seagrasses, since epiphyte growth may attenuate photosynthetically active radiation (PAR) that is crucial for seagrass growth (Dixon 2000). Last but not least, epiphytic communities can be nursing grounds for grazers and predators, the diversity and abundance of which can be affected by the epiphytes themselves or by the impact on host biomass (Jernakoff et al. 1996).

Epiphytes of seagrasses have been studied in terms of biodiversity (Browne et al. 2013), spatial variability (Chung and Lee 2008, Lavery and Vanderklift 2002, Balata et al. 2007, Johnson et al. 2005), nutrient concentration, and anthropogenic stress (Balata et al. 2008, Campbell et al. 2006, Frankovich et al. 2009, Giovannetti et al. 2010). In the Mediterranean Sea, while epiphytes on *Posidonia oceanica* have been studied for decades (e.g. Piazzi et al. 2016, Van der Ben 1971), those of *C. nodosa* are much less known. Also, in some instances, epiphytes were studied at the level of morphological functional-form groups, based on the classification proposed by Littler and Littler (1980) (see Pardi et al. 2006, García-Redondo et al. 2019). However, a classification at a functional group level (e.g., Ecological Status Groups, see Orfanidis et al. 2001, 2011) relevant to nutrient and light levels, key aspects of eutrophication, has not been accomplished so far.

The present research aimed to investigate the diversity and abundance of epiphytes colonizing the seagrass *Cymodocea nodosa* at two reference meadows, and one anthropogenically stressed meadow along the coastline of the Kavala basin (Greece), in order to explore the hitherto-unknown role of anthropogenic stress in epiphyte colonization patterns on the leaves of *C. nodosa*, as well as the potential use of epiphytes as a bioindicator of anthropogenic stress along the coastline in this region.

Materials & Methods

Sampling areas and samples

Sampling was conducted at three meadows of known ecological status in the Kavala Gulf, North Aegean Sea, Greece: Vrasidas (24°19'8.78"E, 40°49'37.53"N), Thasos Island (24°41'46.97"E, 40°46'46.01"N), and Nea Karvali (24°31'7.68"E, 40°57'23.62"N; Figure 1). Vrasidas meadow is located in the inner part of Cape Vrasidas of the Eleutheron Gulf. It is one of the least stressed areas close to the surrounding mainland of Kavala Gulf and has been included in the European Natura 2000 network (code GR1150009). Main anthropogenic activities include fishing and tourism in the wider region, as well as port activities in the nearby town of Nea Peramos. Nea Karvali is an old agricultural and fishing settlement that, since 1981, has seen increased levels of industrial development and, in particular, the establishment of a phosphorus fertilizer plant, the Kavala city wastewater treatment facility, and a crude oil de-sulphurisation complex of the Prinos Oil Field all of which significantly affect the surrounding coastal area. The Thasos Island meadow is located in the north-eastern part of the Kavala Gulf. The island's main income stems from tourism, while the agricultural activities do not significantly influence the coastal water quality because of the relatively high hydro-dynamism of the area. The Vrasidas and Thasos meadows were chosen as reference sites, while the meadow at Nea Karvali was selected as a highly stressed one (see Orfanidis et al. 2011, Papathanasiou and Orfanidis 2018, Papathanasiou et al. 2015).

Preliminary sample collections of *Cymodocea nodosa* (Ucria) Ascherson shoots were conducted at the selected sampling sites, except Thasos Island, in order to qualitatively assess the epiphytic load (Vrasidas: 20/01/2008, 30/01/2008, 23/03/2009 – Nea Karvali: 18/03/2009). For the quantitative analysis, a nested destructive sampling protocol was followed, which allowed comparisons at three spatial scales (Figure S1). In each meadow, two sites were randomly selected that were ca. 500–800 m apart. In total, there were six selected sampling sites: Thasos 1 (TH1), Thasos 2 (TH2), Vrasidas 1 (VR1), Vrasidas 2 (VR2), Nea Karvali 1 (NK1), and Nea Karvali 2 (NK2). All sample collections were conducted on the same day (22 October 2009) by scuba-diving at 1.5 – 3 m depth. All *Cymodocea* shoots were immediately put in bags filled with seawater. Five shoots from each site (30 in total) were fixed in 3-5% formaldehyde solution to be used for the stereoscopic-microscopic analyses. From the meadows at Thasos and Nea Karvali, another five shoots from each site (20 in total) were collected and fixed with ethanol to be used for molecular analyses.

Laboratory Analysis

The first step of the laboratory analysis was the qualitative identification of all the taxa present in each sampled shoot by using a stereoscope (Nikon SMZ1000, equipped with a Sony Exwave HAD color video camera) and microscope (Nikon Eclipse 50i, equipped with a 5-megapixel CCD DS-Fi1 camera).

In order to calculate the epiphyte cover (%) per species, five mature leaves from five randomly collected shoots from each site were analyzed, that is, a total of 30 leaves. From each leaf, seven random, non-overlapping random photos were taken on each side of each leaf, thus a total of 14 from each leaf. As a result, a total of 420 (19.04±4.04 mm^2) photographs were measured and analyzed, as described below.

The oldest part of the leaf that was intact was placed in a Petri dish filled in artificial seawater of 32-35 salinity (Figure S2). Thereafter, the oldest part of the oldest leaf within a given shoot was cut off and the inner and outer side of the leaf were determined. The part of each leaf facing the opening of the leaf sheath was labelled as ′inner′ and the other part as ′outer′. Using the Image Pro-Plus 5.1 software, seven pictures of each side of the leaf were taken. Image Pro-Plus (Media Cybernetics, Rockville, Maryland, USA) was used to calculate the cover of each species of the photo sample leaf area. It should be noted that *C. nodosa* leaves used for taxonomic identification of epiphytes were not included in the quantification of epiphytic cover on leaves.

Ecological Evaluation Index (EEI-c)

EEI-c was calculated according to Orfanidis et al. (2011; www.eei.gr) on two main Ecological Status Groups, ESG I (slow-growing, late-successional species) and ESG II (fast-growing, opportunistic species). To ensure comparability, the EEI-c values were transformed into Ecological Quality Ratios (EQR, i.e., the ratio between the value of the observed biological parameter for a given surface water body and its expected value under the reference conditions) as follows:

$$EEI_{EQR}=1.25*(EEIvalue/RCvalue)-0.25, \text{ where } RC=10.$$

DNA extraction, amplification and sequencing

The samples that were stored in ethanol solution (99 %) were used for DNA extraction. Total DNA was extracted using a modified CTAB protocol (Doyle and Doyle 1987, Surek et al. 1994) from around 20 mg dry weight tissue. Polymerase chain reaction (PCR) was conducted according to an established protocol (PCR; see Marin et al. 1998). The nuclear SSU-ITS region was amplified using the primers EAF3 and ITS055R, and sequenced using additional internal primers, such as 528F, 920F, EBR, 920R, 536R (Marin et al. 1998, Marin et al. 2003) SSU rDNA . PCR products were sequenced using the Sanger method at the Molecular Genetics Facility (MGF) of the UK Natural Environment Research Council (NERC) using primers 82F, 528F, 920F, EBR, 920R and 536R, respectively (Marin et al. 1998). Sequences were deposited in NCBI (Table 1).

Alignments and taxonomic analyses

The 18S sequences obtained were used for taxonomic identification of epiphytic organisms. The forward and reverse sequences were manually aligned and quality-checked using the programs BioEdit (Hall 1999) as well as AliView (Larsson 2014). They were subsequently searched for homologies initially against the GenBank nucleotide database using the BLAST tool (Altschul et al. 1997) and then for a more refined taxonomic search against the databases available via the SILVA platform, specifically Ref NR with taxonomies SILVA, RDP, GTDB, LTP and EMBL-EBI/ENA (Table 1; Yilmaz et al. 2013, Quast et al. 2013).

Statistical Analysis

All statistical analyses were executed using the PRIMER software v. 7. In order to visually assess community differences, we employed non-metric Multidimensional Scaling based on species/taxa cover and Ecological Status Group cover separately. In both cases, the Bray Curtis dissimilarity matrix was used. The hypothesis that community composition was equivalent between four spatial levels (meadow, site, leaf and leaf side) was tested using

Permutational Multivariate Analysis of Variance (PERMANOVA) with 9999 permutations. The factor meadow was treated as fixed, while site was set as random, nested within meadow, and leaf was set as random, nested within site and meadow. Leaf side was treated as fixed and analyzed orthogonally to meadow. When the unique permutations were found to be low, the Monte Carlo procedure was followed, while pairwise analysis was run for the factor meadow. The Bray Curtis dissimilarity was again used, while the analysis was run once for the community matrix based on all the species/taxa cover and once for the two Ecological Status Group covers. Each analysis was accompanied by a Permutational test of Multivariate Dispersion (PERMDISP) for the meadow factor to examine whether the within-group dispersion from the group centroid was similar between the analysis levels. The significant contribution of individual species/taxa in the observed differences and the dissimilarity between meadows was examined using a similarity percentage analysis (SIMPER). Figures were drawn using the package "ggplot" in the R Environment (R Core Team 2020). Analysis of variance of ESGI and ESGII between the three spatial scales was run in the R Environment using the aov() R Core function. The condition of normality and homoscedasticity in our data were not met, even after applied transformations. As such, in order to avoid type I errors, the significance level was set as $a=0.01$. In the results, all descriptive statistics are given with the standard error (mean±SE).

Results

Qualitative analysis of epiphyte diversity

A total of 19 species/taxa were identified from the leaves of *Cymodocea nodosa*, 11 of which belong to the Rhodophyta, 4 to the Heterokontophyta and 4 to the Chlorophyta (Table 2, Figure 2). The presence of a few species of Cyanobacteria was also recorded. The nomenclature and systematic classification of taxa are in accordance with AlgaeBase (Guiry and Guiry 2020).

Figure 3 shows a comparative view of the leaves under the stereoscope in order to visually indicate the differences between the leaves from the three meadows. A number of observations are noteworthy:

At the Vrasidas meadow, *Hydrolithon cruciatum* crusts were bright white and continuous and covered the entire extremity of the leaf of *C. nodosa*. The presence of Anthozoa was observed in all of the specimens at the lateral ends of leaves. It is noticeable that macroalgal epiphytes were observed on the stem. At the younger parts of the leaves, only Anthozoa were observed, whereas on the older parts (towards the leaf apex), the coexistence of *H. cruciatum* and Anthozoa was noticed.

At the Thasos meadow, *H. cruciatum* had strongly lobed or toothed ends, and some individuals were pale white, probably in the early stages of their development.

At the Nea Karvali meadow, *Acrochaetium* sp. grew mostly at the lateral ends of *C. nodosa* leaves, which were often covered by debris or amorphous inorganic constituents that made it difficult to distinguish epiphytes when analyzing photographs. In most cases, it appears that *Feldmannia mitchelliae* did not occur directly on the leaves of *C. nodosa*, but only on *H. cruciatum* crusts.

DNA sequences

Sequencing yielded one sequence per sample. Only two sequences were >99% similar to sequences in GenBank, five corresponded to seagrasses and two sequences corresponded to land plants. BLAST and SILVA searches of partial 18S rDNA sequences revealed the presence of associated organisms (Table 1) – including two red algae of the Ceramiales, and a number of different dinoflagellates, the closest match for which is the genus *Prorocentrum*, but with relatively low similarity levels.

Quantitative analysis at species/taxon level

The abundance of eight taxa was analyzed, using data obtained from the image analysis. All eight taxa were present in the Vrasidas and Thasos meadows. The species *H. cruciatum* and *F. mitchelliae* (81.07±1.80 % and 2.79±0.66 %, respectively) dominated in the Vrasidas meadow, while *H. cruciatum* and Cyanobacteria (52.25±1.54 % and 1.75±0.22 %, respectively) dominated in the Thasos meadow (Table 2, Figure 4). Only six out of the eight encountered taxa were present in Nea Karvali with *H. cruciatum* and *F. mitchelliae* (36.35±1.58 % and 29.95±2.44 %, respectively) being the dominant ones.

The eight taxa identified were not present in the same frequency among all three meadows. Figure 5 shows the number of species/taxa occurrences in the measured leaves per meadow. *Acrochaetium* sp., *Ceramium* sp. and *F. mitchelliae* were more frequent in the Nea Karvali meadow, whereas *Chondria capillaris* and *Cladophora* sp. were absent. In the same meadow, Cyanobacteria were found on only one leaf. The Thasos and Vrasidas meadows showed differences in the number of leaves hosting Cyanobacteria and *F. mitchelliae*.

The non-metric Multidimensional Scaling revealed three clusters at 70% similarity (Figure 6). Cluster A contained all leaves from Nea Karvali together with two leaves from Vrasidas (VR2.1, VR2.2) and one from Thasos (TH1.2) that were more similar to Nea Karvali. Cluster C was formed by almost all other Thasos and Vrasidas leaves, except the leaves VR2.3, VR2.4, VR2.5 and TH1.3 that formed a separate cluster (B). SIMPER analysis revealed that these differences were mainly caused by the two species *H. cruciatum* and *F. mitchelliae* (Table S2). The clusters B and C from Vrasidas and Thasos meadows had the lowest dissimilarity average (26.82) while both had ca. 44% dissimilarity with Nea Karvali (A).

Permutational Analysis of Variance (PERMANOVA) on the community matrix showed that there were statistically significant differences of species/taxa cover (%) between all factors except Leaf side. However, a statistically significant interaction between leaf side and leaf within site within meadow was noticed (Table 3a). Pseudo F was by far highest for the meadow factor (Table 3a), while pairwise analysis revealed that these differences were statistically significant between all three meadows (Table 3b). Data displayed heterogeneous dispersions between meadows (PERMDISP test, F=70.514, p<0.001). The highest dispersion was found at the Nea Karvali meadow, while Vrasidas had the lowest. The pairwise analysis showed that dispersion was statistically different only between Nea Karvali and the other two meadows, while the difference between Thasos and Vrasidas was not significant (*P (perm)*= 0.065; Table S3).

The two sites from Vrasidas meadow showed different diversity as *F. mitchelliae, Acrochaetium* sp.*, Carradoriella elongata* and *Chondria capillaris,* were completely absent from site VR1, that was in turn characterized by the highest *H. cruciatum* cover (82.54±2.93 %). Site VR2 that contained all species/taxa found, was again dominated by *H. cruciatum* (79.60±2.09 %) followed by *F. mitchelliae* (5.50±1.23%).

In the Thasos meadow, *H. cruciatum* and Cyanobacteria were the only epiphytes found in leaves from TH2. *Hydrolithon cruciatum* was again the dominant epiphyte (56.08±2.11 %), and Cyanobacteria had the highest cover among all studied sites (1.92±0.26 %). All species/taxa were found in TH1, with *F. mitchelliae* being second highest in cover (1.83±0.46 %), after *H. cruciatum* (48.42±2.15).

In Nea Karvali, *H. cruciatum* had the lowest mean cover in comparison to the other two meadows (36.35±1.58 %) but was still the dominant species in the meadow, with the highest cover in NK1 (45.23±1.64 %). *Feldmannia mitchelliae* was again the second most abundant species, with highest cover in NK1 (40.00±4.11 %). The two sites were similar in species richness, as *Carradoriella elongata* was present only in NK1 and Cyanobacteria were present only in NK2.

Quantitative analysis at Ecological Status Group level

Among the identified species/taxa, *H. cruciatum* was the only species belonging to ESG I, while the other seven species/taxa belonged to ESG II.

The cover (%) of both ESGI and ESGII was statistically different between all spatial scales of the analysis (Table 4). The highest between group variability was observed at the level of meadow for both ESGs. ESGI had the highest cover (%) in Vrasidas (81.07±1.80 %) and especially in site VR1 (82.54±2.93 %; Figure 7). Nea Karvali had the lowest ESGI cover (36.35±1.58 %) with minimum mean cover measured in site NK2 (27.47±2.26 %). ESGII had the highest cover (%) in Nea Karvali (30.51±2.43 %) and especially at site NK1 where it reached 45.23±1.64 %. Mean cover (%) was considerably lower in the other two meadows with mean cover (%) being 3.89±0.67 % in Vrasidas and 2.77±0.29 % in Thasos. Post hoc analysis (Table S4) showed that, while differences in ESG I cover (%) were statistically significant between all meadows, ESG II cover (%) was significantly different between Nea Karvali and the other two meadows, while the difference between Thasos and Vrasidas was not significant (p= 0.731).

The non-metric Multidimensional Scaling revealed a clear distinction between the cluster of leaves from the degraded meadow at Nea Karvali and the cluster from the other two reference meadows (Thasos, Vrasidas) at 85% similarity (Figure 8). SIMPER analysis showed that the observed differences were mainly attributed to changes in ESG I cover (%) (Table S5). The reference group (A) showed an average dissimilarity of 46.97% from the group (B).

Permutational Analysis of Variance (PERMANOVA) on the community functional matrix showed that there were statistically significant differences of ESG cover (%) between all factors except leaf side. However, a statistically significant interaction between leaf side and leaf within site within meadow was noticed (Table 5a). Pseudo F was by far highest for the meadow factor (Table 5a), while pairwise analysis revealed that these differences were statistically significant between all three meadows (Table 5b). Data displayed heterogeneous dispersions between meadows (PERMDISP test, F=69.943, $p<0.001$). The highest dispersion was found at Nea Karvali meadow, while Vrasidas had the lowest. The pairwise analysis showed that dispersion was statistically significant different only between Nea Karvali and the other two meadows, while the difference between Thasos and Vrasidas was not significant (*P(perm)*= 0.063; Table S6).

Ecological Evaluation Index and anthropogenic stress

Using the EEI-c index and the Ecological Status Class (ESC) boundaries (Orfanidis et al. 2011), the meadows of Vrasidas (EEI-c_{EQR}=0.92± 0.025) and Thasos (EEI-c_{EQR}=0.78± 0.024) were classified in High ESC, while the Nea Karvali meadow as moderate ESC (EEI-c_{EQR}=0.41± 0.020). This classification is in agreement with the cumulative index of the anthropogenic stress MALUSI which was highest in Nea Karvali (8) and lower for Vrasidas (1.25) and Thasos (1) (for the MALUSI estimation see Orfanidis et al. 2020).

Discussion

In this study, for the first time to our knowledge, a combined interdisciplinary, detailed approach has been utilized on the epiphytes of the leaves of *Cymodocea nodosa*. Stereoscope- and microscope-based photographs were combined with accurate qualitative and scale-based quantitative analysis of epiphyte species/taxa cover (%), through an appropriate image analysis software. The approach was complemented further by DNA sequencing.

Methodology

The epiphytic cover (%) on *C. nodosa* leaves was estimated by combining the traditional method of “relevé” (a plot of sufficient size as representative of plant community under examination) with the “Image analysis” technology (Figure S2). While the estimation of crustose species/taxa cover (%) was straightforward, the filamentous species demanded expert judgment, due to protruding thalli. For convenience, we avoided the use of any cover glass above the leaf samples examined under the stereoscope.

Since the spatial variation of epiphytic communities was not known, a random nested sampling design on a hierarchy of spatial scales, ranging from metres (leaf) to hundreds of metres (site) to kilometres (meadow), was undertaken (see O'Neill 1988) by comparing assemblages between one disturbed and two reference meadows (Piazzi et al. 2004). The epiphytic communities on leaf sides (inward, outward) were not statistically different as in Reyes and Sansón (2001), but only at leaf level nested in site and meadow. In contrast, the epiphytes of *Posidonia sinuosa* leaves differed between the two surfaces of the leaf, with the convex surface showing a rich diversity of epiphytic species and higher biomass, including the restriction of some species to this surface only (Trautman and Borowitzka 1999).

The quantitative sampling was realized in autumn (2009) when the epiphytic cover (%) was maximal in older leaves of *Cymodocea*. This is a well-known pattern in blade-shaped leaves, including the genera *Zostera*, *Posidonia*, *Cymodocea*, *Thalassodendron*, and *Thalassia* (Buia et al. 1985, Uku and Björk 2001). Such well-stratified communities of high competition for limited resources like nutrients (Carpenter 1990) are also suitable for indicating anthropogenic stress (Orfanidis et al. 2011).

Epiphyte community as bioindicator

The most important results of this work are shown in Figures 6 and 8. Epiphytic communities studied at species and functional (ESG) levels separated the reference low-stressed meadows (Thasos and Vrasidas) from the degraded one (Nea Karvali), with the functional approach having the highest success. This is an expected result since the ESGs used have been defined using functional traits related to nutrient and light responses (Orfanidis et al. 2011) that indicate the species adaptation under water pollution or eutrophication conditions (Schramm 1999, Cloern 2001, de Jonge et al. 2002, McGlathery et al. 2007). Although both ESG I (*H. cruciatum*) and ESG II (mainly *F. mitchelliae*) cover (%) significantly changed at the meadow scale, the cover (%) of ESG II was significantly higher only in the degraded (Nea Karvali) meadow (Figure 7, Table S4). This result is in agreement with considerations suggesting that eutrophication does not appear to have a direct negative effect on the relative slow-growers (Irving and Connell 2006) but favours the fast-growing species, which tend to overgrow or out-compete slower-growing foliose or encrusting species (Cambridge et al. 2007). On the other hand, this finding disagrees with the result that nutrient enrichment can produce an increase in algal biomass, both in encrusting coralline algae and filament/erect seaweeds, on *Posidonia oceanica* leaves (Balata et al. 2008).

Increase of epiphytes concomitant with higher nutrient levels has been described in the field (Borum 1985, Tomasko and Lapointe 1991, Hemminga and Duarte 2000, Michael et al. 2008, Prado 2018, Schramm and Nienhuis 1996, Frankovich et al. 2009) and has been proven experimentally in the laboratory (Tomasko and Lapointe 1991, Short and Burdick 1996). In particular, the epiphyte load in *Zostera marina* increased by two orders of magnitude along the eutrophication gradient in a Danish estuarine system (Borum 1985). However, this is in contrast to the results of an experimental study by Coleman and Burkholder (1995), in which no significant changes in epiphyte biomass were observed with the addition of nutrients. What they observed, however, was a significant change in the composition of the epiphyte community. This result is in agreement with the results of the present work and indicates that the study of epiphyte flora should be conducted at species or functional level rather than as a general biomass of different species. In this way, the composition of the epiphytic community could be used as environmental indicator (May 1982, May et al. 1978, Michael et al. 2008, Piazzi et al. 2004, Balata et al. 2008, Giovannetti et al. 2010).

Along with nutrient concentrations, epiphyte biomass and / or composition seem to respond to hydrodynamic regimes, depth and light. The interactions between these factors make it difficult to evaluate changes in the composition and biomass of epiphytes (Borowitzka et al. 2006). Heck et al. (2000) experimentally investigated the combination of nutrient and predator enrichment. The correlation between nutrient enrichment and epiphytes may be masked by grazing of macroalgae (Apostolaki et al. 2011) or by global stressors such as ocean acidification (Campbell and Fourqurean 2014).

The biotic index EEI-c (Orfanidis et al. 2011) based on ESGs of epiphytic macroalgae classified the sampled meadows into different Ecological Status Classes: both reference meadows (Thasos, Vrasidas) characterized by less anthropogenic stress were classified in “High” ESC, while Nea Karvali was attributed to the “Moderate” ESC. This result is in agreement with that of other independent approaches. For example, the same meadows were classified as “High” (Thasos), “Good” (Vrasidas) and “Moderate” (Nea Karvali) by the CymoSkew biotic index (Papathanasiou and Orfanidis 2018). The ratio between epiphyte and leaf biomass in *P. oceanica* is one of the metrics in PREI (*Posidonia oceanica* Rapid Easy Index) used to assess the ecological quality of Mediterranean water bodies under the European Water Framework Directive (Gobert et al. 2009).

Scale-based variability

Cymodocea epiphytes showed patchiness in cover (%) of species/taxa or ESGs at the different spatial scales examined (Tables 3, 5). The highest variability was observed at the spatial scale of kilometres (meadow) due to environmental conditions. Indeed, the anthropogenic stress difference among the two reference meadows (MALUSI=1-1.25) and the degraded meadow (MALUSI=8) was high. It indicates that meadow is the adequate spatial scale for assessing the influences of abiotic factors such as water turbidity and nutrients on epiphyte assemblages (see Papathanasiou and Orfanidis 2018) and for applying a biotic index (e.g., EEI-c, see above).

For the small-scale differences in variability observed, we hypothesize that (1) stochastic factors that may affect the dispersal of propagules and select taxa with different requirements and (2) the biotic interactions, such as grazing and competition amongst sessile organisms, are the main drivers responsible (Piazzi et al. 2016). Overall, there has been a small number of studies that have attempted to examine similar spatial patterns of epiphyte distribution in *C. nodosa* leaves. The spatial trends of the epiphytes of different seagrass species do not appear to be similar and they show some differences, which should be studied in more detail in the future. For example, in *Z. marina*, an angiosperm with relatively simple structural complexity, epiphyte populations showed no differences in composition or abundance on the 10-km scale but had significant differences on the kilometer scale (Vanderklift and Lavery 2000, Saunders et al. 2003). In contrast, Piazzi et al. (2007) and Balata et al. (2007) found high variability at the scales of kilometres and 10s of metres and homogeneity at the scale of 100s of metres for the epiphytes of *Posidonia oceanica.*

Epiphyte diversity

The diversity and distribution of epiphytic organisms on the leaves of seagrasses is the result of interactions between many factors and processes. In this study, macroalgal diversity based on quantitative or qualitative (see Table 2, Figure 3) sampling was higher in the reference meadows than in the degraded Nea Karvali meadow, a pattern that was also observed by Uku and Björk (2001) and Campbell and Fourqurean (2014). Another determining factor of epiphyte diversity, besides pollution, is the availability of reproductive stages to colonize any available substrate. The colonizing stages of red and of some brown algae, as well as of diatoms, are non-motile and completely rely on hydrodynamics (Koch et al. 2006). On the other hand, green and brown algal zoids such as those of the genera *Ulva* and *Feldmannia*, are motile, thus they are expected to be more selective as to where they live. The above may explain the small number of algal species growing on the leaves of *Cymodocea* (Reyes and Sanson 1997, Reyes and Sansón 2001), which was also confirmed in the present study. In addition, the overall development of the epiphytic community will be limited by the longevity of the seagrass substrate. One very general rule states that the more persistent and structurally complex species of seagrass tend to bear higher epiphytic biomass, as do the more diverse epiphytic populations discussed by Borowitzka et al. (1990). For example, *Amphibolis griffithii* (fairly long-lived), *Posidonia coriacea* (long-lived) and *Heterozostera tasmanica* (short-lived) co-exist on the sandy shores of Southwest Australia and have been found to carry 90, 60 and 34 epiphyte species, respectively, within one annual cycle.

18S rDNA sequencing clearly confirmed the presence of red algae from the Ceramiales and of dinoflagellates, including a number of different species close to the genus *Prorocentrum*. The latter confirms microscopic observations by Turki (2005) that *C. nodosa* leaves can be an important habitat for harmful dinoflagellates including *Prorocentrum linum*, *Ostreopsis siamensis* and *Coolia monotis*. It should be noted that these dinoflagellates were not observed in the present study by microscopy, underlining that metabarcoding renders microalgal (and potentially cryptic macroalgal) epiphytes detectable. The Ceramiales sequences may well correspond to the *Ceramium ciliatum* or *C. tenerrimum* observed microscopically in many of these samples. An intriguing question is why among the macroalgae only Ceramiales were identified with metabarcoding, and not coralline algae or other groups that were detected using microscopy. This may be due to difficulty in extracting DNA from coralline algae, or a positive bias resulting from the fact that DNA from Ceramiales amplifies more easily under the DNA extraction and PCR conditions applied. Two samples (THB1 and THD1) seem to contain terrestrial plant biomass – which is entirely plausible given that all samples used in this study were collected in inshore locations, which experience lots of terrestrial runoff. However, further in-depth analysis is clearly hampered by the limited sequence coverage of Mediterranean algal diversity, as was also highlighted by a recent review (Bartolo et al. 2020). Therefore, even though the approach of assessing epiphyte diversity on seagrasses by sequencing is highly novel – to our knowledge, the present study is the first ever - the results presented here have to be considered as very preliminary. Further studies of seagrass epiphytes should definitely include molecular techniques with a much higher sequencing depth and including more variable markers (e.g., 5’-COI, ITS), but there is also clearly a need for a much broader taxonomic coverage of barcode sequences for Mediterranean macroalgae and diatoms to be useful in such studies (Bartolo et al. 2020).

Conclusions

This work has shown that the study of *Cymodocea nodosa* epiphytes based on nested sampling design and on an advanced photographic method to quantify epiphyte abundance is a very promising research topic, which may contribute to understanding the mechanisms that influence marine benthic communities under anthropogenic stress. The analysis at the functional group level relevant to anthropogenic stress was valuable to classify the coastal waters in ESCs for the implementation of the European Water Framework Directive. Finally, this study shows that metabarcoding is potentially a promising approach for assessing the diversity of epiphytic organisms across a broad taxon range, without observer bias, provided that a broad taxonomic range of reference sequences are available which, given worldwide efforts to sequence biodiversity for taxonomically informative loci, can be expected to develop in the next few years.

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Figure legends

Figure 1. Map of the three studied meadows of *Cymodocea nodosa* from the Gulf of Kavala, Northern Aegean Sea, Greece.

Figure 2. Epiphytic macroalgae of leaves of *Cymodocea nodosa* in the Kavala Gulf with emphasis on the reproductive structures. a) *Chondria capillaris*: surface view of tetrasporangial axis, scale bar: 100 μm. b) *Chondria capillaris*: immature cystocarp, scale bar: 100 μm. c) *Ceramium tenerrimum*: mature cystocarp surrounded by branches, scale bar: 100 μm. d) *Hydrolithon cruciatum*: germination disc with four central and eight pericentral cells, scale bar: 10 μm. e) *Hydrolithon cruciatum*: conceptacle containing zonate tetraspores, scale bar: 10 μm. f) *Acrochaetium* sp.: branches with stalked monosporangia, scale bar: 10 μm. g) *Feldmannia mitchelliae*: habit, scale bar: 100 μm. h) *Feldmannia mitchelliae*: branch with sessile plurilocular and unilocular sporangia, scale bar: 10 μm. i) *Feldmannia mitchelliae*: branch with stalked plurilocular sporangium, scale bar: 10 μm. j) *Feldmannia mitchelliae*: branch with sessile plurilocular sporangium, scale bar: 10 μm. k) *Polysiphonia* sp.: branches with mature cystocarps, scale bar: 100 μm. l) *Polysiphonia* sp.: branches with tetraspores forming spiral series, scale bar: 100 μm. m) *Ulva polyclada*: habit, scale bar: 10 μm.

Figure 3. Characteristic epiphyte communities on the surface of the leaves of *Cymodocea nodosa*, from the leaf base to the apex, from the three meadows (Thasos, Vrasidas, Nea Karvali) under the stereoscope, scale bar: 1 mm.

Figure 4. Cover (%) of different epiphyte species on the inward (a) and the outward (b) sides of *Cymodocea nodosa* leaves in the six studied sites.

Figure 5. Number of leaves of *Cymodocea nodosa* on which each epiphytic species/taxon was found in the three studied meadows.

Figure 6. Non-metric Multidimensional Scaling of species/taxa cover (%) on leaves of *Cymodocea nodosa* from the three studied meadows (VR-Vrasidas, TH-Thasos, NK-Nea Karvali).

Figure 7. Ecological Status Group cover (%) on leaves of *Cymodocea nodosa* from the six sites from the three studied meadows (VR-Vrasidas, TH-Thasos, NK-Nea Karvali).

Figure 8. Non-metric Multidimensional Scaling of Ecological Status Group cover (%) of epiphytes on leaves of *Cymodocea nodosa* from the three studied meadows.

Graphical abstract

The study of the epiphytes of the leaves of *Cymodocea nodosa* may contribute to the interpretation of the effects of anthropogenic stress. The analysis at the functional group level succeeded to classify the coastal waters in Ecological Status Classes.

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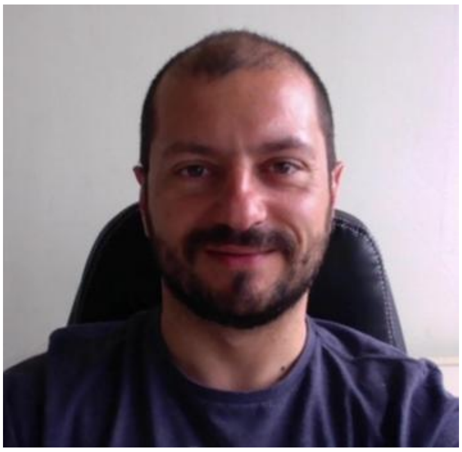

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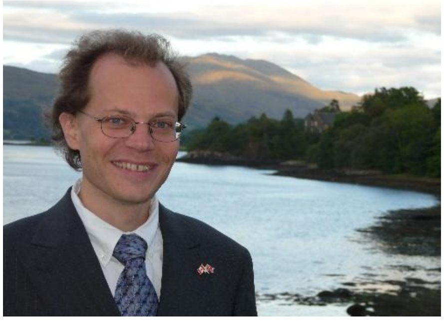

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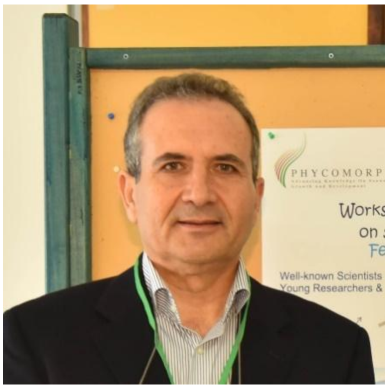

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