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Cymodocea nodosa as a bioindicator of coastal habitat quality: an integrative approach from organism to community scale.

By

VASILLIS PAPATHANASIOU

DOCTOR OF PHILOSOPHY

April 2013

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Cymodocea nodosa as a bioindicator of coastal habitat quality: an integrative approach from organism to community scale.

By

VASILLIS PAPATHANASIOU

A thesis submitted to the University of Plymouth
in partial fulfillment for the degree of

DOCTOR OF PHILOSOPHY

School of Marine Science and Engineering
Faculty of Science and Technology

In collaboration with

Fisheries Research Institute (NAGREF-HAO DIMITER)

April 2013

Abstract

The European Water Framework Directive (WFD) has encouraged considerable research on the development of water quality bioindicators. Seagrasses, that are highly sensitive to direct and indirect anthropogenic stress, and specified as quality elements from the WFD, have been at the center of this effort. In this study the use of *Cymodocea nodosa*, a widely distributed angiosperm in the Mediterranean Sea, as a bioindicator of anthropogenic stress was tested. Key biotic features of two meadows growing in locations of contrasting ecological status in the N. Aegean Sea, Greece, were sampled and analysed following a hierarchical designed approach. Plants from the degraded meadow (Nea Karvali) were found to have significantly ($p<0.05$) longer leaves, higher N and P (%) content and lower C/N ratio in their leaves than the less degraded-pristine (Brasidas and/or Thasos) meadows. The application of chlorophyll fluorescence as an easily measurable indicator of the anthropogenic stress has been tested before with limited success. This study, based on a large amount of measurements (ca.500 per meadow) carried out after a short acclimation period in the laboratory under constant temperature and irradiance conditions, showed significantly higher ($p<0.05$) $\Delta F/Fm'$ and Fm values at plants from the degraded than from the pristine meadows. Three sets of laboratory 8-days experiments were carried out under optimal growth temperature (21 ± 1.5^{o}C) in order to investigate cause-effects relationships between the main local stressors (nutrients-N, P, irradiance and heavy metal-Cu) to shoots collected from differently impacted meadows. High nutrient concentrations (30μM N-NO_3; 2μM P-PO_4) had a significant effect ($p<0.05$) on $\Delta F/Fm'$ only on shoots from the pristine site. Low irradiance (37 μmol photons m^{-2} s^{-1}) resulted in a significant increase ($p<0.05$) of $\Delta F/Fm'$, while high copper concentrations (>4.7 μM) had the opposite effect. Through these experiments light availability and nutrients were identified as the main factor that affects the meadows health.

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I would also like to thank my parents Nikos and Vasiliki, without which I could never have completed this task. Their belief in me and my goals, their nurturing and their love armed me with the necessary strength to overcome any obstacle I came across the way. My wife Stella, who saw purpose in this research and took some of my weariness on her back.

All experiments and most measurements were conducted at the Fisheries Research Institute in Nea Peramos, Kavala. I feel the need to thank all the people working there for their help but especially Stefanos Gounaris, Konstantina Nakou and Tania Tsioli for helping me those countless hours in the water and the laboratory.

I would like to dedicate this work to my newborn daughter Nikoleta and tell her that learning is an extremely difficult process without which we remain hollow throughout our lives.

AUTHOR'S DECLARATION

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award without prior agreement of the Graduate Committee.

During the new route PhD program the postgraduate courses of marine conservation and ecological analysis, marine chemistry and near shore ecology, ocean policy, marine science and regulations, pollution ecology/ ecotoxicology, multivariate analysis for environmental science, laboratory based teaching were undertaken.

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September	2009	Mediterranean Seagrass Workshop 09, Hvar, Croatia.
June	2009	9th International Phycological Congress 2009, Tokyo, Japan.
May	2007	Chlorophyll fluorescence in aquatic sciences- Aquafluo. Nove Hrady Czech republic.
December	2007	Seaweed taxonomy. Heriot- Watt university. Edinburgh, Scotland.
June	2006	Transitional states in transitional and coastal waters. Fisheries Research Institute, Nea Peramos, Hellas.
June	2006	8th Hellenic oceanography and fisheries symposium. Thessaloniki, Hellas.

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Chapter 1: Introduction

Marine phanerogams

Seagrasses are marine angiosperms that 120 million years ago returned to the marine environment and differ from seaweeds in having true leaves, stems, and roots. They are flowering plants from four plant families (Posidoniaceae, Zosteraceae, Hydrocharitaceae and Cymodoceaceae). They form a group that is divided into two monocotyledouous families (Potamogetonaceae, Hydrochariaceae) comprising of 12 genera (*Zostera, Phyllospadix, Heterozostera, Posidonia, Halodule, Cymodocea, Syringodium, Thalassodendron, Amphibolis, Enhalus, Thalassia,* and *Halophila) and* 57 species. They grow in soft sediment in almost all coastal areas around the globe, except Antarctica (Figure 1), in shallow waters (from a few centimeters up to 40m, depending on the species and abiotic parameters such as light), where they form meadows (Den-Hartog, 1970). Globally, they cover an estimated area of 177,000 square kilometers (Orth et al., 2006). This is likely to be an underestimation, since there have been no surveys off the western coasts of Africa and Latin America.

Seven seagrass species can be found in the Mediterranean Sea (Hemminga et al., 2000): *Cymodocea nodosa* (Ucria) Aschers, *Halophila stipulacea* (Forsk.) Aschers, *Posidonia oceanica* (L.) Delile, *Ruppia cirrhosa* (Petagna) Grande, *Ruppia maritima* L., *Zostera marina* L. and *Z. noltii* Hornem. Of these *P. oceanica* is the most common species, while the two *Zostera* spp. alone can be found in the coastal areas of the United Kingdom.

Even though morphology changes between different species, they all share several common features as seen in Figure 2 (Kuo et al., 2000). They are able to survive under complete submersion; they have a sufficient mechanism for attaching themselves to soft sediment, as well as to marine conditions and are able to compete with other marine macrophytes. Some species e.g. *Zostera* spp. can withstand emersion for short periods (Figure 3) while others e.g. *Ruppia* spp. can survive in low salinity environments.

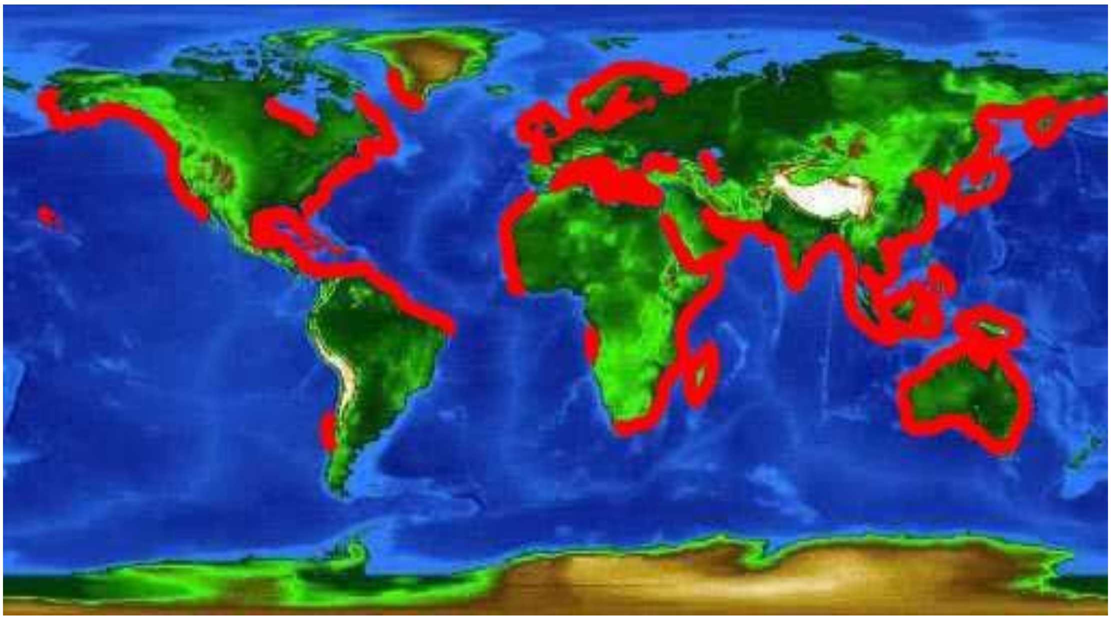

Figure 1. World distribution of seagrasses. 57 species exist worldwide but only 7 of them can be found in the Mediterranean. (source: www.flmnh.ufl.edu)

The basic morphological units are: shoots, nodes, rhizomes, leaves and roots (Figure 2). Rhizomes are horizontal underground stems, from which the leaf sheaths and the leaves are erected. They form a dense network that aids anchorage. They also play an active role in nutrient translocation. True roots branch from rhizomes, with a role similar to that of terrestrial plant roots (nutrient uptake). Leaves (Figure 4) are the main photosynthetic unit but they also absorb nutrients direct from the water column, making the mechanism of nutrient uptake more efficient. Seagrasses have developed air tubes (veins or lacunae) in their leaves which are used for oxygen storage and circulation (Den-Hartog, 1970). They are also responsible for the positive buoyancy that allows leaves to capture incident light for photosynthesis. Air lacunae have a taxonomic value as there is a specific number for each species, e.g. *C. nodosa* has 7- 9 vertical veins (Borum et al., 2004).

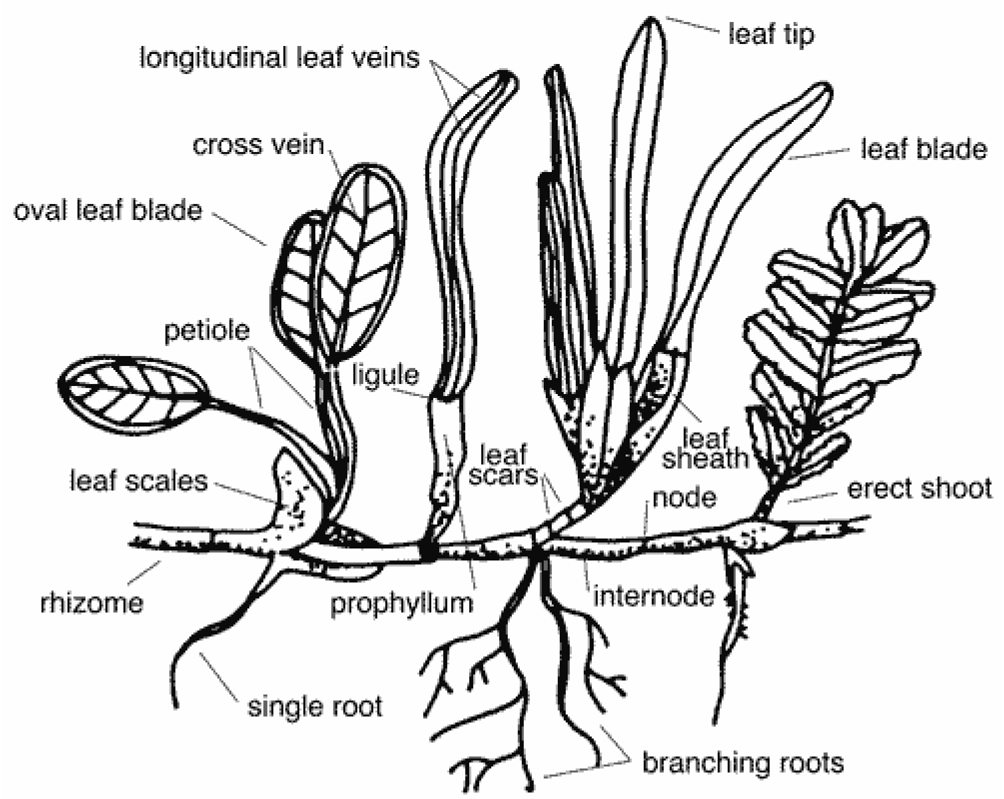

Figure 2. Morphological features of different type of seagrass- composite diagram. The morphology differs among species, depending on the evolution background. However the basic units are repeated to all of them (Larkum et al., 2006).

Seagrass ecology

Seagrass meadows are dynamic ecosystems, characterized by constant changes. Older leaves die, while new ones are formed. Meadows can sustain themselves for long periods, e.g. in the case of *Posidonia oceanica* as long as 4000 years (Mateo et al., 1997). A single shoot, however, has a life cycle of just a few weeks, depending on the species, e.g. *C. nodosa* shoots have a life expectancy of 4 to 22 months, while the life of a leaf is 2-5 months (Reusch et al., 1999). Declining meadows are becoming more common in recent decades (Lee et al., 2007b). A number of parameters result in seagrasses death with burial under the increasing quantities of sediment (Marba et al., 1995), unavailability of sunlight due to turbidity (Enríquez, 2005) and water column and sediment degradation (Terrados et al., 1999) being the most common.

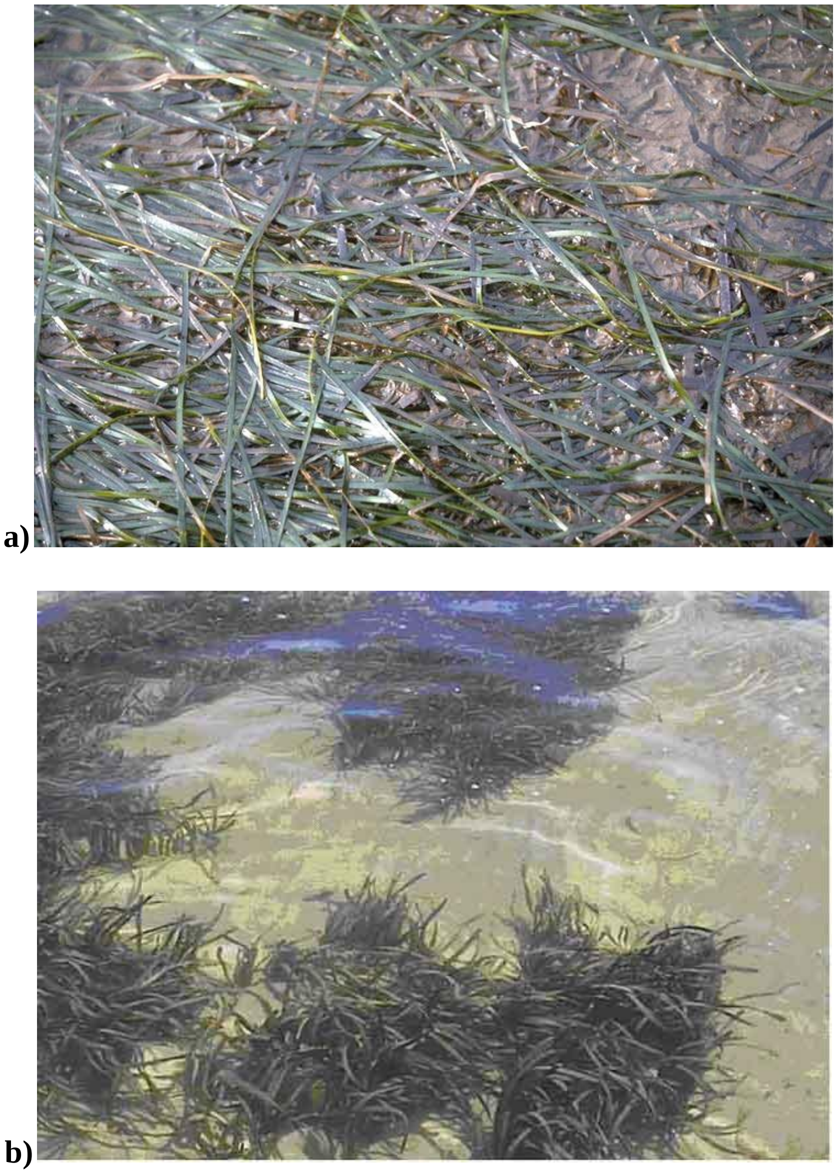

Figure 3. *Zostera marina species* a) emerged and b) submerged (source: www.teamseagrass.blogspot.com).

Figure 4. Different types of seagrass leaves. They vary in shape and length among species but they share the same biology and ecology (Source: www.swfwmd.state.fl.us).

A climax meadow may be extensive and homogenous, but this is uncommon and meadows tend to form patches (Figure 5). Most are heterogeneous as a result of local variability, as well as internal growth. Its common for them to form round structures with low density in the centre (Den-Hartog, 1971; Fonsesca et al., 1998). Intense hydrodynamic activity, such as storms (Patriquin, 1975), play an important role in the formation of such structures, proving the connection between the population's structure and weather conditions (Fonsesca et al., 1998). The patches round shape is a result of the roots geometric model of growth. Roots have the tendency to grow at an angle greater than 30^0, moving in a spiral orbit (Marba et al., 1998), a process that takes place in the outer part of the meadow, where newly formed leaves are expected to be found. Conversely in the centre, older shoots die resulting in a lower density.

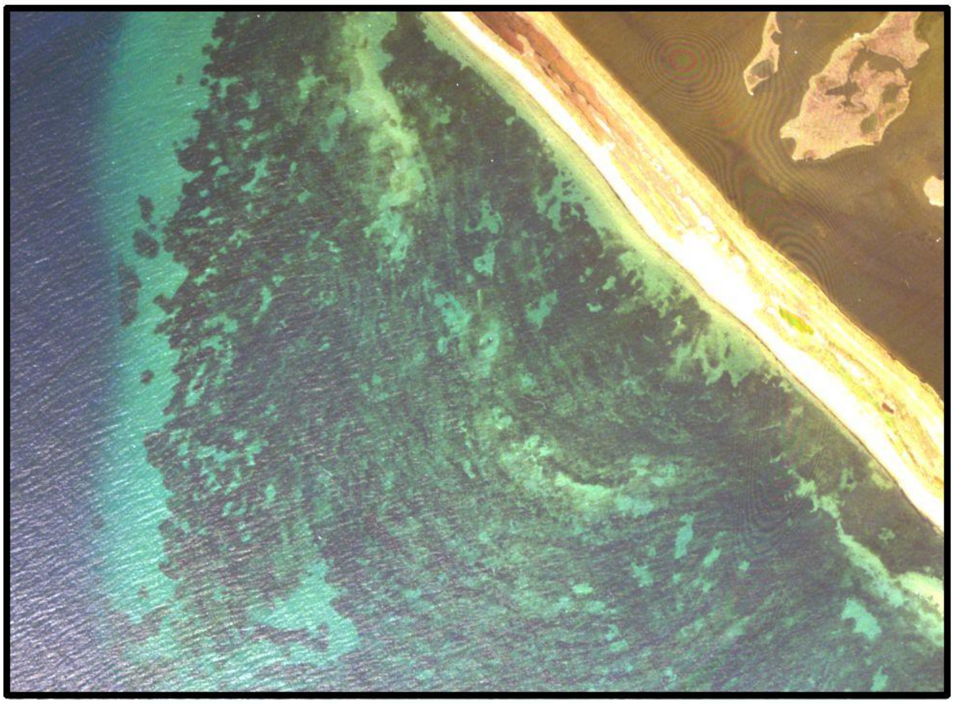

Figure 5. Meadow of *Cymodocea nodosa* in the shores of North Aegean Sea, Hellas. Mosaic distribution can be clearly observed, with some areas appearing dense, while other sparse.

Meadows are in a state of constant change, losing some parts and replacing others, as a result of local scale variability. This is the reason for their characteristic mosaic spatial growth, which can resemble the skin of a tiger (Den-Hartog, 1971). Ultimately, the shape of the meadow depends on the frequency and the scale of disturbances and the meadow's resilience capacity. For instance, frequent disturbance in a meadow with low resilience ability would lead to its extinction, while high resilience would lead to a mosaic type of growth. This dynamic procedure can be easily understood in *C. nodosa* sites, where sand dunes 10-20cm high are formed. When sand dunes are formed roots are left exposed at the lower part of the dune and are destroyed by organisms that feed on them or use them as substratum. At the same time, the part left buried by the dune continues to grow (Marba et al., 1995). This phenomenon is not witnessed in *P. oceanica* sites, because of longer leaves in this species that can still thrive without mortality under sand dunes of 30cm height.

Figure 6. Seagrasses form one of the most productive ecosystems, with a high biodiversity. A *Posidonia oceanica* meadow sustains a biodiversity of up to 500 species (Borum et al., 2004).

Seagrasses form a unique productive habitat (Figure 6). Their leaves and roots are the only hard substratum that organisms can find to attach to in a soft sediment environment. They stabilize the sediment and provide food and shelter (Pollard, 1985; Harmelin-Vivien et al., 1995; Edgar, 1999a, b). Leaves and roots form three different subhabitats; one in the water column between the leaves, a second on the sediment above the roots and a third in the sediment, between the roots and rhizomes. Moreover, leaves that break due to intense wave action are washed onto the shore, where they form another ecosystem in the super littoral zone with high biodiversity (Figure 7).

Figure 7. Washed out seagrass leaves form a new habitat, quite different from the meadow itself that has its own biodiversity (source: www.waterwatchadelaide.net.au).

	gCm-2yr-1
Coral reefs	2000-5000
Rocky shores	-
Kelp systems (subtidal)	1000
Fucoid systems (intertidal)	100
Seagrass communities	300-1000
Coastal phytoplankton	50-250

Table 1. Photosynthetic production in different benthic communities (Mann, 1982).

Seagrass meadows have a high rate of production (Table 1). High biomass means that there is increased production of oxygen through photosynthesis. Dead leaves decompose at a slow rate, thus enriching the sediment with organic matter over a long period. Even though they are responsible for only 1% of world oxygen production, they store 12% of ocean carbon matter (Borum et al., 2004). This difference between the amount of carbon that is stored and the amount that is released through respiration is indicative of the significant role they play in world carbon cycle (Touchette et al., 2000a). Epiphytes and macrophytes that are associated with seagrass meadows also have high rates of production and therefore contribute to this very productive ecosystem, which is comparable to terrestrial forests.

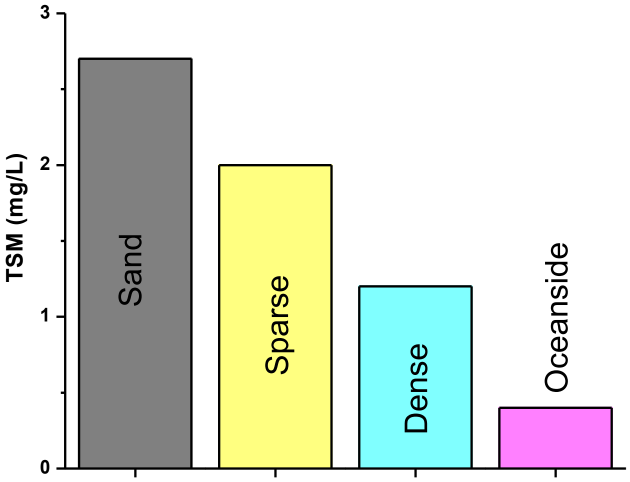

Figure 8. Amount of Total Suspended Matter (mg*L^{-1}) as measured from satellite in an area without benthic vegetation (sand), one with sparse seagrass meadow, another with a dense seagrass meadow and along the Oceanside. A dense meadow seems to stabilize the sediment, lowering the TSM. Image reproduced from (Dierssen et al., 2010)

Another important function of seagrass meadows is their ability to increase water quality. Leaves form a net that decreases the severity of hydrodynamic action and prevents the sediment from re-suspending and filtering the water that reaches the coast (Figure 8), while the capacity of the meadow to trap suspended matter increases due to the activity of filter feeders (Fonseca, 1989). In this way light availability increases, allowing the meadow and other photosynthetic organisms to thrive. Seagrasses take up nutrients from the water column through their leaves as well as from pore water through their roots. This mechanism is responsible for high productivity values in oligotrophic environments that allow them to compete with planktonic and macroalgae that are the main producers in marine ecosystems and that can significantly affect seagrass beds when in high biomass by depleting all nutrients from the water column and lowering irradiance (Short, 1987).

The network of roots and rhizomes stabilizes the sediment, prevents the retention of particles, because of wave action or currents (Fonseca et al., 1996) and minimizes coastal erosion. Leaves can act as a barrier that dissipates wave energy (Fonseca et al., 1992). In enclosed areas without river systems the sustained organisms in a meadow can become the only source of new sediment.

Human impact on seagrass meadows

The coastal zone is characterized by high anthropogenic pressure, such as excessive pollution from sewage discharge, oil and runoff, dredging, uncontrolled bait digging, boat propellers (Figure 9) and anchors and inappropriate fishing (Ruiz et al., 2003). Seagrasses have high phenotypic plasticity and show a variety of morphological and physiological adaptations in order to cope with both natural and anthropogenic stress (Jensen et al., 2001). For example leaf death leads to a more sparse meadow, but in an effort to replace this loss new rhizomes are produced, mainly at the peripheral area, so that new individuals can be formed further distant to the pressure source. This forms the main strategy of resilience for the population, as well as for spatial growth; however, patch production is a slow process for most seagrasses (Table 2). At the same time seeds are produced, to ensure the plant's survival (Duarte et al., 1990). However, because of the low number of seeds that are being produced and their high death rate (Vidondo et al., 1997; Olesen, 1999), only a few germinate and flower. Shoot replacement has been examined for different species and is highly variable. For example *Z. marina* has a rate of 5x10^{-3} shoots.ha^{-1}yr^{-1} (Olesen et al., 1994), *C. nodosa* 5x10^{-3} shoots.m^{-2}yr^{-1} (Duarte et al., 1990) and *P. oceanica* 3x10^{-4} shoots.ha^{-1} yr^{-1} (Meinesz et al., 1984).

The basic strategy for spatial enlargement of a meadow is through root growth (Duarte et al., 1990). New roots are formed in the outer part of the meadow, where sufficient space is available and they can find the space required for their spatial growth. At the early stages of their formation, nutrients are transported from older leaves through the root system, so that growth rate is increased (Duarte et al., 1996).

Figure 9. Propeller scars in intertidal seagrass meadows and channel dredging in coastal ecosystems (Borum et al., 2004).

Species	Rate of formation	Source
Zostera marina	1000 patches ha-1yr-1	Olesen &Sand-Jensen 1994
Cymodocea nodosa	45 patches ha-1yr-1	Duarte & Sand-Jensen 1990
Posidonia oceanica	3 patches ha-1yr-1	Meinesz & Lefevre 1984

Table 2. Examples of seagrass patch formation rates. Different species have different formation rates. A meadow of *Posidonia oceanica,* which is the most abundant seagrass of the Mediterranean, has the lowest growth rate, meaning that after a stress incident it needs a lot of effort to return to its previous condition. The same does not apply for fast growing species, such as *Z. marina* and *C. nodosa*. From (Borum et al., 2004)

Water framework directive 2000/60 EC of the European parliament of the council

The WFD is the latest policy of the EU to protect aquatic ecosystems; it explains the protection required by member states for all aquatic environments (rivers, lakes, coastal and underground waters) within the EU. One of the main goals is to ensure that all these ecosystems acquire or at least maintain a “good” ecological quality until the year 2015, through intercountry pollution control and management plans (Table 3). The directive introduces some novel views in the field of water management, such us the link between the water quality and the supported ecosystem and the relevance of organisms in the definition or evaluation of water quality (Romero et al., 2007). It also includes the different habitat types that because of their ecological importance and sensitivity need to be protected; the need for use of reference conditions, ecological status classes and indicative parameters (quantified) of the quality elements (for the coastal ecosystem these are benthic plants and animals and phytoplankton).

The purpose of this Directive is to establish a framework for the protection of inland surface waters, transitional waters, coastal waters and groundwater which among others:

a) *Prevents further deterioration and protects and enhances the status of aquatic ecosystems and, with regard to their water needs, terrestrial ecosystems and wetlands directly depending on the aquatic ecosystems;*
b) *Aims at enhanced protection and improvement of the aquatic environment inter alia through specific measures for the progressive reduction of discharges, emissions and losses of priority substances and the cessation of phasing-out of discharges, emissions and losses of the priority hazardous substances;*

  ....

  and thereby contributes to:

- ....
- *The protection of territorial and marine waters, and*
- *Achieving the objectives of relevant international agreements, including those which aim to prevent and eliminate pollution of the marine environment, by Community action under Article 16(3) to cease or phase out discharges, emissions and losses of priority hazardous substances, with the ultimate aim of achieving concentrations in the marine environment near background values for naturally occurring substances and close to zero for man-made synthetic substances.*

Table 3. Part from the Water Framework Directive (EEC, 2000).

According to the WFD, the ecological quality of an ecosystem is quantified by a comparison with a pristine ecosystem (reference conditions). Only after reference condition sites have been chosen, ecological indicators can be developed so that aquatic environments are classified. However, this task has implications, since different Member States of the EU have different environmental conditions and must form a different approach to their reference sites. The real problem occurs when different countries have to compare their results. This is why the WFD makes use of “classes of ecological status" for every biological quality element, so that all indicators can be calibrated against (Table 4).

The WFD states that ecological status should be measured with the use of bioindicators. McCarty and Munkittrick (1996) have related the concept of biomarkers and bioindicators in one definition, which considers bioindicators as, "anthropogenically-induced variation in biochemical, physiological, or ecological components or processes, structures or functions (i.e. biomarker) that has been either statistically correlated or causally linked, in at least a semiquantitative manner, to biological effects at one or more of the organism, population, community, or ecosystem levels of biological organization".

Species that are used as bioindicators should be sedentary, of ecological importance, widespread and widely studied and sensitive to environmental variations (Molfetas et al., 1981). Biomarkers are cellular, molecular and biochemical changes induced by chemical pollutants, measurable in biological systems such as tissues, cells and biological fluids (McCarthy et al., 1990; Lagadic et al., 1997, 1998). Biomarkers are more specialized in their terminology and they can offer more relevant information on the potential impact of toxic pollutants on the health of organisms.

Today, ecological status of a marine ecosystem is measured mainly by using benthic macroinvertebrates, fish, macrophytes and plankton as indicative groups (Gibson et al., 2000). Some examples of such bioindicators are BENTIX (Simboura et al., 2002), BC (Ponti et al., 2004), ISD (Reizopoulou et al., 2007), BQI (Rosenberg et al., 2004) etc. The WFD however recognizes marine angiosperms as a biological quality element and a sensitive ecosystem. They seem to be more appropriate for the use of bioindication and biomonitoring, due to certain aspects of their biology and ecology (Orfanidis et al., 2001; Reizopoulou et al., 2004; Orfanidis et al., 2005a). However, only a few ecological indicators or monitoring techniques exist, based on marine plant assemblages and even less on seagrasses, something that seems to be changing the past two decades (Figure 10).

	High Status	Good Status	Moderate Status
Macroalgae and angiosperms	All disturbance sensitive macroalgal and angiosperm taxa associated with undisturbed conditions are present.	Most disturbance sensitive macroalgal and angiosperm taxa associated with undisturbed conditions are present.	A moderate number of disturbance sensitive macroalgal and angiosperm taxa associated with undisturbed conditions are absent.
	The levels of macroalgal cover and angiosperm abundance are consistent with undisturbed conditions.	The level of macroalgal cover and angiosperm abundance show slight signs of disturbance.	Macroalgal cover and angiosperm abundance is moderately disturbed and may be such as to result in an undesirable disturbance to the balance of organisms present n the water body.

Table 4. Definition of three quality classes of coastal waters based on macroalgae and angiosperms (source WFD, Annex V, §1.2.4).

Seagrasses are sensitive to disturbances (Delgado et al., 1999; Francour et al., 1999; Ruiz et al., 2003), widely spread, especially in the Mediterranean coasts (Coyer et al., 2004) and there is sufficient background study on their biology and ecology (Romero et al., 2005) as well as of specific responses of species to anthropogenic disturbances (Pergent et al., 1999; Campanella et al., 2001; Cancemi et al., 2003; Ruiz et al., 2003; Vizzini et al., 2004). Since they are sessile organisms, they have to adapt by changing morphological (leaf length and width) and functional features (density, number of leaves per shoot), which can be quantified and used as indicators. Moreover, they are at the bottom of the food chain, reacting more rapidly to the presence of pollutants than organisms living at higher trophic levels. Their faster response to pollutants is trivial when it comes to the formulation of counter-pollution management plans. Even though macrophytes are widely used as bioindicators, seagrasses aren’t. Two examples are the depth limit of *Z. marina* (Krause-Jensen et al., 2005) and *P. oceanica* Multivariate Index (Romero et al., 2007). Another positive aspect of seagrasses as bioindicators is their capacity to accumulate a wide range of pollutants such as organo-chlorine compounds (Chabert et al., 1984), artificial radionucleides (Florou et al., 1985) and particularly heavy metals such as Cd, Pb, Cu, Zn, Se (Malea et al., 1989).

Monitoring an ecosystem's health must be carried out periodically. Thus, the bioindicators employed should be based on a simple, inexpensive methodology (EEC, 2000). Seagrass indicators seem to comply with these conditions, since the plants are found at shallow depth and they are easy to collect (although SCUBA diving might be necessary), with no need for sophisticated sampling devices or research vessels, making them ideal for long term monitoring (Figure 11).

Cumulative evidence indicates that impacts are best investigated at the population or community level (Lobban et al., 1994; Crowe et al., 2000). This requires an approach that integrates an ecological assessment into the more traditional chemical and physical evaluation (Gibson et al., 2000). However, the diagnosis of the ecological status is often a difficult task because of spatial and temporal variability in community features as a result of changes in physical and chemical conditions (Orfanidis et al., 2001). This problem can be overcome by studying an ecosystem at the community level from a functional point of view. At this level communities appear to be much more temporally stable and predictable than when examined at the species level (Steneck et al., 1982; Steneck et al., 1994).

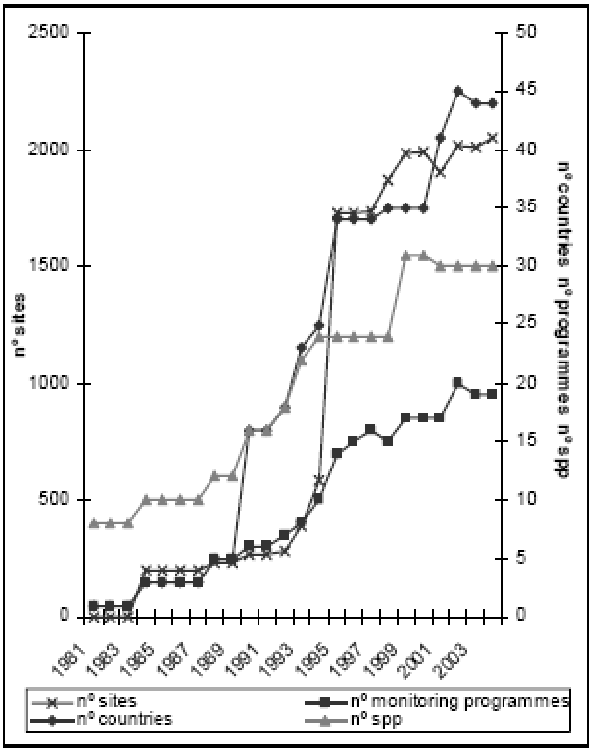

Figure 10. Evolution of seagrass monitoring programmes in the last two decades. All the more attention is focused on seagrasses in the past two decades, mainly because of further understanding their ecology and biology (Borum et al., 2004).

Figure 11. Monitoring and sampling protocols for seagrasses are quite easy procedures, with no need for sophisticated sampling devices (source: www.teamseagrass.blogspot.com).

An indicator focused on a higher level of organization (community, population) is more suitable to describe the impacts of pollution hence one that is based on lower levels (species morphology, enzymes, biomarkers) can explain the true nature of the pollutant (Orfanidis et al., 2001). For example, the EEI by Orfanidis et. al. that can measure the ecosystem’s quality in an early stage, taking in account the synthesis of the benthic seaweeds. However, this is not sufficient to form a management plan for a degraded area and in order to find out the exact source of disturbance and exploit the entire ecosystem's information we need to examine what is happening at a lower level of biological organization, where certain chemicals or cell attributes might be affected by specific pollutants (Munkittrick et al., 1995).

Cymodocea nodosa as a potential bioindicator

C. nodosa is the second most important seagrass species in the Mediterranean in terms of ecological importance and abundance (Barbera et al., 2005). In addition to the benefits that seagrasses have as bioindicators, *C. nodosa* seems to be even more appropriate due to its fast growth rates, especially in comparison to *P. oceanica* a species on which many bioindicators are based, and as a result it's more sensitive to environmental changes with a faster response time. It can be found in very shallow subtidal areas (50cm- 6m), so sampling is very easy and its smaller size makes it suitable for laboratory analysis (Borum et al., 2004). However, seagrass studies have mainly focused on *P. oceanica* and there is insufficient knowledge on the ecology and biology of *C. nodosa* under impacted conditions (Barbera et al., 2005).

Aims of the study

Transitional and coastal waters are some of the most productive ecological systems on Earth and have a high value to human society. However, they are being threatened by anthropogenic pressure (Crooks et al., 1999). In order to face this problem its critical to identify the key biological signals (impacts) that indicate the intensity of anthropogenic stress these coastal environments suffer and the impact on the ecological status.

A further study of the relationships at physiological, individual, population, and community levels in *C. nodosa* meadows, relatively to patterns in contaminant loading along different spatial and temporal scales gradient, will give us a better understanding of the species ecology and adaptation mechanisms. Through these studies, the mechanistic basis between environmental stressors and stress responses of the species could be unravelled (Chesworth et al., 2004), contributing to ecological impact assessment of coastal water resources and the protection of marine biodiversity. The development of an early warning bioindicator of ecosystem quality that is based on a top-bottom approach, would also contribute to the race that the Water Framework Directive has signalled, towards a complete intercountry management plan for coastal water ecosystem's sustainability.

So far there are two bioindicators based on this seagrass: CYMOX (Oliva et al., 2011) based on multiple parameters such as $\delta^{15}N$, $\delta^{34}S$, root weight ratio and heavy metal content etc., and CymoSkew (Orfanidis et al., 2009b), based on measuring the skewness index of ln transformed relative frequencies of leaf length values. In this study the relatively new technology of chlorophyll- *a* fluorescence of *C. nodosa* leaves as an ecological indicator of anthropogenic stress will be tested. Previous studies indicate that such a parameter could be used after further research (Beer et al., 2000; Durako et al., 2002 ; Horn, 2006). Seagrasses increase chlorophyll biosynthesis under nutrient induced stress (Lee et al., 2007b), thus increased photosynthetic yield is expected in sites with high nutrient concentrations. At the same time morphological and dynamic parameters of the meadow will be measured, since they provide a good indication of the meadow's health (Orfanidis et al., 2007) and their correlation to anthropogenic stress will be studied. In order to better clarify the species reaction to the main identified stressors (nutrients, light and heavy metals) in our study area, laboratory experiments will be carried out.

The hypothesis that two populations acclimated in different environments (a pristine and a degraded meadow) should have different reaction to stressors will be tested.

The aims of this study are to:

- describe the morphology and physiology of *C. nodosa* under pristine and degraded conditions and combining them, contributing to the ecological and biological knowledge of the species.
- assess the physiological responses to elevated heavy metal and macronutrient exposure as well as light irradiance.
- evaluate chlorophyll- *a* fluorescence as a bioindicator of anthropogenic stress by comparing the photosynthetic efficiency of the species under different levels of anthropogenic stress.

Chapter 2: General Methodologies

Introduction

About twenty three per cent of human population lives within 100km of the ocean, while highest population density is located in the first 10km (Nicholls et al., 2002). Overgrowth of nearest coastal cities is accompanied with the development of artificial structures in both terrestrial and marine ecosystems (harbors, piers etc.) as well as the necessary infrastructures that produce resources (food, energy, freshwater etc.). As a result, inputs of nutrients, organic matter and contaminants has increased worldwide (Nixon et al., 2009), leading to a deterioration of coastal environment quality. The effect has been documented in many key ecosystems, such as seagrass meadows that have been declining at an alarming rate (Waycott et al., 2009).

Many researchers have identified and explained the important functions of seagrasses in a habitat, as well as their global role (Larkum et al., 2006) and the need to plan towards their conservation (Orth et al., 2006). Moreover, their high sensitivity to environmental changes (both in the water column and the sediment), their fast growth rates and their widespread global distribution has established them as suitable bioindicators (Marba et al., 2012) that have been used in many policies aiming to the improvement of marine ecological quality, such as the Water Framework Directive (WFD, 2000/60/EC) in Europe and the Clean Water Act (CWA) and National Estuary Program in the USA.

Their establishment as bioindicators has led to the production of numerous methodologies, based on different seagrass species, representing different structural and functional levels and spatial scales. Some of the most common metrics employed are meadow distribution and extent, abundance, shoot morphology, chemical composition of the plants, population and growth dynamics (Krause-Jensen et al., 2005; Romero et al., 2007; Orfanidis et al., 2009b). Marba et. al. (2012) identified 51 metrics employed in seagrass bioindicators, 61% of which are based on one single metric.

The variety of existing bioindicators reflects the difference distribution of species that in turn means different dynamics between ecoregions, while at the same time diverse scientific traditions and local knowledge play a key role in the choice of indicators. Even though many indicators have been created, there is still room for further research, since a common methodology, easy to apply, with an early warning behavior that can be easily integrated across regions has yet to be established. At the same time new technologies offer new research angles, adding to the existing knowledge on seagrasses.

Photosynthesis is a primary mechanism of all plants. Seagrass growth is dependent on the quality and quantity of light available, while light limitation has been linked to seagrass degradation as well as massive seagrass die-offs (Short et al., 1996.). Photosynthetic efficiency has been linked to many abiotic factors, such as temperature (Masini et al., 1995), dissolved organic nutrients (Alcoverro et al., 2001a), dissolved oxygen and water movement (Sand-Jensen, 1989), as well as biotic factors like chlorophyll content (Drew, 1978), age of leaves (Drew, 1978) and epiphytic load (Bulthuis et al., 1983). Therefore study of photosynthetic activity in a specific meadow can provide a total measure of stress that the plants are receiving.

In this study the use of PAM fluorometry, a method that has been used increasingly the past 10 years, is tested as a stress index of *Cymodocea nodosa* meadows in the North Aegean Sea, while at the same time an effort is made to gather knowledge about the differences of morphological (leaf length, width, leaves per shoot) and physiological aspects along a pollution gradient. Analysis of accumulated carbon, nitrogen and phosphorus in shoots along a pollution site gave insight on the extent of stress the plants were under and allowed us to link nutrient load to physiological responses in the field.

Lee and Park (2007a) suggested that in order to use PAM as an indicator of meadow health, further research is needed in order to link physiological responses to the complicated combined action of stressors. To this end experiments helped determine the effect of irradiance, heavy metal and nutrient concentrations on photosynthesis and growth of *C. nodosa*. Chlorophyll effective quantum fluorescence yield and leaf elongation were the measured parameters.

Pulse amplitude modulated fluorometry (PAM)

One of the most characteristic and important functions in seagrasses, as in all photoautotrophic organisms, is photosynthesis. Photosynthesis is the metabolic pathway through which all plants convert light energy captured into chemical. Light enters the chloroplasts and is captured by the chlorophyll pigments in the thylakoid membrane. The energy is then transported to the reaction centers of photosystems I and II, where it's utilized in the photochemical reactions that will eventually produce carbon molecules and oxygen. The overall equation used to describe photosynthesis inside water is:

$$2n\ CO_2 + 2n\ H_2O + \text{photons} \rightarrow 2(CH_2O)n + 2n\ O_2 \quad \textbf{equation (1)}$$

Photosynthetic measurements of seagrasses have been used to provide direct and indirect information about growth, as well as responses to environmental stressors (Ralph et al., 1995). Until recently the most common technique used for measuring photosynthesis was gas exchange, which is based on measuring the O_2 release seen in equation (1) during photosynthetic process. However, the method is time consuming and unfit for large-scale spatial analysis of meadows.

A new method based on chlorophyll *a* fluorescence has been developed over the past two decades that allows rapid measurements of different photosynthetic parameters. The method is frequently referred to as Pulse Amplitude Modulation fluorometry (Schreiber et al., 1986). The energy captured by the chlorophyll pigments is led to the reaction centers were it would be used in photochemistry. While the electrons are transported to the reaction centers in the chloroplasts two competitive pathways of de-excitation also take place, heat dissipation and chlorophyll fluorescence. Chlorophyll fluorescence is the emission of photons by the radiative de-excitation of excited chlorophyll molecules. Since energy cannot be lost, the sum of photochemistry (P), heat dissipation (D) and fluorescence (F) must equal the energy of the photons absorbed. This sum is steady and complementary and described by equation 2:

$$F+D+P=1 \quad \textbf{equation (2)}$$

Heat dissipation is low and constant and can be ignored, meaning that fluorescence increases proportionally as photochemistry efficiency decreases. Thus, a measurement of the first would lead to an indirect estimation of the second. Even though chlorophyll fluorescence is very small (1-2% of light energy absorbed; (Maxwell et al., 2000)) its measurement is quite simple and it gives us a fast and valuable estimation of photosynthetic efficiency or photosynthetic rates.

The measuring principle for chlorophyll fluorescence is simple (Figure 12). The leaf rests in darkness for a period of 10-20 minutes, depending on the species, so that all reaction centers of photosystem II become “open”, meaning that they don’t have any electrons resting on them. Then it’s exposed to a pulsating measuring light and the fluorescence yield, termed Fo is measured. A period of 0.5-1sec of a saturating light (2000- 3000 μmol photons $m^{-2}s^{-1}$) follows that “closes” all reaction centers of PSII. During this period the fluorescence yield reaches its maximum value, Fm, which is measured right after the saturation light stops again with the pulsating measuring beam. From these two values the **maximum quantum yield (Fv/Fm)** can be calculated:

$$Fv/Fm= (Fm-Fo)/Fm, \quad \textbf{equation (3)}$$

$$Fv=Fm-Fo$$

Maximum quantum yield can be measured only in dark- adapted leaves in order to ensure that all reaction centers are open, Fo is minimum and Fm maximum. Fm is a sensitive parameter that decreases under different types of stress factors, such as photoinhibition, salt stress, high and low temperature, presence of toxicants etc. (Maxwell et al., 2000). Fv/Fm measures photochemical efficiency and it can be used to assess the physiological state of PSII, as well as plant responses to certain stressors (Ralph et al., 1995; Dawson et al., 1996; Ralph et al., 1998a; Björk et al., 1999; Mallick et al., 2003).

When the same measurements are taken under ambient light the effective quantum yield, ΔF/Fm’ is measured. Usually when measuring ΔF/Fm’ the saturating pulse (where the Fm’ is recorded) is emitted after only a short period of constant actinic light with a steady level of fluorescence yield (Ft), allowing the photosynthetic rate under a certain level of light stress to be determined. ΔF/Fm’ is a more sensitive and at the same time more complex parameter than Fv/Fm (Genty et al., 1989).

$$\Delta F/Fm' = (Fm'-Ft)/Fm' \quad \textbf{equation (4)}$$

When measuring with the PAM on seagrasses a problem arises as to where exactly to measure. There is large variability within shoots and leaves. Durako and Kunzelman (2002) showed how Fv/Fm varies within a shoot, reaching maximum values at the middle of the leaf in *T. testudinum,* while at the same time they measured minimum values in younger leaves. Since seagrasses grow from the basal meristem, it’s only logical that this region will have lower chlorophyll content and lower yield values.

Preliminary measurements in *C. nodosa* showed that the second adult leaf of a shoot, at 2cm above the stem, yielded more stable ΔF/Fm' measurements, so it was chosen as the standard point of PAM measurements.

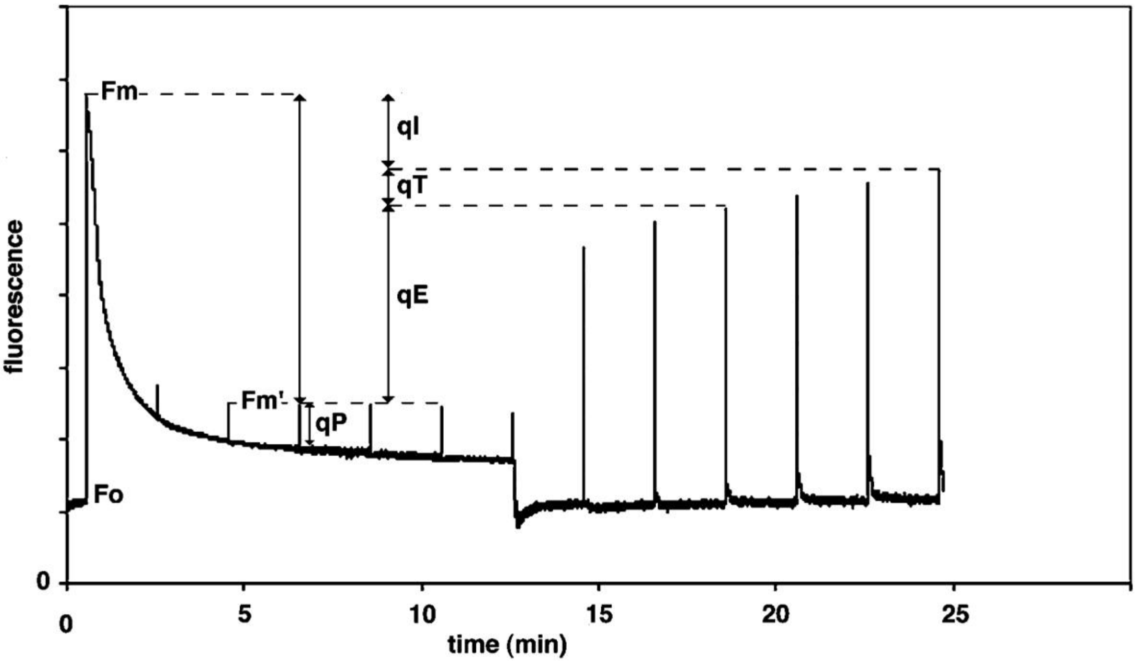

Figure 12. Chl fluorescence measurement from an Arabidopsis leaf. In the presence of only weak measuring light the minimal fluorescence (F_o) is seen. When a saturating light pulse is given, the photosynthetic light reactions are saturated and fluorescence reaches a maximum level (F_m'). Upon continuous illumination with moderately excess light (750 μmol photons $m^{-2}sec^{1}$; growth light was 130 μmol photons m^{-2} sec^{-1}), a combination of qP and NPQ lowers the fluorescence yield. NPQ (qE + qT + qI) can be seen as the difference between F_m and the measured maximal fluorescence after a saturating light pulse during illumination (F_m'). After switching off the light, recovery of F_m' within a few minutes reflects relaxation of the qE component of NPQ. (Müller et al., 2001)

According to the PAM manual, temperature and ambient light can influence the instruments measurements to a certain degree (PAM, 1998). In order to ensure that all measurements would be taken under the same conditions and therefore could be safely compared, samples were transported to the lab, were they were acclimated in plastic basins containing artificial seawater of 35 psu in 21^{0}C for 1-2 hours before any measurements were taken. All measurements were taken inside a Haake open cryo-thermostat circulator that kept water at 21^{0}C. Dark-adapted parameters (Fv/Fm and Fm) were taken after 10-15minutes of dark acclimation, using the clips provided by the instrument.

Morphological measurements

C. nodosa belongs to the group of seagrasses with a distinct erect stem and strapped shaped leaves borne at the top of an erect stem (Kuo et al., 2006). The roots arise from the lower part of the rhizome, usually close to the nodes. Roots often have characteristic features between different genera (Kuo et al., 2006) but their morphology hasn't been connected to any external stress factors. The same can be said about the rhizomes that are cylindrical and herbaceous. From each node an erect stem arises that is formulated by older leaves after senescence. Older leaves are attached to the stem, while new ones are formed inside it.

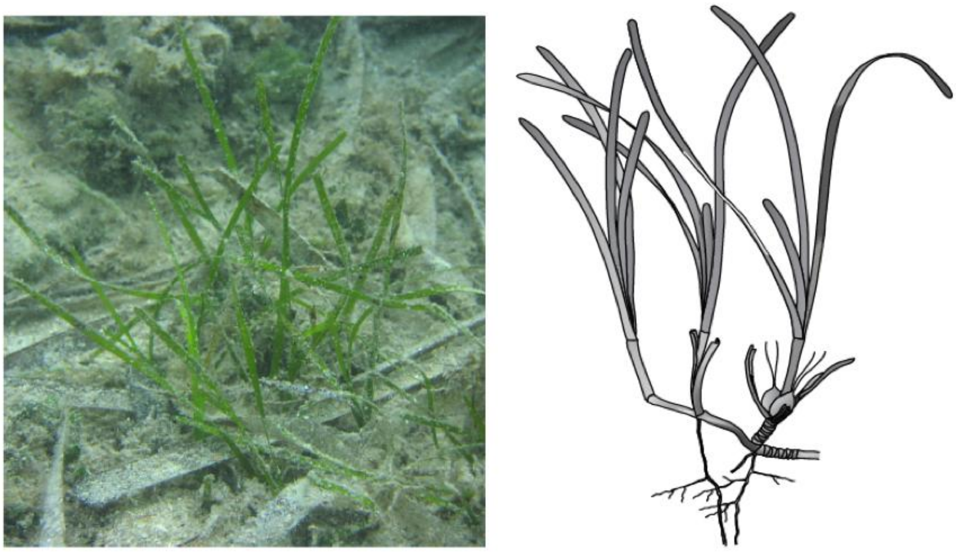

Figure 13. *Cymodocea nodosa* shoots drawn and in the field.

Leaves are usually 2-5 per shoot and can reach from 10-45 cm (Borum et al., 2004), however values outside that range have frequently been observed. Measuring the morphological features of seagrasses and specifically *C. nodosa* is a quite straightforward method. Each shoot was carefully separated to its distinct leaves, and number of leaves per shoot was measured. Length and width were measured using a ruler. Width showed variability along the leaf, so measurements were taken at its central part.

Chlorophyll- *a* measurements

Chlorophyll-a content in leaves was measured according to the methodology described in chapter 20 of global seagrass research methods (Granger et al., 2001). Leaves used for the analysis were cleaned of epiphytes using a microscope slide and stored at -75^{0}C. Prior to the analyses samples had to thaw first at room temperature. A 2cm sample was extracted from the central part of each leaf. The sample was weighted using a microbalance. Since *C. nodosa* doesn’t have thick leaves, there was no need to presoak the material in acetone.

Pigment extraction took place in a dark room, so that pheopigments that are a degraded product of chlorophyll wouldn’t interfere with the measurements. The samples were grinded using a mortar and a pestle with the addition of 90% acetone and a small amount of clean sand to help the grinding process. The sample reached a pale-green, flocculent slurry and was quantitatively transferred to 10ml glass test tubes and brought to volume using 90% acetone. The test tubes were finally loaded to a centrifuge for 10 minutes under dark conditions and then chlorophyll *a* was measured using spectrophotometry.

An UV-1800 phasmatophotometer by Shimadzu Corporation was employed. Absorbances were measured at 647nm, 664nm and 750nm (blank). Chlorophyll *a* was calculated according to the following equation:

$$\text{Chl } a\ (\mu\text{g ml}^{-1})=11.93E_{664}-1.93E_{647}, \text{ equation } \mathbf{(5)}$$

Where E stands for the corrected to the blank absorbency (absorbency at wavelength-absorbency at 750nm). The result was multiplied by 10, which was the acetone volume used and divided with the sample weight in order to express the result as μg Chl a*g^{-1}.

C/N analysis

An EA1110 Elemental Analyzer was used to determine C and N content in different structural units of *C. nodosa.* The analyzer uses the “Dumas” method that is referred to the instantaneous and complete oxygenation of the sample using flash combustion. Products are separated by a chromatographic column and detected by a Thermal Conductivity Detector (TCD).

Samples were separated to Roots, Stems and Leaves, then freeze dried, using a B.Braun Biotech CHRIST LOC-1 m freeze drier and stored at -75^{o}C inside aluminum foil, in order for them to be transferred from Greece to the UK. Prior to the analysis they were thawed in room temperature. From each sample 2mg were weighted using a five-figure Mettler AT201 balance inside 6X4 tin cups, labeled and loaded to a suitable plastic tray.

The instrument is consisted by an autosampler, a combustion reactor, a chromatographic column and a T.D.C. detector. Samples are loaded to the carousel autosampler and the instrument switched on. Immediately all of its compartments are flushed with helium. Helium is the preferred medium to carry the combustion products through the analytical system to atmosphere and to purge the instrument, since its chemically inert relative to tube packing chemicals, and it has a very high coefficient of thermal conductivity.

Once the samples are loaded into the combustion chamber it’s filled with pure oxygen that is ignited at 975^{o}C. At the same time all sensors are calibrated with helium that flows through the instrument. The samples are then guided to a mixing volume, where a reduction tube converts oxides of nitrogen to molecular nitrogen and removes oxygen. In the mixing volume sample gasses are thoroughly homogenized at a precise volume, temperature and pressure and the sample is then released through the sample volume into the thermal conductivity detector.

Three sets of pairs of conductivity cells are set in order to measure the combustion products. At the first one, an absorption trap removes water. The differential signal read before and after the trap reflects the amount of the samples water and therefore its hydrogen. At the second pair, a trap removes carbon dioxide and a similar measurement is made that indicates carbon content. At this stage the gas left is consisted by helium and nitrate. It passes through a conductivity cell and the measurement taken is compared to a reference cell through which pure helium flows. Results were expressed as percentages of C or N of dried weight (%DW). Every ten samples a blank was run in order to avoid instrumental drifts.

Phosphorus measurements

Samples used in the analysis of phosphorus content in leaves were freeze dried in Greece, transferred to Plymouth University within a portable cooler and digested prior to the analysis. Inductively coupled plasma optical emission spectroscopy (ICP-OES) was used to analyze the digested samples and a Varian 725-ES spectrometer was employed. The method aims to the production of excited atoms and ions that emit radiation at characteristic wavelengths for each element.

Sample digestion

All glassware used for sample digestion was first cleaned with 5 – 10% nitric acid strength to ensure that minimal contamination occurs. Samples were weighed inside a beaker. The beaker was put on an empty scale, the scale was tared, then the sample was added to the beaker and its weight was measured. 2ml of nitric acid was added and the beaker was covered with watch glass to stop evaporation. The beaker was put inside a ducted fume cupboard and left there for an hour in room temperature in order for digestion to begin. Still inside the ducted fume cupboard the beakers were placed upon a hotplate and slowly brought to boil. The samples were left to simmer for no less than an hour.

Digestion was over when brown fumes stopped and the beakers where left to cool. The samples were then transferred quantitatively into pre-cleaned volumetric flasks of 25ml and brought to volume using 2% nitric acid. Blank samples were prepared following the same procedure, without adding the sample.

ICP-OES

The ICP-OES analyzer has two parts, an ICP torch and an optical spectrometer. The ICP torch is used to create plasma and prepare the sample for specter measurements. It consists of 3 quartz glass tubes that are included in a “work” coil of a radio frequency generator (RF). The RF generator creates an intense electromagnetic field in the coil creating charged particles, while at the same time argon gas flows within the coil. The gas is ignited with a Tesla unit and stable high temperature plasma (7000K) is created as the neutral argon atoms collide with the particles.

The sample is introduced directly inside the plasma flame, but it’s first transformed to mist in a nebulizer. The sample mist collides with electrons and charged ions in the coil and its molecules break down to atoms. These atoms lose electrons and recombine repeatedly in the plasma, giving off radiation that is characteristic for each element in a specific wavelength. At this point the plasma with the sample meets an optical interface, where its cooled by a constant argon flow and driven to the optical chamber.

Within the optical chamber the light is separated into its different wavelengths, and their intensities are measured with photodetectors. A wavelength is chosen according to the element examined. The intensity is the compared to standard measurements of known concentration elements and the concentration within the sample is calculated using the calibration lines.

In order to produce calibration curves the following calibration standards of P were used: 0.5, 1, 2 and 5 mg/L. The wavelength at which P was determined was 213.6 nm. Every 10 samples check standards were run in order to ensure that there was no instrumental drift. The results taken from the calibration curves (mg/L) were multiplied by the volume of the digest (25ml) and then divided by the weight of the material digested (g), so that the resulting units would be mg/Kg.

Experimental culture conditions

Experiments were designed to test the physiological responses of *C. nodosa* to stress caused by a heavy metal (copper), macronutrients (nitrogen and phosphorus) as well as irradiance. Experiments were repeated with shoots from two meadows of different conservation status (known by previous studies (Orfanidis et al., 2009b)) in order to test how acclimation in different habitats influences the reaction to the mentioned parameters. Since there wasn’t enough space in the laboratory, experiments weren’t run simultaneously but consecutively.

Samples were collected by Self Contained Underwater Breathing Apparatus at 2-3m deep and transferred to the lab within 30-60 minutes from collection in plastic containers with seawater from the sampling site. Shoots with similar morphological traits were chosen, in order to ensure similar uptake rates, especially for Cu whose uptake is area dependent (Ralph et al., 2006). 4cm of rhizome was left attached to the shoot and a single, 2cm root. In the lab shoots were left to acclimate for no 1- 2 hours and effective quantum yield was measured. Shoots were then placed into plastic basins (one shoot per basin) that were filled with 1L artificial seawater and covered with watch glass in order to prevent evaporation. The solution Münster sea salt (Meersalz) was used to create the artificial seawater, and salinity was kept at 35 psu. In each replicate the studied element was added (Cu or nutrients) and replicates moved in a controlled temperature chamber (at 21^0C±1.5). Six replicates per treatment were studied (Figure 14), as well as six blank replicates. Rubber tubes were fit in the containers from a small hole in the lid, providing air in the medium and ensuring it stayed oxygenated. Photoperiod was set at 14 h light: 10 h dark.

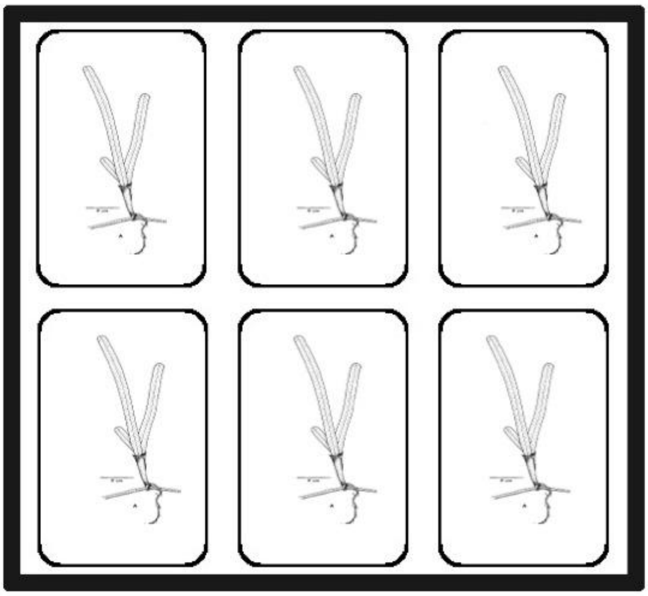

Figure 14. Six replicates per treatment were studied in all experiments.

In the case of copper exposure experiments three concentrations were chosen 1.6, 4.7 and 7.9 μM. Macronutrients were also added to the medium in order to avoid starvation (0.3μM N and 0.02μM P), reflecting a low nutrient environment in the Kavala Gulf,. The effect of nutrients was studied by comparing the following four treatments of rising nutrient concentrations:

Solution A: 0.3μM $N\text{-}NO_3^-$ 0.02μM $P\text{-}PO_4^{2-}$

Solution B: 10μM $N\text{-}NO_3^-$ 0.5μM $P\text{-}PO_4^{2-}$

Solution C: 20μM $N\text{-}NO_3^-$ 1μM $P\text{-}PO_4^{2-}$

Solution D: 30μM $N\text{-}NO_3^-$ 2μM $P\text{-}PO_4^{2-}$

Light irradiance is usually controlled with shades. Since they were not available, low light conditions were achieved by shutting off half of the lamps in the CT chamber. Using a Li-250 light meter by Li COR, high conditions when all lamps were switched on was measured at 65μmol photons $*m^{-2}*s^{-1}$, while low PAR (50% of the lamps) at 37 μmol photons $*m^{-2}*s^{-1}$. These measurements were taken at the bottom of water filled aquaria. The effect of high and low irradiance on photosynthetic efficiency was studied in combination to increasing P concentration in the medium. Four $P\text{-}PO_4^{3-}$ concentrations were used 0.02, 0.5, 1 and 2μM, with six replicates each. In each container 30μM of $N\text{-}NO_3^-$ was also added. For this experiment 4 concentrations X 6 replicates each X 2 light conditions= 48 replicates were used.

Analysis of data

All samplings followed the random nested design in order to view variability in more than one spatial scale. Field data were collected from two meadows of known ecological condition, one degraded and one with almost no anthropogenic influence. Each meadow was separated into two sites (100's of m apart) and each site into two areas (10's of m apart). From each area 4 25X25 quadrats were sampled with SCUBA at 2-4m depth. The two meadows reflected the two states of ecological status that were studied and were treated as fixed variables. Sites, areas and quadrats however, were chosen randomly and were treated as such.

Proper statistical analysis is always a great challenge. In this research the R environment was used and all data were analyzed using Linear Mixed Models that employ both random and fixed factors. The first affect the variance of the dependent variable while the second only the mean value. The package "nlme" was used.

Even though normality of data isn't a strict rule when using Mixed Models (Fitzmaurice et al., 2004) heterogeneity and independence are two issues that need to be taken into account. Heterogeneity or heteroscedasticity happens if the spread of the data isn't the same at each X value. Plotting the fitted values of the model against the standardized residuals tests the existence of heteroscedasticity. When data are plotted the spread should be roughly the same across the range of the fitted values (Figure 15). Probably the most serious problem is independence of data, which happens when the dependent value Yi in Xi is influenced by another Xi (Quinn et al., 2002). The second important test is plotting the fitted values against the dependent variable. The data need to show a linear relationship between the two, which means that their relationship is deterministic and the model can predict the dependent variable at any moment (Figure 16).

Every model that was created was tested for the above limitations before being accepted but also tested against the null model using analysis of variance. The null model is the same model without any fixed parameters but only the intercept. In order for a model to be accepted it must vary significantly from the null model.

Seasonal changes have long been known to strongly influence seagrass beds (Drew, 1978). Since the extent of this influence changes according to species and geographic region, samples were collected both during cold and hot months. Two sampling efforts were taken per period (hot and cold) so that statistical variability could be ensured. February and March were chosen to represent a cold period, since during those months *C. nodosa* receives the maximum amount of natural stress due to extreme temperatures, increased hydrodynamic activity, higher nutrient inputs and low light availability. On the other hand, during June and July, the highest productivity values have been witnessed. The parameter "period" (Hot and Cold) was treated as fixed.

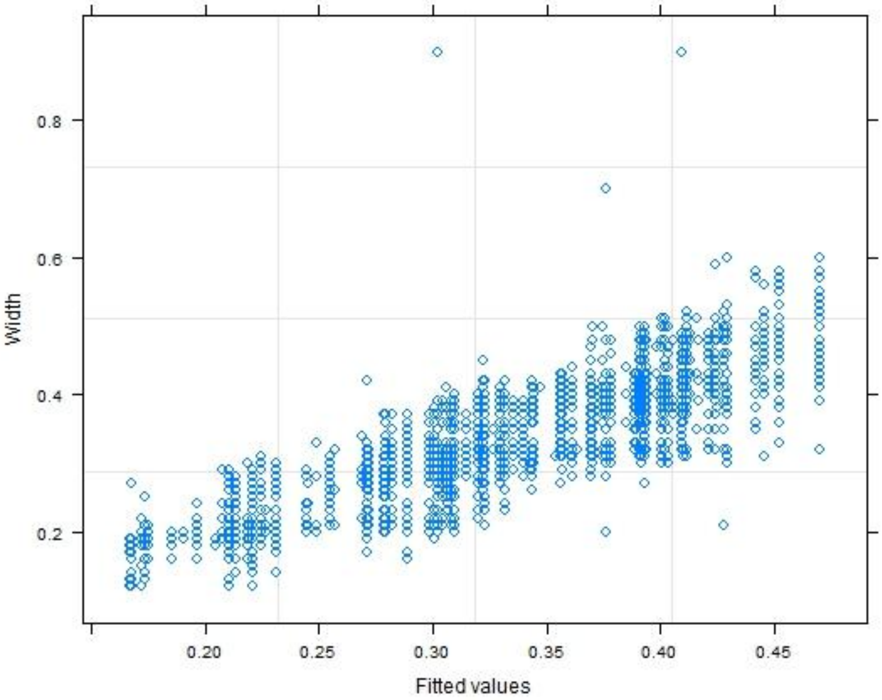

Figure 15. Example of fitted values against the measured variable. A linear relationship between the two can be observed.

Since spatial data are usually susceptible to spatial correlation, the factor *corRatio (form= ~1|Site/Area/Quadrat, nugget=T)* was added to the model. In all models a weighting factor was also added in order to eliminate heteroscedasticity presented in the fitted values Vs standardized residuals plots. In all cases the weighting variable varPower() was used, except number of leaves per shoot, C/N and chl-a in leaves that varConstPower() was preferred and P content in leaves that the best model was given by varExp().

The model created was: lm*e(Parameter ~1+Period+Meadow, random=~1|Site/Area/Quadrat, control = lmeControl(maxIter = 10000, msMaxIter = 5000, niterEM = 500, msMaxEval = 500, msVerbose = TRUE, opt = "optim") , data=Data, weights=*<u>*varPower()*</u>*, correlation =corRatio (form= ~1|Site/Area/Quadrat, nugget=T),method="ML").*

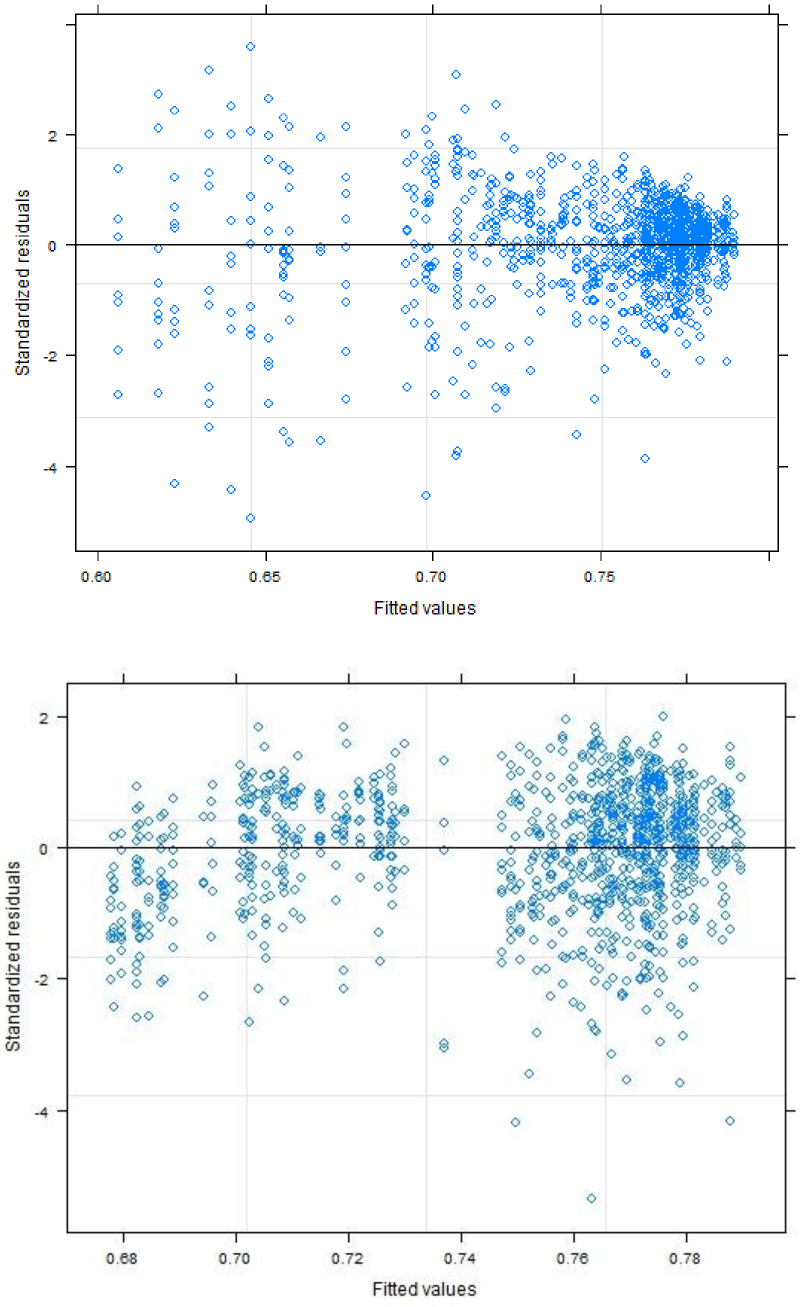

Figure 16. Example of heterogeneity. In the first graph we observe a steadily increasing variance for lower fitted values, which means there is heterogeneity and a correcting function needs to be added to the model. In the second graph there is no apparent correlation between the two axes after the factor varPower() has been applied.

Chapter 3: Application of key metrics of *Cymodocea nodosa* as bioindicators

SUMMARY

Anthropogenic pressure is known to affect seagrass beds both directly and indirectly. Since *Cymodocea nodosa* shows high phenotypic plasticity, it changes and adapts according to the given environmental conditions. In order to link these changes with anthropogenic stress, physiological (ΔF/Fm’, Fv/Fm, Fm’), morphological (Leaf Length and Width, Number of leaves per shoot) and structural (C, N, P and Chlorophyll-a content) measurements were taken from a pre-established trend of ecological quality in the Kavala Gulf, North Aegean Sea. Two well-described meadows, one pristine and one under significant anthropogenic stress, were sampled hierarchically at three spatial scales ranging from tens of meters (area) to hundreds of meters (site) to kilometers (meadow) seven times (from June 2007 to June 2009) and data were analysed using the R environment. Statistically significant differences were identified between cold (January, March) and hot (June, July) months for almost all parameters measured, suggesting that temperature as well as naturally induced stress during winter play an important role on how *C. nodosa* is responding in the field. From the parameters measured, ΔF/Fm’, Fm, N and P content in leaves, leaf length and width varied significantly between the two differently impacted meadows, a variation that became more evident during the cold months.

INTRODUCTION

Seagrasses are rhizomatous marine angiosperms that form extensive meadows in temperate to tropical regions. These meadows form some of the most productive ecosystems in Earth, providing high value ecosystem services, comparable even to terrestrial habitats (Naidoo et al., 2008). Their ability to influence many functional levels of an ecosystem makes them a habitat of high ecological value. With their leaves they form a “net” that traps sediment and nutrients, while at the same time they modify currents and waves (Hemminga et al., 2000). Biodiversity inside the meadows is higher than most other marine habitats and many economically important species use the safety they offer to spend their early life (Beck et al., 2001). Seagrassess also provide an enormous source of carbon, part of which is buried within the seagrass sediment (Duarte et al., 2005) and part is exported to the deep sea (Suchanek et al., 1985).

The key role of seagrass beds in the marine ecosystem has been recognized worldwide. That and the fact that seagrass loss has increased tenfold over the past 40 years (Orth et al., 2006) has led many countries to plan and act towards their conservation and recovery where its needed. Currently, there are 19 monitoring programs with the aim of protecting 30 seagrass species in 44 countries around the globe (Orth et al., 2006).

More recently the function of seagrasses as biological sentinels, or bioindicators has gained attention. Bioindicators have been defined as components or measures of ecologically relevant phenomena that provide simple and efficient information to quantify the ecosystem health, changes, or to set environmental goals (Heink et al., 2010). Seagrasses live in shallow coastal waters where most nutrient and sediment inputs occur, and since they are sessile they have to adjust to varying water qualities. They are considered to be suitable bioindicators due to their sensitivity to a wide range of stressors, including eutrophication, light limitation, chemical pollution, mechanical disturbances, biological interactions etc., and respond to disturbances in the water column as well as the sediment (Orfanidis et al., 2007). Moreover, the significant knowledge that exists on their biology and ecology (Larkum et al., 2006), as well as their wide distribution range, allows researchers to develop more efficient management tools.

The bioindicators that have been developed have been used in different ways. Some are focused on specific disturbances such as nutrients, metals or dredging (Campanella et al., 2001; Ferrat et al., 2003; Lee et al., 2004), while others employ metrics to asses the overall water quality (Krause-Jensen et al., 2005; Orfanidis et al., 2009b). It has been suggested that each indicator can be applied only within the biogeographical distribution of the species or community that it’s based on. However, the need to compare data from different regions and to compare similar methodologies has led to the development of more complex indicators that employ a combination of metrics (Montefalcone et al., 2006; Romero et al., 2007; Lopez y Royo et al., 2010). This produces highly complex methodologies that are difficult to apply, and have still to be tested in different regions.

Throughout their evolution seagrassess have shown genetic, physiological and morphological adaptations to withstand variations in naturally occurring stressors, such as temperature, salinity and irradiance fluctuations, and nutrient limitation (Duarte, 2002), which have permitted them to thrive in different ecoregions, from temperate to tropical ecosystems. Concurrently, seagrasses are characterized by high phenotypic plasticity (Marba et al., 1995) that allows for a single species to adopt different phenologies throughout its own biogeographical distribution (Borum et al., 2004). As a result, different species have shown different behaviors when exposed to certain stressors, while various responses to the same stressors can be found in the literature even in researches from different biogeographic regions (Martinez-Crego et al., 2008). This makes it difficult to study the ecological behavior of seagrasses, as findings for a particular species can’t be extrapolated to others, while sometimes results from one species refer to only a specific biogeographical region of its distribution.

In order to safely use seagrass species as bioindicators, more research on their responses to stressors is needed, specially focusing on multiple stressors and field studies (Orth et al., 2006). Many researchers have managed to link specific abiotic factors with plant responses, from both laboratory and field experiments. For example, *Zostera marina* was found to have larger leaves under high nutrient conditions (Short, 1983), while Udy et. al. (1997) described how nutrient enrichment significantly increased growth rates, biomass, canopy height and shoot density for *Halodule uninervis* and *Zostera capriconi* but had no significant effect on *Cymodocea serrulata*. However, studies of species responses to overall ecosystem degradation are lacking.

Research on individual stressors provides valuable knowledge on response mechanisms and patterns for seagrasses. However, the large number of factors affecting seagrass behavior, as well as the complex relationships among them, makes the development of a simple prediction model in the field difficult to achieve. The continuous and methodical monitoring of well-described seagrass meadows of known conservation status could provide insight on when, how and why seagrasses resolve to specific physiological, morphological and structural changes, and which of those changes are particularly suitable for assessing the overall degree of stress that an ecosystem is receiving.

Three such meadows were chosen in the N. Aegean Sea, two pristine and one under significant anthropogenic influence. Specific morphological and physiological metrics (leaf length and width, number of leaves per shoot, carbon, nitrogen and phosphorus concentrations of leaves, stems and roots, chlorophyll-*a* concentration in leaves and chlorophyll-*a* fluorescence parameters) were monitored in a sequence of seven sampling periods. Since seasonality is known to influence seagrass in many functional levels (physiology, growth, morphology etc.) (Malea et al., 1995a), three samplings took place during the colder months of January and March and four during the hotter period of June and July.

The use of PAM fluorometry as a biomarker of ecological health was also investigated. PAM fluorometry is a fast, non-invasive methodology that can be used in-situ and has become very popular over the past decade. It has been suggested that since photosynthesis is one of the main functions of autotrophs, its efficiency should reflect the overall conditions under which the plants are growing (Maxwell et al., 2000). The use of PAM fluorometry as a biomarker of ecosystem status has been recently tested for *P. oceanica* with interesting results (Gera et al., 2012).

MATERIALS AND METHODS

Since the spatial variation of the measured indices was not known, a random nested sampling design on a hierarchy of spatial scales, ranging from tens of meters (area) to hundreds of meters (site) to kilometers (meadow), was utilized. The approach followed is based on hierarchical theory which regards ecosystems as hierarchically organized systems (O`Neill, 1988) that are regulated by a relatively small set of principles. A hierarchical sampling design ensures that both large scale and local variability can be identified.

Two meadows of known ecological quality were chosen for sampling in the N. Aegean Sea. Brasidas meadow reflected a site of minimum anthropogenic impact and Nea Karvali meadow a highly impacted site in the Aegean Sea. Each meadow (kilometers apart) was divided into two sites hundreds of meters apart. Each site was again divided into two areas tens of meters apart. In each area four 25 x 25cm quadrat samples were collected (Figure 17) using SCUBA at 2-3m depth in the morning (usually between 09.00 and 10.00am). Samples were collected quantitatively and all rhizomes, roots and shoots of *C. nodosa* were transferred in labeled plastic bags to the laboratory, within 30 min of their retrieval.

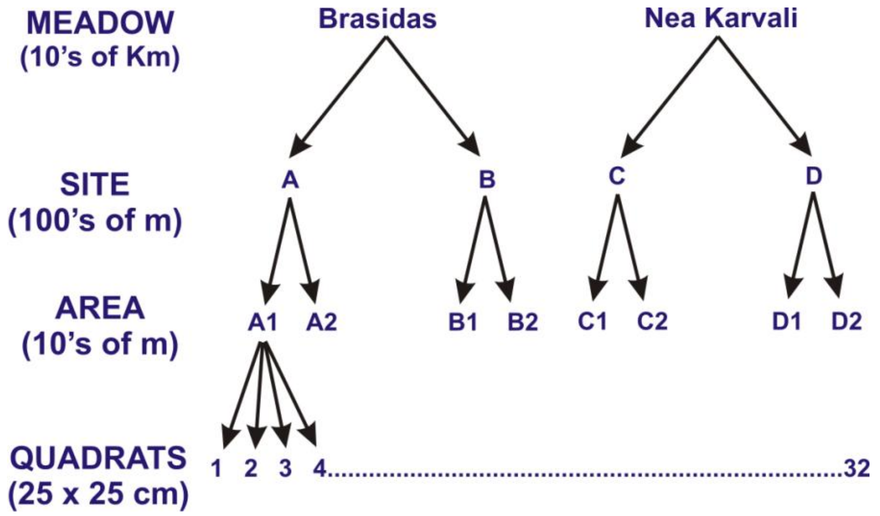

Figure 17. Hierarchical sampling design, for all *Cymodocea nodosa* collections throughout this study (Meadow A: Brasidas, Meadow B: Nea Karvali).

Seven sampling efforts were undertaken. The first took place in June 2007 and since it was the first sampling, a reliable measuring strategy for *C. nodosa* plants was also established. In January 2008 the second effort was conducted, in order to acquire general information about photosynthetic performance, carbon and nitrogen concentrations in shoots and description of morphological characteristics of plants from different meadows. Morphological and physiological parameters were studied throughout the next three sampling efforts (March, June and July 2008). In March 2009 a study was conducted of one area per meadow, with greater replication from each area and samples were also collected from around the Island of Thasos.

The final collections were obtained from Thasos and Brasidas during June 2009, in order to compare these two meadows. Over the four years of sampling Brasidas meadow and the wider area of Nea Peramos, were subjected to increasing domestic development, as well as a significant increase in tourism. Observations made during this period of a decreased lower depth limit and reduced coverage of the meadow raised questions about the suitability of Brasidas as a reference meadow. Therefore, it was decided that another, more remote meadow with less anthropogenic influence (i.e. at Thasos Island), be included in the sampling regime to permit direct comparisons with Brasidas.

The fact that by 2008 the meadow at Brasidas had shifted to shallower waters (0-2m depth), from depths of 2-3m from where all previous samples had been taken, raised questions about the influence of change in depth on the measured parameters. In order to test this hypothesis another field experiment was conducted in summer 2008. A third site was chosen between sites A and B, approximately 70-80m from each. Site E was located in the middle of Brasidas meadow, where *C. nodosa* grew down to 6m depth. From this site material was collected in order to test the hypothesis that small changes in depth (2-3m) don't significantly influence the measured parameters.

A large scale monitoring program of *C. nodosa* conducted over a period of four years has rarely been attempted and never in Greek coastal waters. Therefore, there was no existing information to draw on and hence the need to modify the planned analyses during the study.

Study area: The study was conducted in two sheltered sandy habitats in N. Aegean, Greece. Both sites are enclosed in Kavala Gulf (40.52^o N; 24.25^o E), a semi-enclosed coastal water body, that communicates with the N. Aegean Sea through the Thasos Island channel and plateau (Figure 18). Temperatures range from 11^{o}C in winter to 26^{o}C in summer. Seasonal changes in salinity are strongly influenced by the flow of low salinity water from the Black Sea, with coastal waters of Macedonian and Thrace ranging from 33psu in spring and summer to 35psu in winter.

These two meadows were chosen as representatives of two water bodies that are well described and clasified using the Ecological Evaluation Index (EEI). The concept of the EEI is based on the obvious and universal pattern that "anthropogenic disturbance, e.g. pollution-eutrophication, shifts the ecosystem from pristine where late-successional species is dominant to degraded state, where opportunistic species is dominant". Marine benthic macrophytes (seaweeds, angiosperms) are used as bio-indicators of ecosystem shifts, from the pristine state with late-successional species (Ecological State Group I) to the degraded state with opportunistic species (ESG II). In moderately impacted coasts slow growing, shade-adapted calcareous species (ESG IC) and opportunistic macroalgae often co-dominate. Based on the EEI, Nea Karvali has been classified as moderate ecological status (Emoderate e 0.17SE, Moderate ESC) and Brasidas as good (E and Br +/-0.11, Good-High ESC) (Orfanidis et al., 2005b).

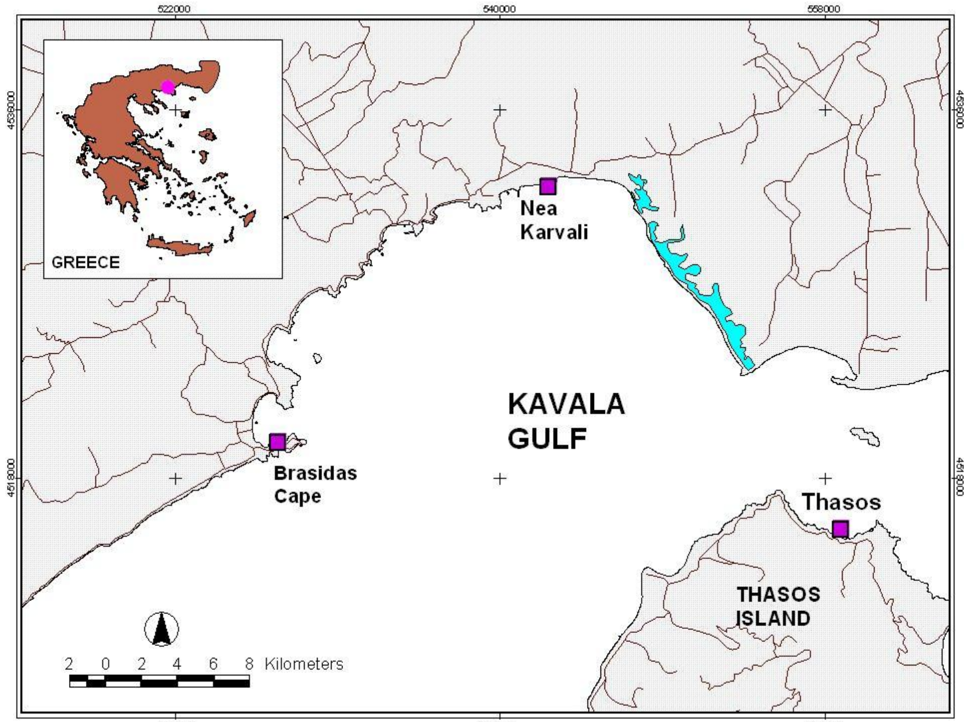

Figure 18. Map of the two studied meadows of Brasidas Cape and Nea Karvali in Kavala Gulf, North Aegean, Hellas.

The impacted site of Nea Karvali (Figure 19) 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 and a crude oil de-sulphurization complex , which is affecting the once undisturbed coastal zone (Xeidakis et al., 2010). Concurrently, a wastewater treatment facility has been installed to the west of Nea Karvali. The meadow is located at the eastern part of the town, where two freshwater streams reach the sea. A hierarchial sampling plan was implemented for the study. The meadow was subdivided into two ‘sites’ (C and D) that were ca. 1km apart and each ‘site’ was further subdivided into two ‘areas’ (C1, C2, D1, D2) that were ca. 150 - 200m apart. Site C was located in front of a small camping facility that is occupied during the summer but deserted for most of the rest of the year, with C1 and C2 were located at the eastern and western ends of the site, respectively. Site D was located further to the east of Nea Karvali, close to a small land-based fish farming facility, with D1 further east than D2 and closer to the stream. All samples were collected from a depth of 2-3m.

The meadow near Brasidas is located in the inner part of Cape Brasidas on the Eleutheron Gulf (Figure 20). Its one of the least impacted areas on the mainland of the Kavala Gulf and has been included in the European Natura 2000 network (code GR1150009). The town of Nea Peramos, which is located about 3Km to the east, is characterized by fishing and port activities together with a steadily growing urbanization and increasing tourist industry. The location around the meadow is visited only by amateur fishermen and very few tourists. Sites A and B were at the western and eastern ends of shoreline, ca. 1Km apart. Areas A1 and A2 and B1 and B2 were approximatelly 100-150m apart. All samples were collected from a depth of 1-3m. To assess possible differences with water depth within a meadow, an additional site (E) was established in Brasidas and samples collected from 2-3m.

Figure 19. Nea Karvali meadow as seen in Google Earth©.

Figure 20. Brasidas Meadow as seen in Google Earth©.

The background concentrations of key dissolved macronutrients (N and P) and metal cocnetrations in sediments are shown in Table 5 (Sylaios et al., 2004). Mean values of total dissolved inorganic nitrogen (TDIN) and soluble reactive phosphorus (SRP) measured relatively close to the sampling sites, at the 10 m isobath, increased from Brasidas (TDIN = 6.7 µmol l^{-1}, SRP = 1.1 µmol l^{-1}) to Nea Karvali (TDIN=11.9 µmol l^{-1}, SRP=2.57 µmol l^{-1}). On the basis of the Ecological Evaluation Index (EEI) of upper infralittoral seaweed communities, Orfanidis and Panayotidis (2005a) have classified Nea Karvali as of “moderate” and Brasidas as of “good” ecological status class.

Figure 21. Site Th at Thasos Island as seen on Google Earth©

Subsequent to commencement of sampling at the meadows near Nea Karvali and Brasidas it was decided to establish a sampling station at a second reference meadow located at Thasos Island in the eastern part of Kavala Gulf, approximately 10 km from the mainland (Figure 21). Waters surrounding the island are characterized by high coastal diversity, with calm and shallow waters to the north and stony, rocky seashores to the south, where there is greater exposure to wave action. Thasos has a population of 13,765 people with the main income being tourism. Agricultural activities do not influence significantly the coastal ecological quality, because of the increased hydro-dynamism. *Cymodocea nodosa* is only found at the northern part of the island in one sheltered beach with an east-north orientation. Few tourists visit this area and, furthermore, an eastern current from the Black Sea moves through the Thasos plateau affecting water circulation. Within the meadow, one site was sampled (Th), that was then separated into two areas tens of meters apart (Th1, Th2). Nutrient concentrations measured at Thasos reflected an oligotrophic environment (Table 5).

Abiotic Parameters	Nea Karvali (*Highly impacted*)	Brasidas (*non-impacted*)	Thasos (*non-impacted*)
Main human Pressures	Domestic (ca. 80.000 inhabitants), Industrial & Agricultural effluents	Domestic (ca. 20.000 inhabitants) & Agricultural effluents	Domestic (ca. 13765 inhabitatns
Seawater (surface)			
Temperature (^oC)	17.4	17.4	18.95
Salinity (PSU)	33.7	33.7	34.1
$N\text{-}NO_3$ (μmol/l)	8.8	5.41	1.625
$N\text{-}NO_2$ (μmol/l)	0.18	0.15	0.305
$N\text{-}NH_4$ (μmol/l)	2.89	1.15	0.135
$P\text{-}PO_4$ (μmol/l)	2.57	1.13	0.98
Sediment			
Cu (ppm)	39.9	9.63	NS

Ni (ppm)	43.8	11.67	NS
Zn (ppm)	102.9	97.12	NS
Pb (ppm)	31.97	34.51	NS
Cd (ppm)	1.5	0.09	NS
Cr (ppm)	162.3	32.96	NS

Table 5. Mean concentrations of key macronutrients in the water column and metal concentrations in sediments sampled at 10m depth adjacent to the studied meadows (Sylaios et al., 2004).

Sample handling. In the first two samplings efforts (June 2007 and January 2008), the plants collected from each quadrat were divided into two equal halves upon their arrival at the laboratory. One half was used for morphological and PAM measurements, while the other material was stored at -75^{0}C to await further analysis. From the first half, eight individual plants were randomly chosen for PAM analysis, 5 of them for measurements of ΔF/Fm' and 3 for Fm and Fv/Fm. The remaining material was stored in 4% formaldehyde until such time those morphological parameters could be measured. In all subsequent collections no such division of material took place. Instead, ten shoots were chosen from each quadrat and all measurements were taken on these, to allow direct comparisons of individuals. The remainder of the material from the quadrats was used for all other analyses (CHN, chl-*a* and P contents in leaves).

All areas were sampled during the summer months but during winter a problem occurred while trying to sample Site D (Nea Karvali). Sampling was impossible due to high sediment run-off that buried the meadow almost entirely. Leaves tended to be longer where burial had occurred but in some cases only a few centimeters were observed protruding from the sediment (e.g. only 5cm of a 30-40cm leaf) and any effort to collect the samples resulted in the destruction of the shoot. Therefore, during winter months only site C was sampled from Nea Karvali.

In March 2009, only one area per meadow was sampled. In June 2009, Thasos and Brasidas were sampled, so that the suitability of Brasidas as a reference meadow could be assessed. Due to the small extent of the meadow, only one site (Site Th) was sampled at Thasos, which was subdivided into two areas (Th1 and Th2) to allow for a nested analysis to be carried out at two spatial scales. Table 6 provides a summary of the variables measured at each sampling period.

Sampling Period	Sampled Sites	Variables measured
June 2007	All	ΔF/Fm', Fm, Fv/Fm, Total Leaf length, Leaf Width, Number of leaves per shoot, Chlorophyll-a content in leaves, C/N
January 2008	All-Except Site D	ΔF/Fm', Fm, Fv/Fm, Total Leaf length, Leaf Width, Number of leaves per shoot, C/N
March 2008	All-Except Site D	ΔF/Fm', Fm, Fv/Fm, Total Leaf length, Leaf Width, Number of leaves per shoot
June 2008	All-plus Site E	ΔF/Fm', Fm, Fv/Fm, Total Leaf length, Leaf Width, Number of leaves per shoot, C/N
July 2008	All-plus Site E	ΔF/Fm', Fm, Fv/Fm, Total Leaf length, Leaf Width, Number of leaves per shoot
March 2009	All-Except Site D, plus Site Th	ΔF/Fm', Fm, Fv/Fm, Total Leaf length, Leaf Width, Number of leaves per shoot, Chlorophyll-a content in leaves, C/N, P content in leaves
June 2009	Only Sites A and Th	ΔF/Fm', Fm, Fv/Fm, Total Leaf length, Leaf Width, Number of leaves per shoot

Table 6. Sites and variables measured during the seven sampling efforts conducted in the Kavala Gulf.

PAM analysis. Prior to any measurements shoots were cleaned of all visible epiphytes using a microscope slide. Temperature can affect PAM and must be stable prior to measurements being taken (Beer et al., 1998). All shoots were acclimated in 21^{0}C seawater for 10 minutes, using a Haake open cryo-thermostat circulator (Figure 22). All measurements of shoots were taken in the water tank and then shoots were stored at 4% formaldehyde for morphological analysis or at -75°C for CHN, P and chl-*a* analysis.

Measurements of effective quantum yield (ΔF/Fm’), maximum quantum yield (Fv/Fm) and maximum fluorescence (Fm) were taken on the second leaf of the shoot at a distance of 2 cm above the stem, since the first leaf was usually covered with epiphytes. The need for a standard point for fluorescence measurements because of the high variability of measured parameters along an individual leaf, has been discussed by many researchers (Beer et al., 1998). ΔF/Fm’ was measured in 5 randomly selected shoots, while Fv/Fm and Fm were determined from 3 shoots that had been dark adapted for 10 minutes using clips provided with the fluorometer. This method was followed only in June 2007 and January 2008, while in all subsequent sampling events 10 shoots were chosen randomly and all measurements were taken from the same shoots.

Figure 22. Laboratory set up of the PAM fluorometer and the Haake open cryo-thermostat circulator for measuring photosynthetic activity of *Cymodocea nodosa* shoots.

Morphological analysis. Material stored in 4% formaldehyde was used for morphological measurements. Only plants from three of the four quadrats sampled were used for this analysis. Leaves were separated from each shoot from the sample and leaf length and width were measured using a ruler, while the number of leaves on shoots was counted. Since leaf width can vary slightly along the length of the leaf, all measurements were taken from the central section, where width is maximal. When counting the number of leaves per shoot, all leaves were accounted for, regardless of age and condition.

Morphometric analysis of shoots used for PAM analysis was also carried out immediately following measurements of fluorescence parameters, so that any relationships between the morphological and physiological factors could be identified.

Chlorophyll-a content in leaves. Leaves used for determining chlorophyll-a concentrations were kept in a darkened refrigerator at -75^{0}C. From each leaf, a 2cm long portion of the central part was removed and weighed using a microbalance. Pigments were extracted using a mortar and a pestle, with clean sand and 10ml of 90% acetone. The final extract was transferred to a test tube and loaded into a refrigerated centrifuge at 10^4 rpm for 10 minutes. Absorbances of extracts were measured at 647nm, 664nm and 725nm (blank) and concentrations calculated using the following equation:

$$\text{chl } a\ (\mu\text{g*ml}^{-1}) = 11.93E_{664} - 1.93E_{647}$$

where E is the absorbency at the chosen wavelength minus absorbency at 725nm. Values were expressed as μg Chl *a* *g^{-1} of leaf by multiplying the given value by the volume of acetone used (10ml) and then dividing to the weight of material used for the analysis.

C/N analysis. Plants from three of the four quadrats per area were analyzed for total carbon (C) and nitrogen (N) and the C/N determined. Shoots were separated into leaves, stems and roots upon their arrival in the laboratory and all leaves were cleaned of epiphytes using a microscopy slide. The different plant portions were freeze dried separately (B.Braun Biotech CHRIST LOC-1 m freeze drier), labeled and stored at -75°C so that they could be transferred in a portable freezer to Plymouth University for analysis. Samples were thawed at room temperature, 0.2 μg of material weighed, loaded into the CHN analyzer using a microbalance, and volatilized under high temperatures (975°C) to produce CO_2 and N_2. The gases were then measured by a chromatograph equipped with a thermal conductivity detector. From knowing the volume of the gases mass and relative abundance was then calculated as percentages of dried weight (%DW).

Phosphorus content in leaves. Freeze dried samples, clean of epiphytes, were transported from Greece to Plymouth University using a portable freezer. Prior to analysis samples of known weight were digested placed in 5ml glass beakers to which was added 2ml of 2% HNO_3 acid. Beakers were covered with watch glass and placed in a ducted fume cupboard for 1 h, after which they were transferred to a hot plate, slowly brought to the boil and left to simmer for 1 h until brown fumes stopped and the beakers were left to cool. Once cooled, sample extracts were transferred to 25ml volumetric flasks and brought to volume using 2% nitric acid.

Phosphorus concentrations were then determined using inductively coupled plasma optical emission spectroscopy (ICP-OES; Varian 725-ES). The method uses a radio frequency generator and a tesla unit to ignite charged argon molecules and create plasma. The sample is introduced to the plasma in the form of a mist and is guided to an optical chamber were light is separated to its wavelengths and the intensity of each wavelength is measured in photo-detectors. The method is based on the ability of excited atoms and ions to emit electromagnetic radiation at characteristic wavelengths for each element. The intensity of this emission is proportional to the concentration of the element. Phosphorus was measured at 213.6nm. Calibration curves were prepared using 0.5, 1, 2, 5 mg/L from a standard stock solution of P. Blanks were run every 10 samples in order to ensure that there was no instrumental drift. Results were multiplied with the sample volume (25ml) and divided by the weight of the material digested so that results were given as mg kg^{-1}d wt.

Statistical analysis. All data were analyzed using the R environment, which is provided by the R Foundation. Multiple sampling efforts per period (3 in winter = cold period, and 4 in summer = hot period) were taken in order to ensure statistical variability and period was treated as a fixed factor. The two meadows were treated as fixed variables, since they were chosen to reflect two specific ecological conditions, a degraded meadow and one with almost no anthropogenic impact. Site, Area and Quadrat were treated as random variables. Since both fixed and random variables were present a mixed model was created using the function “lme” from the “nlme” package in the R environment.

Each model that was created had to significantly differ from the null model. The validity of each analysis was also tested by plotting the standardized residuals versus the fitted values given by the model, as well as the fitted versus the measured values (a linear relationship needs to be identified). In the first case any conical shapes in the graph was translated as heteroscedasticity and the model was redrawn using a weighting variable. For all parameters the weighting variable varPower() was used, except for number of leaves per shoot, C/N and chl-*a* concentrations of leaves for which varConstPower() was preferred and P content in leaves for which the best model was given by varExp(). Since spatial data often suffer from spatial correlation, the factor *corRatio (form= ~1|Site/Area/Quadrat, nugget=T)* was added to the model.

The model created was: lm*e(Parameter ~1+Period*Meadow, random=~1|Site/Area/Quadrat, control = lmeControl(maxIter = 10000, msMaxIter = 5000, niterEM = 500, msMaxEval = 500, msVerbose = TRUE, opt = "optim") , data=Data, weights=varPower(), correlation =corRatio (form= ~1|Site/Area/Quadrat, nugget=T),method="ML").*

In order to calculate the components of variance for each analysis the function “lmer” from the package “lme4” was used. Each parameter was tested against the hierarchical nested spatial scales, cross-nested to period according to the model: lmer(Parameter~(1|Meadow/Site/Area/Quadrat)*(1|Period), Data)->components of variance.

RESULTS

Variation between and within the two meadows at Nea Karvali and Brasidas in two sampling periods.

Physiological parameters

All PAM parameters varied significantly ($p<0.001$) between the two sampling periods. Only effective quantum yield (ΔF/Fm') and maximum fluorescence (Fm) showed significant differences between the two meadows as well, while the interaction between meadow and period was significant for all parameters. As shown in Figure 23, typically where significant differences occurred values were higher for Nea Karvali, a difference that became more obvious during winter months, while in general higher values were obtained during summer.

Effective Quantum Yield. In all analyses, the graph of standardized residuals versus fitted values was produced. In order for the analysis to be accepted the shape had to be a random formation, resembling a cloud as much as possible. Figure 24 shows the graph produced from the analysis of effective quantum yield. The graph was similar when plotted for all other parameters, changing only in the number of observations. Since it was neither linear nor had a conical shape all analyses were accepted.

Significant differences for effective quantum yield mean values were identified between periods, meadows and their interaction ($p<0.001$). Mean values measured during the hot period were higher than those measured during the cold period by 0.079±0.005 (Table 7). Higher values of ΔF/Fm' were taken from Nea Karvali than from Brasidas in almost all sampling efforts (Figure 25). During cold period ΔF/Fm' mean values ranged from 0.502 in area A1 to 0.793 in area B2, while during the hot period the same range was from 0.647 in area A1 to 0.810 in area D2. Components of variance calculated showed highest variance at the level of period (Table 9), while from the spatial scales at the level of quadrats.

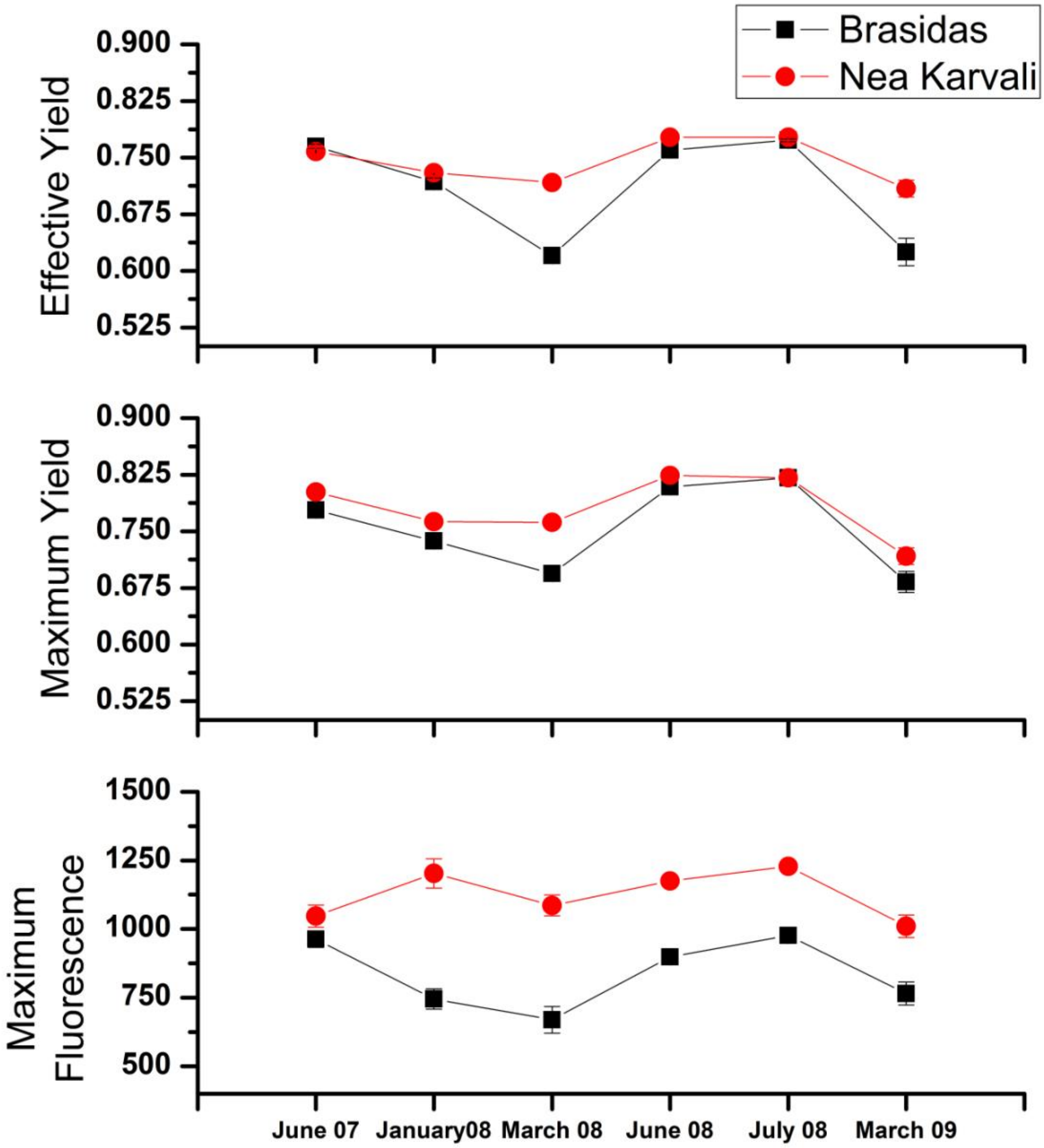

Figure 23. Mean values of physiological parameters (effective quantum yield - ΔF/Fm’, maximum quantum yield - Fv/Fm and maximum fluorescence - Fm) ± standard error (n=139-400) measured in leaves of *Cymodocea nodosa* collected from two meadows in Kavala Gulf, over 7 sampling periods. N.B. Standard error is too small where it’s not shown.

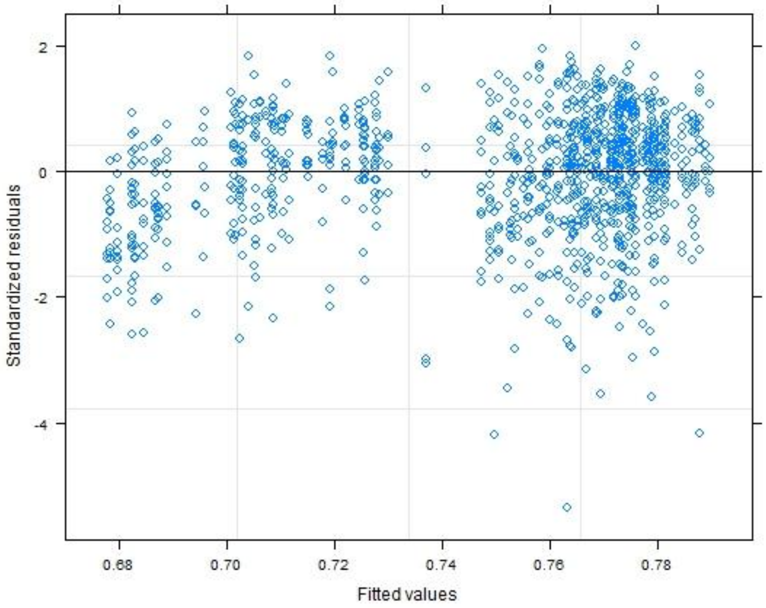

Figure 24. Standardized residuals versus fitted values of the mixed model used to predict the effective quantum yield -ΔF/Fm’ for *Cymodocea nodosa* plants that were sampled from the meadows of Nea Karvali and Brasidas at two time periods, one hot (June and July) and one cold (January and March). The two clusters seen represent the two sampling periods, while no linear relationship could be identified.

Factor	Value	Std.Error	df	t-value	p-value
Intercept	0.688	0.005	972	126.227	0.000*
Nea Karvali	0.032	0.007	2	4.339	0.049
Hot Period	0.079	0.005	131	14.712	0.000*
Meadow*Period	-0.026	0.007	131	-3.704	0.000*

Table 7. Mixed model analysis of ΔF/Fm’ measured in *Cymodocea nodosa* leaves, in samples from two meadows (Brasidas and Nea Karvali) in N. Aegean Sea, in two different sampling periods (Hot and Cold). * p<0.001

Period	Meadow	Mean	S.E.	Variance	Minimum	Maximum	Valid N
Hot	Brasidas	0.766	0.001	0.0006	0.647	0.804	400
	Nea Karvali	0.773	0.001	0.0005	0.647	0.810	399
Cold	Brasidas	0.674	0.005	0.0042	0.502	0.793	175
	Nea Karvali	0.720	0.003	0.0012	0.571	0.790	139

Table 8. Descriptive statistics of ΔF/Fm’ measured in C*ymodocea nodosa* leaves, in samples from two meadows (Brasidas and Nea Karvali) in N. Aegean Sea, into two different sampling periods (Hot and Cold).

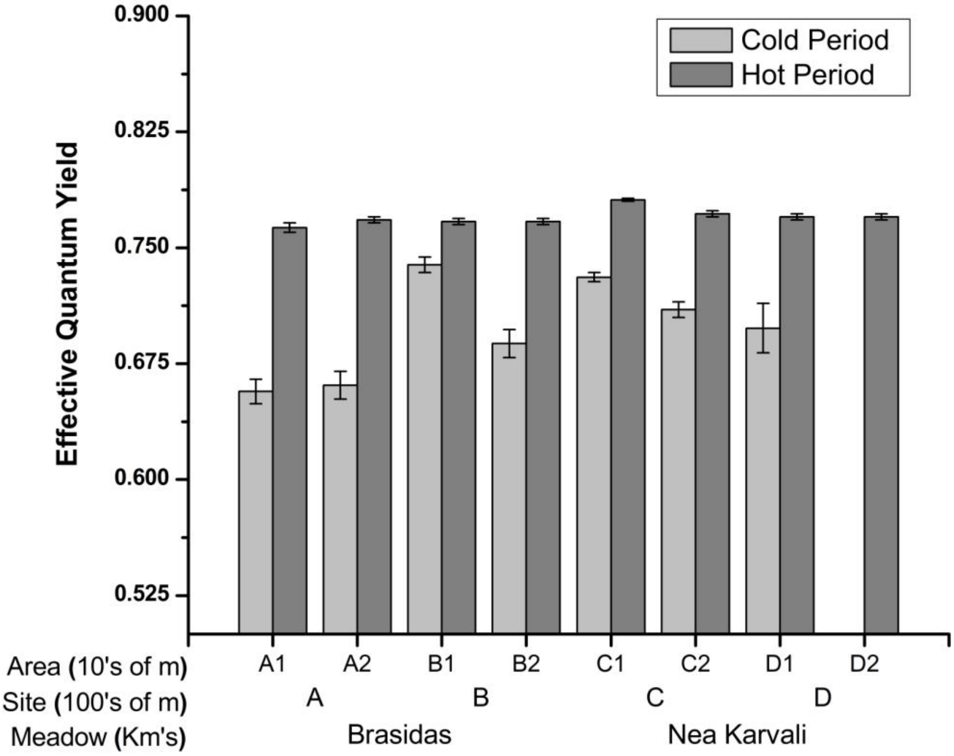

Figure 25. Mean values of ΔF/Fm’±Standard Error (n = 56-100) measured in *Cymodocea nodosa* leaves according to a hierarchical sampling design of three spatial scales (Meadow, Site and Area) in two meadows (Brasidas and Nea Karvali) in the N. Aegean Sea, sampled during a hot and a cold period.

Level of analysis	Components of Variance		
	ΔF/Fm'	**Fv/Fm**	**Fm**
Quadrat:(Area:(Site:Meadow))	0.000480	0.000412	24004.84
Area:(Site:Meadow)	0.000023	4.5E-18	415.09
Site:Meadow	0.000120	0.000128	5083.25
Temperature	0.002444	0.002599	7599.15
Meadow	0.000002	0.000107	31694.32
Residual	0.000780	0.000445	26858.74

Table 9. Components of variance from the ΔF/Fm’, Fv/Fm and Fm analysis measured in leaves of *Cymodocea nodosa* collected from two meadows (Nea Karvali and Brasidas) in the N. Aegean Sea, in two periods (Hot and Cold).

Maximum Quantum Yield. There was no significant difference between the two meadows ($p = 0.074$), while the interaction between meadow and period and period were significant ($p<0.05$). Mean values measured during the hot period were significantly higher than those measured during cold conditions by 0.082±0.007 (Table 10). Higher values of Fv/Fm were taken from Nea Karvali than from Brasidas in almost all sampling efforts (Figure 26). Highest Fv/Fm mean values were measured in Area D1 (mean Fv/Fm= 0.822±0.002) during the hot period, while the lowest values in Area A1 (mean Fv/Fm= 0.705±0.011), during the cold period (Table 11). During cold period Fv/Fm mean values ranged from 0.705±0.011 in area A1 to 0.762±0.005 in area C2, while during the hot period the same range was from 0.800±0.004 in area A1 to 0.822±0.002 in area D1. Components of variance calculated showed highest variance at the level of period, while from the spatial scales at the level of quadrats (Table 9).

Factor	Value	Std.Error	df	t-value	p-value
Intercept	0.724	0.007	750	98.988	0.000*
Nea Karvali	0.034	0.010	2	3.479	0.074
Hot Period	0.082	0.007	131	12.030	0.000*
Meadow*Period	-0.023	0.009	131	-2.552	0.012

Table 10. Mixed model analysis of Fv/Fm measured in *Cymodocea nodosa* leaves, in samples from two meadows (Brasidas and Nea Karvali) in N. Aegean Sea, in two different sampling periods (Hot and Cold). * p<0.001

Period	Meadow	Mean	S.E.	Variance	Minimum	Maximum	Valid N
Cold	Brasidas	0.717	0.005	0.0024	0.604	0.798	89
	Nea Karvali	0.751	0.004	0.0012	0.628	0.798	66
Hot	Brasidas	0.809	0.001	0.0007	0.616	0.859	368
	Nea Karvali	0.820	0.001	0.0002	0.716	0.863	368

Table 11. Descriptive statistics of Fv/Fm measured in *Cymodocea nodosa* leaves, in samples from two meadows (Brasidas and Nea Karvali) in N. Aegean Sea, in two different sampling periods (Hot and Cold).

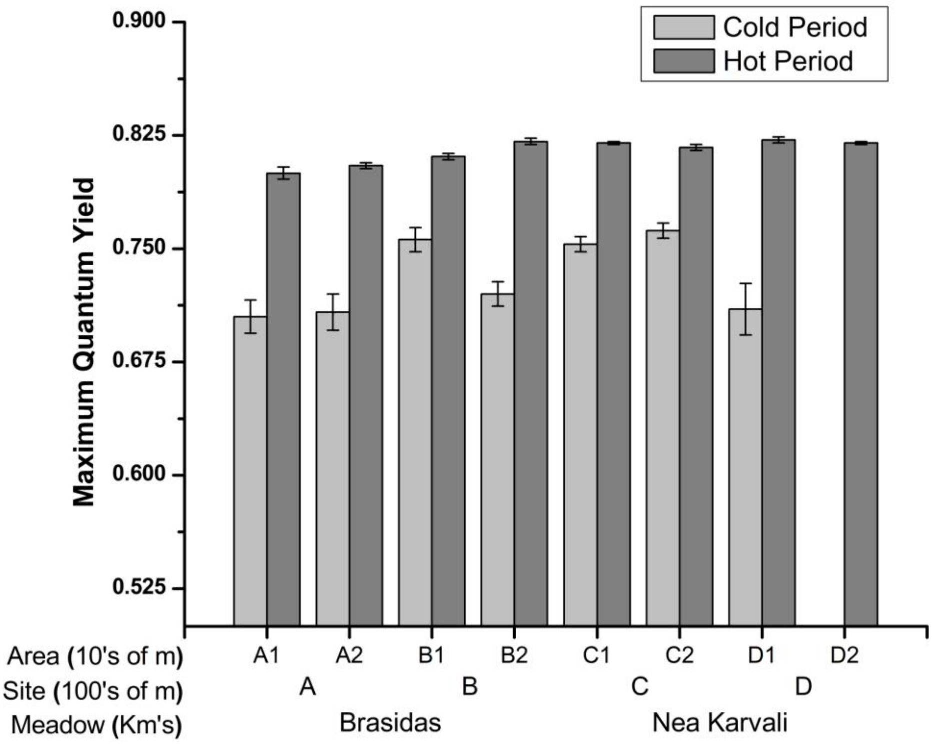

Figure 26. Mean values of Fv/Fm±Standard Error (n =20-92) measured in *Cymodocea nodosa* leaves according to a hierarchical sampling design of three spatial scales (Meadow, Site and Area) in N. Aegean Sea, sampled during a hot and a cold period.

Maximum Fluorescence. Significant differences for maximum fluorescence mean values were identified between periods, meadows and their interaction ($p<0.001$) (Table 12). Mean values measured during the hot period were higher than those measured during the cold period (Figure 27) by 212.361±41.687. Fm ranged from a minimum of 287 in Area A2 to 1784 in Area C2 during the cold period. During the Hot period minimum value was 239 in Area A2 and the maximum 1974 in Area D2. Components of variance calculated showed highest variance at scale of meadow (Table 9).

Factor	Value	Std.Error	df	t-value	p-value
Intercept	728.381	47.000	750	15.498	0.000*
Nea Karvali	408.882	73.276	2	5.580	0.031
Hot	212.361	41.687	131	5.094	0.000*
Meadow*Period	-194.624	66.652	131	-2.920	0.004

Table 12. Mixed model analysis of Fm measured in *Cymodocea nodosa* leaves, in samples from two meadows (Brasidas and Nea Karvali) in N. Aegean Sea, in two different sampling periods (Hot and Cold). * $p<0.001$

Period	Meadow	Mean	S.E.	Variance	Minimum	Maximum	Valid N
Cold	Brasidas	730.876	24.343	52146.603	287	1306	89
	Nea Karvali	1104.636	27.242	48238.050	603	1784	66
Hot	Brasidas	940.446	12.663	58849.502	239	1864	368
	Nea Karvali	1181.538	11.079	45045.537	324	1974	368

Table 13. Descriptive statistics of Fm measured in *Cymodocea nodosa* leaves, in samples from two meadows (Brasidas and Nea Karvali) in N. Aegean Sea, into two different sampling periods (Hot and Cold).

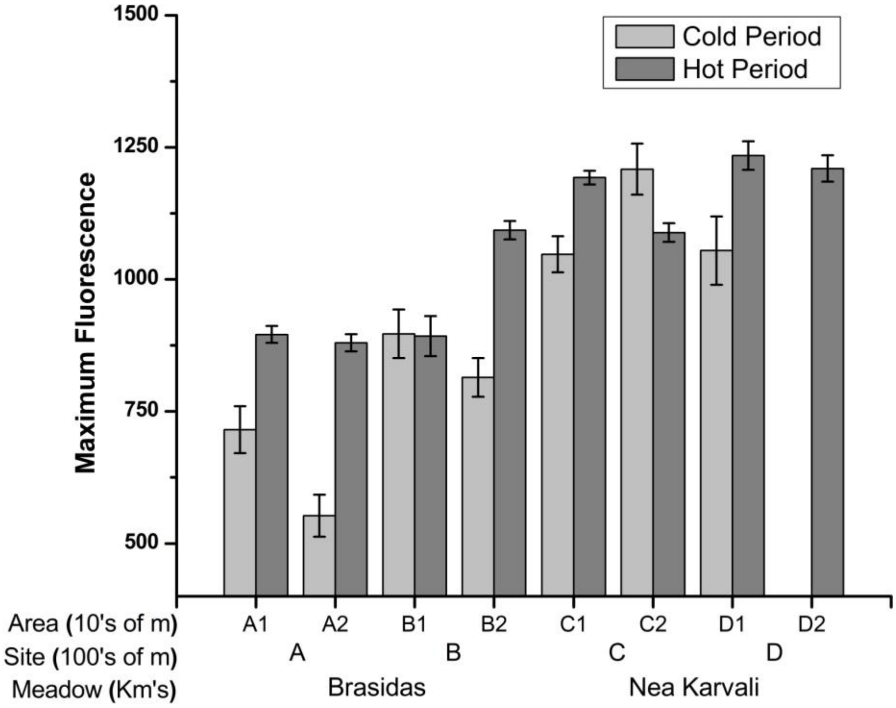

Figure 27. Mean values of Fm ± Standard Error measured in *Cymodocea nodosa* leaves between two meadows (Nea Karvali and Brasidas) according to a hierarchical sampling design of three spatial scales (Meadow, Site and Area) in N. Aegean Sea, sampled during a hot and a cold period.

Morphological parameters

Total Leaf Length. Significant differences for total leaf length mean values were identified between periods, meadows and their interaction ($p<0.001$) (Table 14). Mean values measured during the hot period were higher than those measured during the cold period (Figure 29) by 3.249±0.949 cm. Higher values of total leaf length were taken from Nea Karvali than from Brasidas in almost all sampling efforts (Figure 28) by 12.915±2.070 cm.

Factor	Value	Std.Error	df	t-value	p-value
Intercept	8.182	1.441	3060	5.679	0.000*
Nea Karvali	12.915	2.070	2	6.239	0.025
Hot Period	3.249	0.949	95	3.425	0.001
Meadow*Period	5.146	1.389	95	3.705	0.000*

Table 14. Mixed model analysis of *Cymodocea nodosa* leaf length, from two meadows (Brasidas and Nea Karvali) in N. Aegean Sea and two different sampling periods (Hot and Cold). * $p<0.001$

During the cold period *C. nodosa* had much shorter leaves as seen in Table 15. At that point, total leaf length ranged from 5.921±0.373 cm in Area B2 to 24.113±1.187 cm in Area C2. During the hot period leaf length ranged from 8.050±0.179 cm in Area A2 to 32.558±0.854 cm in Area C2. Components of variance calculated showed highest variance at scale of meadow (Table 16).

Period	Meadow	Mean	S.E.	Variance	Minimum	Maximum
Cold	Brasidas	8.424	0.347	20.496	0.7	24.9
	Nea Karvali	20.574	1.022	152.450	0.4	65.7
Hot	Brasidas	11.371	0.16	37.064	0.4	31
	Nea Karvali	29.445	0.428	254.769	0.6	79.4

Table 15. Descriptive statistics of *Cymodocea nodosa* total leaf length, for samples from two meadows (Brasidas and Nea Karvali) in N. Aegean Sea, in two sampling periods (Hot and Cold).

Level of analysis	Components of Variance		
	Leaf Length	Leaf Width	No of Leafs per shoot
Quadrat:(Area:(Site:Meadow))	20.10	0.00128	
Area:(Site:Meadow)	5.16	0.00062	0.059
Site:Meadow	1.7E-07	0.00000	0.000
Temperature	11.54	0.00978	1.181
Meadow	154.47	0.00395	0.019
Residual	116.34	0.00244	0.465

Table 16. Components of variance from the Leaf length and width and number of leaves per shoot analysis measured in leaves of *Cymodocea nodosa* collected from two meadows (Nea Karvali and Brasidas) in the N. Aegean Sea, in two periods (Hot and Cold).

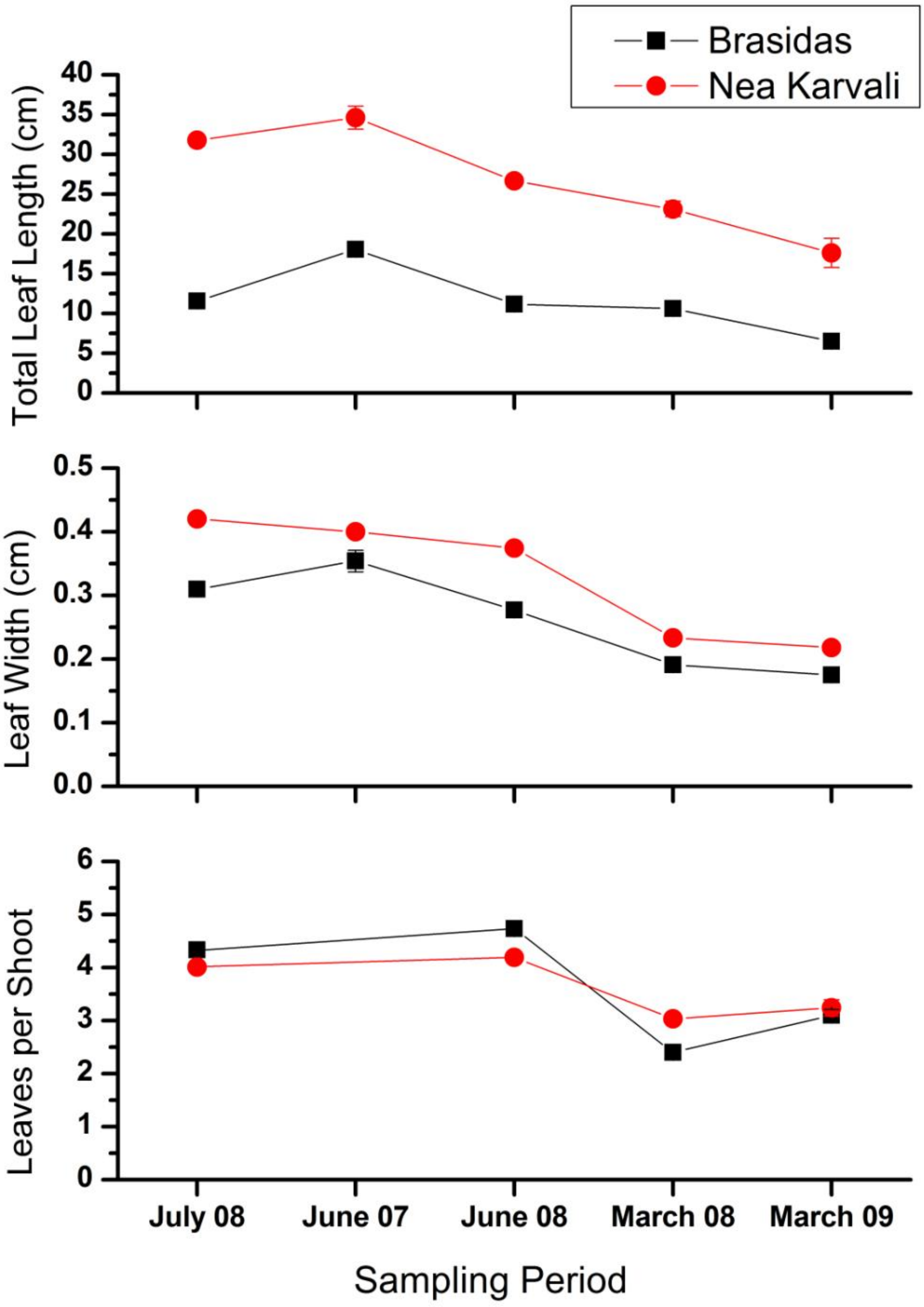

Figure 28. Mean values of total leaf length (cm), width (cm) and number of leaves per shoot ± Standard Error (n=100-1430), of *Cymodocea nodosa* plants sampled from two meadows (Brasidas and Nea Karvali) in N. Aegean Sea, at 5 sampling periods. N.B. Standard error is too small where it’s not shown.

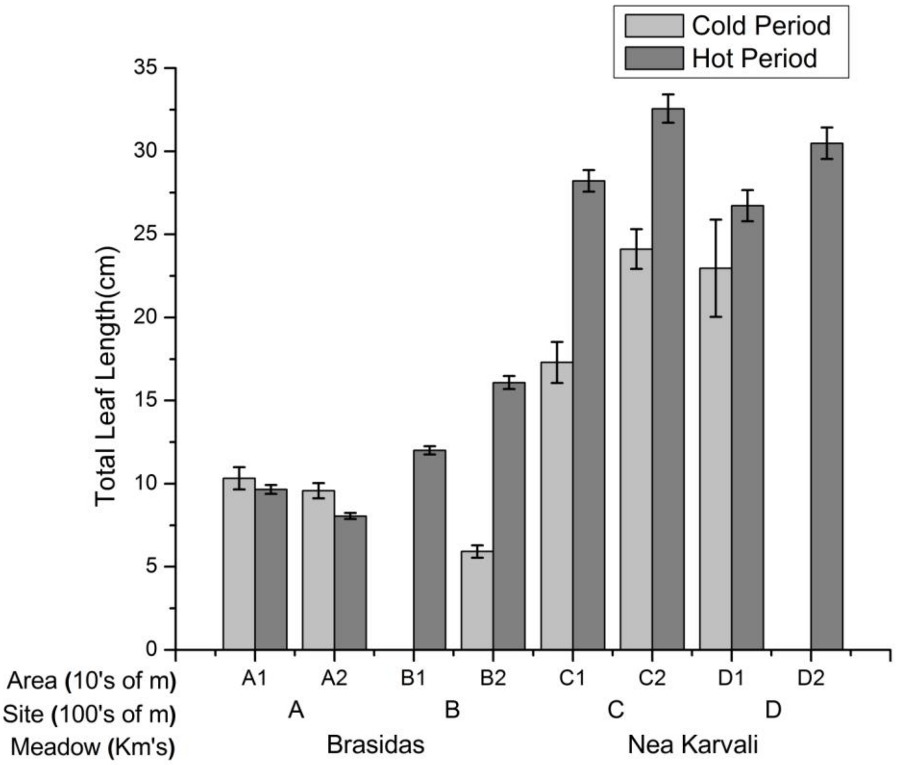

Figure 29. Mean values of *Cymodocea nodosa* Leaf Length (cm) ±Standard Error (n=100-1430) according to a hierarchical sampling design of three spatial scales (Meadow, Site and Area) in two meadows (Brasidas and Nea Karvali) in N. Aegean Sea, sampled during a hot and a cold period.

Leaf Width. Leaf width showed significant variability between the two periods ($p<0.001$) and the interaction between meadow and period (Table 17), with thicker leaves during the hot period (Table 18). Width became minimum in Area B2 (mean Width= 0.182±0.006 cm) and maximum in Area C2 (mean Width= 0.231±0.008 cm) during the cold period. Width was minimum in Area A2 (0.12cm in both periods), while maximum in Area C1 (0.9cm during the hot period and 0.34cm during the cold). Components of variance calculated showed highest variance at the level of period (Table 16), while from the spatial scales at the level of quadrats. Figure 30 shows the mean values variability of leaf width between the two meadows.

Factor	Value	Std.Error	df	t-value	p-value
Intercept	0.185	0.017	2150	10.790	0.000*
Nea Karvali	0.046	0.025	2	1.860	0.204
Hot Period	0.112	0.015	94	7.590	0.000*
Meadow*Period	0.054	0.021	94	2.592	0.011

Table 17. Mixed model analysis of *Cymodocea nodosa* leaf width, from two meadows (Brasidas and Nea Karvali) in N. Aegean Sea and two different sampling periods (Hot and Cold). * p<0.001

Period	Meadow	Mean	S.E.	Variance	Minimum	Maximum	Valid N
Cold	Brasidas	0.185	0.004	0.001	0.120	0.290	78
	Nea Karvali	0.227	0.005	0.002	0.160	0.340	67
Hot	Brasidas	0.292	0.002	0.004	0.120	0.900	1077
	Nea Karvali	0.396	0.002	0.006	0.200	0.900	1032

Table 18. Descriptive statistics of *Cymodocea nodosa* leaf width, from two meadows (Brasidas and Nea Karvali) in N. Aegean Sea and two different sampling periods (Hot and Cold).

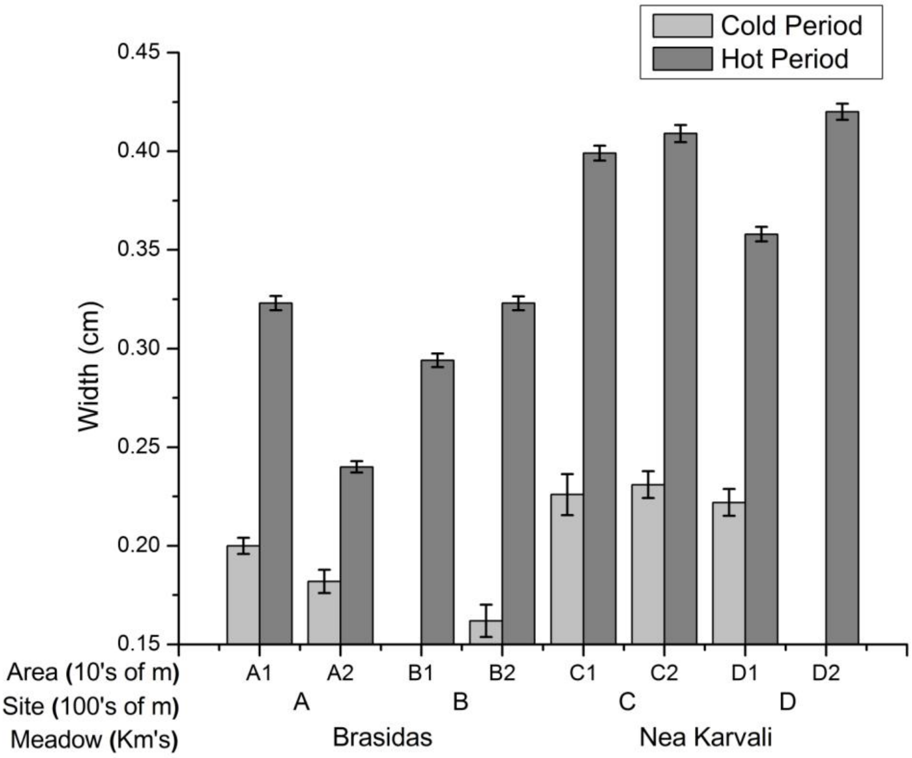

Figure 30. Mean values of *Cymodocea nodosa* Leaf Width (cm) ±Standard Error (n=18-296) according to a hierarchical sampling design of three spatial scales (Meadow, Site and Area) in two meadows (Nea Karvali and Brasidas) in N. Aegean Sea, sampled during a hot and a cold period.

Number of Leaves per shoot. Significant variance existed between the two periods (p<0.001) and the interaction between meadow and period (p<0.001; Table 19), with more leaves per shoot during the hot period (Figure 31). The two meadows had no significant difference between them (p statistic=0.161). During the cold period shoots had from 0.162±0.008 (Area B2) to 0.222±0.007 (Area D1) leaves per shoot, while during the hot period from 0.240±0.003 (Area A2) to 0.420±0.004 (Area D2). The parameter showed highest variability in Brasidas meadow, especially during the hot period (Table 20). Components of variance calculated showed highest variance at the level of period (Table 16), while from the spatial scales at the level of area.

Factor	Value	Std.Error	df	t-value	p-value
Intercept	2.759	0.125	841	22.001	0.000*
Hot Period	1.736	0.138	841	12.583	0.000*
Nea Karvali	0.400	0.184	2	2.179	0.161
Meadow*Period	-0.813	0.198	841	-4.096	0.000*

Table 19. Mixed model analysis of *Cymodocea nodosa* leaves per shoot, from two meadows (Brasidas and Nea Karvali) in N. Aegean Sea and two different sampling periods (Hot and Cold). * p<0.001

Period	Meadow	Mean	SE	Variance	Minimum	Maximum	Valid N
Cold	Brasidas	2.591	0.061	0.409	2.000	4.000	110
	Nea Karvali	3.079	0.061	0.374	2.000	4.000	101
Hot	Brasidas	4.538	0.042	0.569	3.000	6.000	320
	Nea Karvali	4.103	0.036	0.413	2.000	6.000	320

Table 20. Descriptive statistics of *Cymodocea nodosa* leaves per shoot, from two meadows (Brasidas and Nea Karvali) in N. Aegean Sea and two different sampling periods (Hot and Cold).

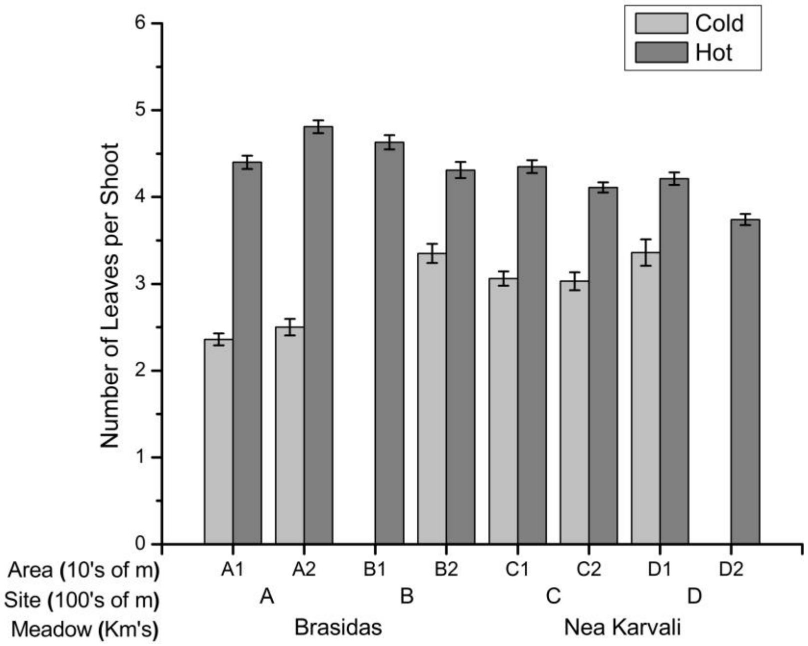

Figure 31. Mean values of *Cymodocea nodosa* Leaves per Shoot ± Standard Error (n=20-80) according to a hierarchical sampling design of three spatial scales (Meadow, Site and Area) in two meadows (Brasidas and Nea Karvali) in N. Aegean Sea, sampled during a hot and a cold period.

Chlorophyll-a. Chlorophyll- *a* content differed significantly between the two sampling periods (Table 21). During the cold period leaves had 0.302±0.030μg chl-*a* per g of leaf more than they did in the hot period (Figure 32, Table 22). Chl-*a* didn't vary significantly between meadows (p statistic=0.815). It ranged from 0.254±0.027μg/g in Area B2 to 0.475±0.046μg/g in Area C1 during the hot period and from 0.544±0.092μg/g in Area D1 to 0.715±0.038μg/g in Area C1 during the cold period. Components of variance calculated showed highest variance at the level of period (Table 23), while from the spatial scales at the level of area.

Factor	Value	Std.Error	df	t-value	p-value
Intercept	0.650	0.069	167	9.438	0.000*
Hot Period	-0.400	0.073	167	-5.462	0.000*
Nea Karvali	-0.042	0.096	2	-0.435	0.706
Meadow*Period	0.135	0.095	167	1.411	0.160

Table 21. Mixed model analysis of chlorophyll-*a* content in *Cymodocea nodosa* leaves, from two meadows (Brasidas and Nea Karvali) in N. Aegean Sea and two different sampling periods (Hot and Cold). * p<0.001

Period	Meadow	Mean	S.E.	Variance	Minimum	Maximum
Hot	Brasidas	0.254	0.027	0.011	0.114	0.450
	Nea Karvali	0.341	0.015	0.024	0.125	0.925
Cold	Brasidas	0.642	0.026	0.019	0.367	0.894
	Nea Karvali	0.633	0.050	0.063	0.114	0.967

Table 22. Descriptive statistics of chlorophyll *a* content in *Cymodocea nodosa* leaves, from two meadows (Brasidas and Nea Karvali) in N. Aegean Sea and two different sampling periods (Hot and Cold).

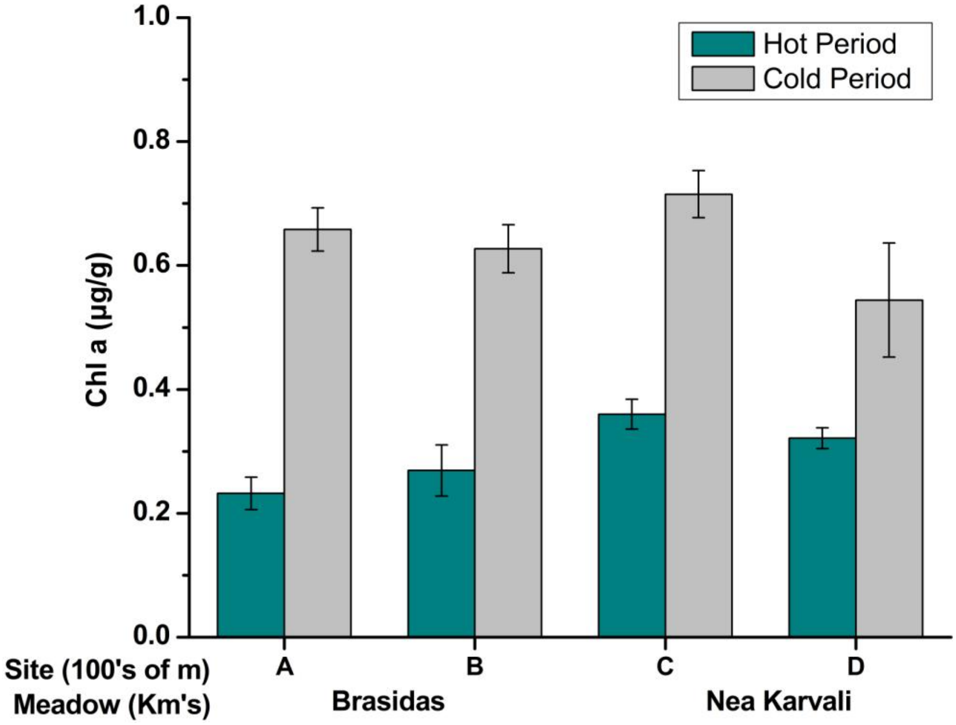

Figure 32. Mean values of Chlorophyll-*a* content (μg/g)± Standard Error (n=15-108) in leaves of *Cymodocea nodosa* according to a hierarchical sampling design of two spatial scales (Meadow, Site) in N. Aegean Sea, sampled during a hot and a cold period.

Level of analysis	Components of Variance
Area:(Site:Meadow)	0.0050
Site:Meadow	0.0000
Period	0.0442
Meadow	0.0000
Residual	0.0228

Table 23. Components of variance in the nested hierarchical analysis of *Cymodocea nodosa* chlorophyll *a* content in leaves for samples collected in two meadows (Nea Karvali and Brasidas) in N. Aegean Sea and in two periods (Hot and Cold).

CHN

Leaves. Nitrogen content in leaves varied significantly between the two meadows and the two periods (Table 25). Nea Karvali had higher values than Brasidas, a difference that became more intense during the cold period (Table 24), during which leaves from Nea Karvali had 1.287 (Area D1) to 4.714 (Area C1) %DW of nitrogen, while leaves from Brasidas 1.186 (Area B2) to 2.149 (Area A1) %DW. Lower values were measured during the hot period (Figure 33), where leaf nitrogen in Brasidas ranged from 0.463 (Area B2) to 1.963 (Area B1) %DW and in Nea Karvali from 1.483 (Area D2) to 6.287 (Area D1) %DW.

Carbon in leaves varied between the two periods and the interaction between period and meadow (Table 25). Mean leaf carbon was 25.896±0.946%DW during the cold period and 31.836±0.626%DW during the hot period for Brasidas meadow. Nea Karvali had a mean value of 30.531±0.757%DW during the cold period and 31.960±0.737%DW during the hot period.

The carbon to nitrogen ration varied significantly between the two meadows and the interaction between meadows and periods (Table 25). Its mean value varied from 15.145±0.737 and 10.635±1.101 %DW for the Brasidas and Nea Karvali meadows respectively during the cold period to 27.007±2.041%DW and 14.562±0.767%DW during the hot one.

Period	Meadow	N	S.E.	C	S.E.	C/N	S.E.
Cold	**Brasidas**	1.739	0.045	25.896	0.946	15.145	0.737
	Nea Karvali	3.262	0.193	30.531	0.757	10.635	1.101
Hot	**Brasidas**	1.323	0.069	31.835	0.626	27.007	2.041
	Nea Karvali	2.412	0.16	31.96	0.737	14.562	0.767

Table 24. Mean values ± standard error of nitrogen, carbon and C/N content in leaves of *Cymodocea nodosa* sampled in two meadows (Brasidas and Nea Karvali) in N. Aegean Sea, in two periods (Hot and Cold).

	Factor	**Value**	**Std.Error**	**df**	**t-value**	**p-value**
N	Intercept	1.772	0.132	101	13.405	0.000*
	Hot	-0.464	0.150	101	-3.090	0.003
	Nea Karvali	1.511	0.300	2	5.038	0.037
	Period*Meadow	-0.442	0.338	101	-1.308	0.194
C	Intercept	25.860	1.206	101	21.450	0.000*
	Hot	6.070	1.272	101	4.771	0.000*
	Nea Karvali	3.958	1.721	2	2.300	0.148
	Period*Meadow	-3.823	1.810	101	-2.112	0.037
C/N	Intercept	30.072	3.253	50	9.245	0.000*
	Hot	56.563	23.603	50	2.396	0.020
	Nea Karvali	-7.916	4.589	2	-1.725	0.227
	Period*Meadow	7.036	32.572	50	0.216	0.830

Table 25. Mixed model analysis of C, N and C/N content in *Cymodocea nodosa* leaves, from two meadows (Brasidas and Nea Karvali) in N. Aegean Sea and two different sampling periods (hot and cold). * p<0.001

Level of analysis	**Components of Variance**		
	N	**C**	**C/N**
Area:(Site:Meadow)	0.000	0.000	1.403
Site:Meadow	0.000	3.607	0.406
Period	0.192	8.209	31.296
Meadow	0.796	0.000	40.830
Residual	0.492	14.921	54.871

Table 26. Components of variance in the nested hierarchical analysis of *Cymodocea nodosa* N, C and C/N content in leaves sampled from two meadows (Nea Karvali and Brasidas) in N. Aegean Sea in two periods (Hot and Cold).

Roots. All three variables varied significantly only between periods (Table 28), which was also the component of higher variability (Table 29). During the hot period both carbon and stored in roots were higher, while nitrogen and C/N lower. Brasidas had an overall mean value of 0.753±0.058%DW nitrogen, 29.137±1.107%DW carbon and 53.230±7.879 C/N, while Nea Karvali 1.153±0.153%DW nitrogen, 29.433±1.167 %DW carbon and 62.016±18.140 C/N. Table 27 shows the mean values of all three parameters for the two meadows between the two periods.

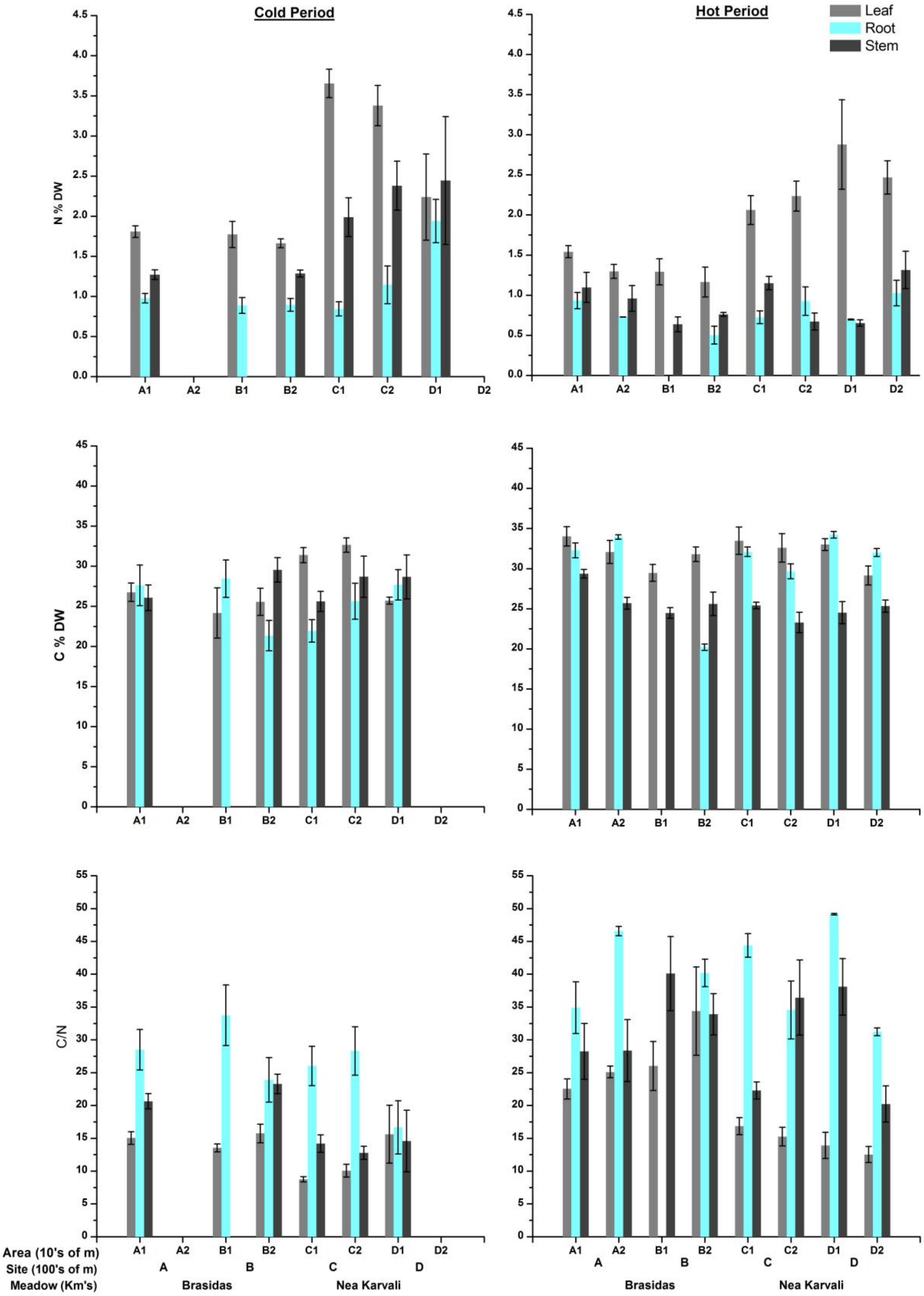

Figure 33. Mean values of Carbon, Nitrogen and C/N (%DW) ±Standard Error (n=3-15) content in *Cymodocea nodosa* leaves, roots and stems according to a hierarchical sampling design of three spatial scales (Meadow, Site and Area) sampled from two meadows (Nea Karvali and Brasidas) in N. Aegean Sea, during a hot and a cold period.

Period	Meadow	N	S.E.	C	S.E.	C/N	S.E.
Cold	**Brasidas**	0.924	0.050	27.321	1.558	30.431	2.077
	Nea Karvali	1.542	0.198	26.661	1.446	22.475	2.989
Hot	**Brasidas**	0.765	0.086	30.518	2.624	40.624	2.764
	Nea Karvali	0.845	0.085	31.642	1.156	39.642	3.943

Table 27. Mean values ± standard error of nitrogen, carbon and C/N content in roots of *Cymodocea nodosa* sampled in two meadows (Brasidas and Nea Karvali) in N. Aegean Sea, in two periods (Hot and Cold).

	Factor	Value	Std.Error	df	t-value	p-value
N	Intercept	0.979	0.112	37	8.743	0.000*
	Nea Karvali	0.574	0.286	2	2.010	0.182
	Hot	-0.285	0.123	37	-2.321	0.026
	Meadow*Period	-0.480	0.282	37	-1.700	0.098
C	Intercept	27.301	1.536	37	17.778	0.000*
	Nea Karvali	-0.714	2.286	2	-0.312	0.784
	Hot	3.128	2.472	37	1.266	0.214
	Meadow*Period	1.980	3.314	37	0.597	0.554
C/N	Intercept	29.655	3.178	37	9.333	0.000*
	Nea Karvali	-7.979	5.088	2	-1.568	0.257
	Hot	11.460	4.815	37	2.380	0.023
	Meadow*Period	7.664	6.873	37	1.115	0.272

Table 28. Mixed model analysis of C, N and C/N content in *Cymodocea nodosa* roots, from two meadows (Brasidas and Nea Karvali) in N. Aegean Sea and two different sampling periods (Hot and Cold). * p<0.001

Level of analysis	Components of Variance		
	N	C	C/N
Area:(Site:Meadow)	0.098	0.000	20.995
Site:Meadow	0.000	0.000	32.128
Period	0.205	15.383	1645.173
Meadow	0.029	0.000	0.000
Residual	0.194	30.661	1655.323

Table 29. Components of variance in the nested hierarchical analysis of *Cymodocea nodosa* N, C and C/N content in roots from samples collected in two meadows in N. Aegean Sea.

Stem. CHN analysis on stems showed that there wasn't any significant variation between the two meadows. Nitrogen content varied significantly between the two periods and the interaction of period and meadow (Table 31). During the cold period nitrogen varied from 1.013 (Area A1) to 1.504%DW (Area A1) in stems from Brasidas meadow and from 1.005 (Area C2) to 3.808 (Area D1) %DW in stems from Nea Karvali. During the hot period minimum value of stem nitrogen in Brasidas meadow was 0.465%DW, measured in Area B1 and reached to 1.446 %DW in Area A1. At the same time minimum value for samples from Nea Karvali was met in Area C2 and was 0.461%DW when the maximum value was 1.780%DW in Area D2.

Carbon to nitrogen ratio wasn't significant at any level (Table 31) and it varied from 22.158±1.003 in the cold period to 32.645±2.428 during the hot period for Brasidas and from 13.753±0.948 to 29.255±2.942 for Nea Karvali at the same time.

Carbon content in stems had a slightly significant variation between the two meadows (p=0.056) but it didn't vary at any level (Table 31). A mean value of 27.139±1.164 %DW was measured in Nea Karvali and 28.078±1.148%DW in Brasidas. In the hot period mean values were lower. Nea Karvali had a mean value of 24.629±0.510%DW and Brasidas 26.278±0.681%DW.

Period	Meadow	N	S.E.	C	S.E.	C/N	S.E.
Cold	**Brasidas**	1.279	0.035	28.078	1.148	22.158	1.003
	Nea Karvali	2.193	0.191	27.139	1.164	13.753	0.948
Hot	**Brasidas**	0.863	0.078	26.278	0.681	32.645	2.428
	Nea Karvali	0.947	0.105	24.629	0.510	29.255	2.942

Table 30. Mean values ± standard error of nitrogen, carbon and C/N content in stems of *Cymodocea nodosa* sampled in two meadows (Brasidas and Nea Karvali) in N. Aegean Sea, in two periods (Hot and Cold).

	Factor	Value	Std.Error	df	t-value	p-value
N	Intercept	1.273	0.090	53	14.219	0.000*
	Hot	-0.410	0.118	53	-3.466	0.001
	Nea Karvali	1.008	0.297	2	3.394	0.077
	Period*Meadow	-0.939	0.301	53	-3.116	0.003
C	Intercept	27.636	1.579	53	17.506	0.000*
	Hot	-1.240	1.866	53	-0.664	0.510
	Nea Karvali	-0.347	2.066	2	-0.168	0.882
	Period*Meadow	-1.420	2.327	53	-0.610	0.544
C/N	Intercept	21.884	1.559	53	14.033	0.000*
	Hot	10.583	3.829	53	2.764	0.008
	Nea Karvali	-8.330	2.050	2	-4.064	0.056
	Period*Meadow	5.843	4.820	53	1.212	0.231

Table 31. Mixed model analysis of C, N and C/N content in *Cymodocea nodosa* stems, from two meadows (Brasidas and Nea Karvali) in N. Aegean Sea and two different sampling periods (Hot and Cold). * $p<0.001$

Level of analysis	**Components of Variance**		
	N	**C**	**C/N**
Area:(Site:Meadow)	0.000	0.000	11.030
Site:Meadow	0.000	0.000	2.236
Period	0.338	1.707	87.733
Meadow	0.169	0.160	16.225
Residual	0.318	17.728	38.396

Table 32. Components of variance in the nested hierarchical analysis of *Cymodocea nodosa* N, C and C/N content in stems from samples collected in two meadows in N. Aegean Sea.

Phosphorus concentration in Leaves. Significant differences in P content in leaves were identified between meadows (p=0.045; Table 34). Figure 34 shows the mean values of P concentration in leaves between the four sampled areas. Area C1 had the highest mean value (mean=2516.900±168.607mg/Kg) and area B2 from Brasidas meadow the lowest (mean=1343.400±78.548mg/Kg) (Table 33). Components of variance calculated showed highest variance at the level of meadow (Table 35).

Site	**Mean P (mg/Kg)**	**S.E.**	**S.D.**	**Minimum**	**Maximum**
A	1723.200	146.620	463.654	1232	2428
B	1343.400	78.548	351.276	787	2128
C	2516.900	168.607	533.182	1754	3319
D	2238.636	144.025	477.678	1506	3151

Table 33. Descriptive statistics of P content (mg/Kg) in C. nodosa leaves sampled from 4 sites (A, B, C, and D) in N. Aegean Sea in March 2009.

Factor	**Value**	**Std.Error**	**df**	**t-value**	**p-value**
Intercept	1501.067	116.394	47	12.896	0.001*
Nea Karvali	868.536	191.615	2	4.533	0.045

Table 34. Mixed model analysis of phosphorus content in *Cymodocea nodosa* leaves, from two meadows (Brasidas and Nea Karvali) in N. Aegean Sea, sampled in March 2009. *, p<0.001

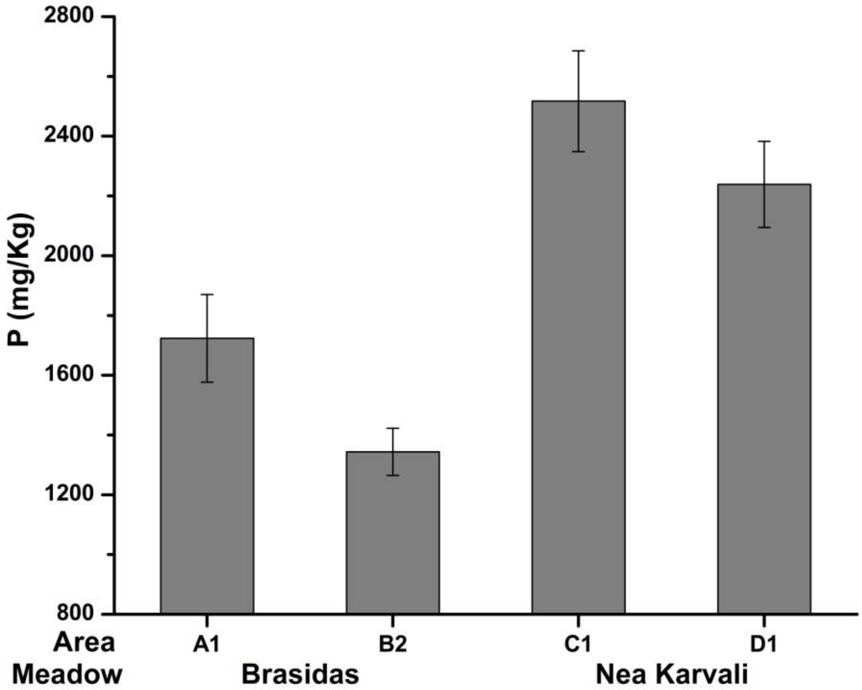

Figure 34. Mean values of phosphorus content (mg/Kg) ± Standard Error (n=10-20) in *Cymodocea nodosa* leaves sampled from four areas (A1, B2, C1, D1) of two meadows (Brasidas and Nea Karvali) in N. Aegean Sea, during March 2009.

Level of analysis	Components of Variance
Site:Meadow	20598.000
Area:(Site:Meadow)	20598.000
Meadow	340127.000
Residual	193797.000

Table 35. Components of variance in the nested hierarchical analysis of *Cymodocea nodosa* P content in leaves from samples collected in two meadows (Nea Karvali and Brasidas) in N. Aegean Sea.

Depth correlation

The two sampled sites A and B (1-2m deep) were compared to the deeper site E (2-3m deep) in Brasidas meadow. The analysis showed that small depth changes do not have any significant effects on any of the measured variables (Table 36). Mean values and p statistics, from Area E compared to Area A and B are shown in Table 36.

Variable	**Area A**		**Area B**		**Area E**		**p statistic**	
	Mean	**S.E.**	**Mean**	**S.E.**	**Mean**	**S.E.**	**Site B**	**Site E**
ΔF/Fm'	0.767	0.002	0.766	0.002	0.770	0.003	0.609	0.842
Fv/Fm	0.810	0.002	0.819	0.001	0.830	0.001	0.168	0.056
Fm	892.219	12.375	981.856	24.271	982.823	20.600	0.468	0.517
Length	9.373	0.213	15.818	0.404	13.959	0.657	0.070	0.193
Width	0.322	0.022	0.324	0.003	0.307	0.008	0.935	0.510

Table 36. P statistic and mean values ± Standard Error of key physiological and morphological parameters of *Cymodocea nodosa,* sampled in three sites (A, B, E) of Brasidas meadow in N. Aegean Sea, during June and July 2008.

Comparison between Thasos and Brasidas meadow.

The hypothesis that Brasidas meadow remains non-impacted despite observations to the contrary and that it could be compared to Nea Karvali safely, was tested by comparing the measured variables with a site at Thasos Island. All measured parameters didn’t vary between the two meadows (Table 37), while significant variability was found between the two periods.

Figure 35 shows the mean values of all physiological parameters between the two meadows. There wasn’t any significant variation between the four sampled areas, especially during the hot period, when the two meadows showed similar mean values. Area A1 had lower physiological values than all other areas. During the cold period minimum mean Fm was 544.000±68.527 (area A1), while maximum mean value was 812.526±42.928 area B2. Minimum maximum quantum yield was measured in area A1 (mean=0.609±0.028) and was maximized in area Th2 (mean=0.725±0.019) that also had maximum effective yield. The later variable ranged, from 0.589±0.018 in area A1, to 0.706±0.019 in area Th2.

During the hot period all parameters had similar mean values, except area Th2 that had a lower mean Fm value (Figure 35). Fm ranged from 964.500±74.272 in area Th2 to 1344.171±271 in area Th1. Minimum mean Fv/Fm was measured at area Th1 (mean Fv/Fm=0.777±0.009) and maximum at area A1 (mean=0.791±0.003). Effective quantum yield had an even smaller variation, from 0.756±0.008 in Area Th1 to 0.763±0.005 in Area A1.

Variable	Factor	Value	Std.Error	df	t-value	p-value
Fm	**Intercept**	690.880	76.566	56	9.023	0.000*
	Thasos	27.965	109.316	3	0.256	0.815
	Hot Period	454.846	100.024	56	4.547	0.000*
Fv/Fm	**Intercept**	0.684	0.018	56	38.730	0.000*
	Thasos	-0.007	0.010	3	-0.689	0.541
	Hot Period	0.105	0.018	56	5.961	0.000*
ΔF/Fm'	**Intercept**	0.640	0.027	56	23.632	0.000*
	Thasos	-0.015	0.013	3	-1.182	0.322
	Hot Period	0.137	0.023	56	6.043	0.000*
Length	**Intercept**	6.908	0.524	178	13.171	0.000*
	Thasos	4.341	0.766	1	5.664	0.111
	Hot Period	-1.500	0.642	178	-2.338	0.021
Width	**Intercept**	0.172	0.014	164	12.357	0.000*
	Thasos	-0.021	0.020	1	-1.063	0.481
	Hot Period	0.086	0.014	164	5.993	0.000*

Table 37. Mixed model analysis morphological and physiological parameters in *Cymodocea nodosa* samples from two meadows (Brasidas, Thasos) in N. Aegean Sea, during two periods (Hot and Cold). * p<0.001

Meadow	Fm	S.E.	Fv/Fm	S.E.	ΔF/Fm'	S.E.	Length	S.E.	Width	S.E.
Brasidas	828.811	45.633	0.709	0.013	0.668	0.014	6.285	0.327	0.213	0.007
Thasos	969.077	58.282	0.751	0.010	0.729	0.010	10.152	0.529	0.230	0.007

Table 38. Mean ±SE values of *Cymodocea nodosa* morphological and physiological parameters, measured in samples from two meadows (Brasidas and Thasos) in N. Aegean Sea.

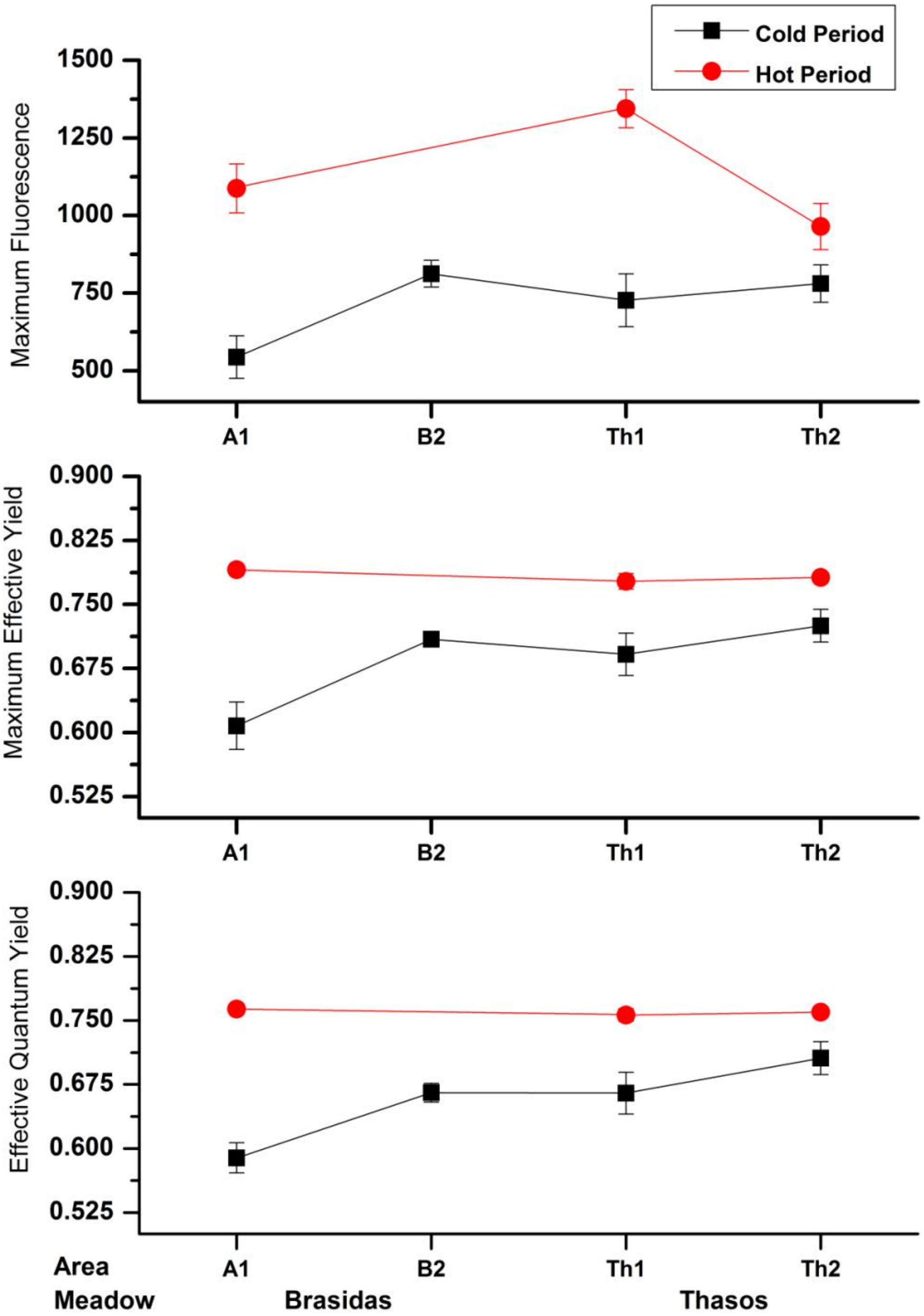

Figure 35. Mean values of ΔF/Fm', Fv/Fm and Fm ± Standard Error (n=6-10) measured in *Cymodocea nodosa* leaves, in four areas (A1, B2, Th1, Th2) nested in two meadows (Brasidas and Thasos) in N. Aegean Sea, sampled in two periods (hot and cold).

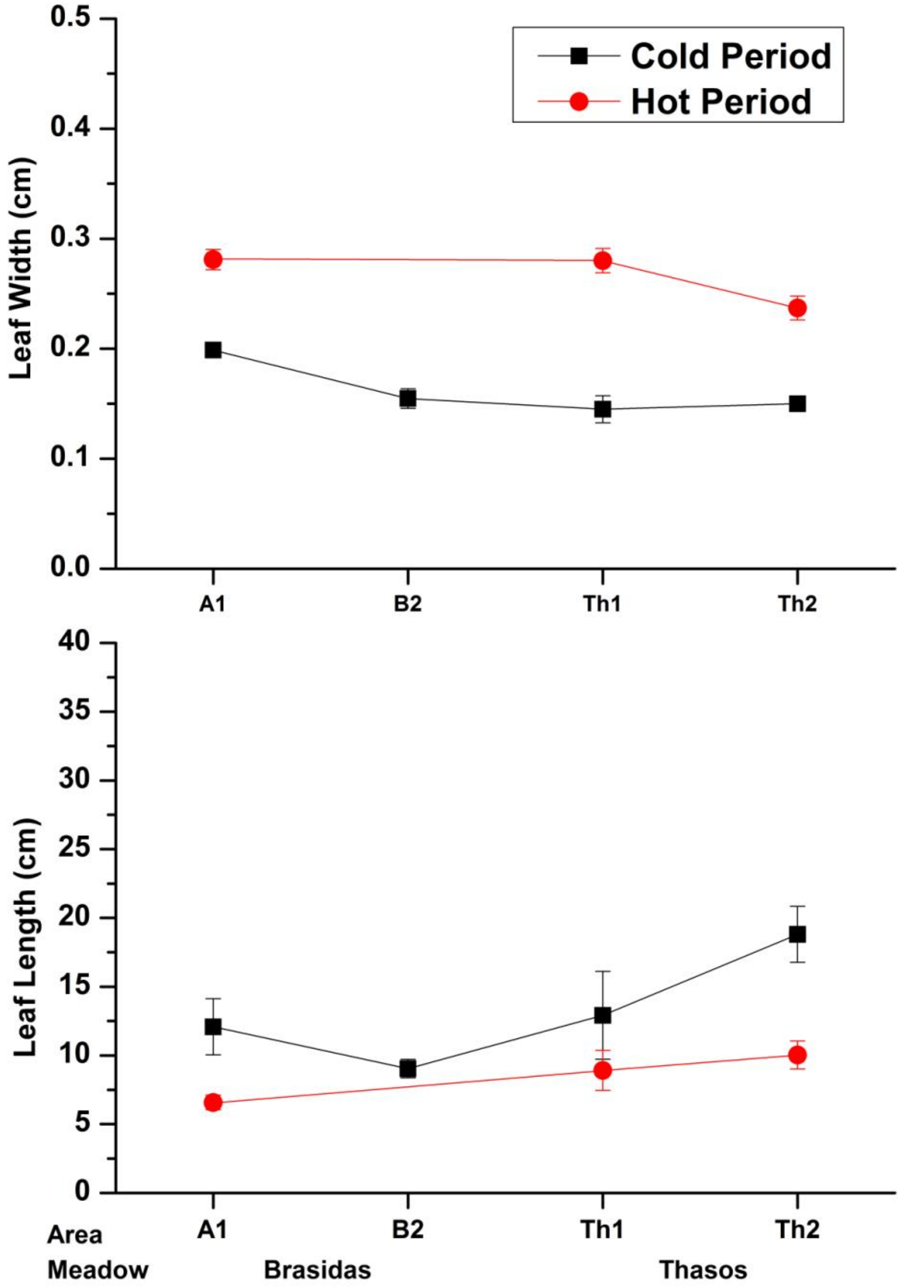

Figure 36. Mean values of *Cymodocea nodosa* leaf length and width (cm) ± Standard Error (26-43), in four areas (A1, B2, Th1, Th2) nested in two meadows (Brasidas and Thasos) in N. Aegean Sea, sampled in two periods (hot and cold).

Both morphological parameters had a significant variation between the two periods (Table 37). Thasos had longer and wider leaves than Brasidas. During the hot period, leaf length ranged from 5.44±0.34cm (Area A1) to 10.27±0.78cm (Area Th1), while during the cold from 6.35±0.47cm (Area A1) to 11.97±2.32cm (Area Th2). Leaf width ranged from 0.11 to 0.27cm and from 0.12 to 0.2cm during the cold period in Brasidas and Thasos respectively. During the hot period higher values were observed (Table 38) starting from 0.22 to 0.32cm in Brasidas and from 0.18 to 0.42cm in Thasos.

DISCUSSION

There were significant differences in *Cymodocea nodosa* populations that grow under different degree of anthropogenic stress in the measured morphological, physiological and biochemical parameters (Table 39). Leaves from the highly stressed environment of Nea Karvali were 1.3 times wider and 2.3 times longer than those from Brasidas, they had significantly more N and P assimilated in their leaves and had higher photosynthetic efficiency. All measured parameters had significant variation between a hot (June- July) and cold (January- March) period, highlighting the importance of seasonal variation for this species.

Biological organization level	**Parameter**		**Meadow**	**Period**	**Meadow*Period**	**Weights**	**Level of higher variance**
Physiological level	**ΔF/Fm'**		X	X	X	Power	Period
	Fv/Fm			X	X	Power	Period
	Fm		X	X	X	Power	Meadow
	Chl *a*			X		Exp	Period
	Leaves	**N**	X	X		Power	Meadow
		C		X	X	No	Period
		C/N	X		X	Exp	Meadow
	Roots	**N**		X		ConstPower	Period
		C				Power	Period
		C/N		X		ConstPower	Period
	Stems	**N**		X	X	ConstPower	Period
		C				Power	Period
		C/N		X		ConstPower	Period
	P in leaves		X			Exp	Meadow
Population	**Length**		X	X	X	No	Meadow
	Width			X	X	No	Period
	Number of leaves/ shoot			X	X	Power	Period

Table 39. Results of all mixed model analysis between two meadows, two periods and their interaction. Weights are the weight factor used for the construction of each model using R, while the level of the analysis with the higher component of variance was calculated using the "lmer" function and are presented in the last column. "X" indicates that p statistic was lower than 0.05.

Variable	Hot				Cold			
	Brasidas		Nea Karvali		Brasidas		Nea Karvali	
	Mean	S.E.	Mean	S.E.	Mean	S.E.	Mean	S.E.
ΔF/Fm'	0.766	0.001	0.773	0.001	0.674	0.005	0.720	0.003
Fm	239.622	6.888	388.049	6.348	128.955	10.727	335.833	17.263
Chl-a (μg/g)	0.254	0.027	0.341	0.015	0.642	0.026	0.633	0.050
Length (cm)	11.371	0.160	29.445	0.428	8.424	0.347	20.574	1.022
% N in Leaves	1.323	0.069	2.412	0.160	1.739	0.045	3.262	0.193
P in Leaves (mg/Kg)					1470.000	77.615	2371.143	111.760

Table 40. Mean values ± Standard Error of measured parameters with statistical significant differences between the two studied meadows of Nea Karvali and Brasidas and the two sampling seasons, hot (June- July) and cold (January- March).

Differences between the two meadows

Effective quantum yield and maximum fluorescence were sensitive to anthropogenic stress (Tables 7 and 12). They both had significantly higher values in Nea Karvali meadow, a difference that became more apparent during the cold period (Figure 23). Though the two studied meadows had different nutrient regimes, caution is needed when trying to interpret PAM parameters, since most of them are influenced by multiple factors with no existing knowledge on their combined effect on seagrass physiology (Gera et al., 2012).

There is evidence that high nutrient concentrations can lead to increased photosynthetic efficiency in seagrasses (Agawin et al., 1996; Lee et al., 1999a), however short time laboratory experiments from chapter 4 indicated that this is not the case with *C. nodosa* as no significance response was noted. Increased nutrient availability often leads to increased biomass of phytoplankton as well as seaweeds, that in turn have an impact on seagrass meadows by reducing the available light (Ralph et al., 2007). Under these low light conditions, that were also met in Nea Karvali meadow (ca. 66% of reference site irradiance at 3 m depth; unpublished data), seagrasses have been found to increase ΔF/Fm’, Fv/Fm and Fm values while lowering them under high light stress (Dawson et al., 1996; Ralph et al., 1998b; Longstaff et al., 1999b; Enriquez et al., 2002; Major et al., 2002; Durako et al., 2002 ; Ralph et al., 2005).

Seagrass growth is highly related to the quantity and quality of light that they receive (Zimmerman et al., 2006). In general, due to their high biomass per unit area they have high minimum light requirements and are thus very sensitive to light reductions (Dennison et al., 1993). In order to adapt to low light they need to increase their photosynthetic efficiency, so that they can capture more photons and sustain growth, a mechanism reflected by the increased ΔF/Fm'. In order for a plant to increase its photosynthetic efficiency it must reduce the minimum quantum requirements for photosynthesis ($1/\Phi_{max}$) to approach the theoretical minimum of 8 photons (Govindjee, 1999) and enhance its leaf absorptance (Ralph et al., 2007), usually by increasing the chlorophyll content (Longstaff et al., 1999a).

High light irradiance is known to cause damage to PSII, mainly by damaging the D1 protein, which lowers ΔF/Fm' (Flanigan et al., 1996). Ralph et. al. (1995) showed that a decline of Fm is associated to the amount of reaction centers closed due to high light stress. Lower Fm values in plants from Brasidas meadow are indicative of chronic exposure to higher light conditions than Nea Karvali and that they trade photosynthetic efficiency for photoprotection. Such a mechanism has also been reported for tropical seagrasses (Campbell et al., 2003). Since water depth was similar between the sampled areas, changes in PAR measured reflected the degree of suspended matter (both organic and inorganic) that was in turn related to the anthropogenic gradient (nutrients and effluents).

Chlorophyll *a* had indeed higher mean values in Nea Karvali than in Brasidas meadow but only during the hot period (Table 22), and it still wasn't significant. Even though increased chlorophyll *a* content has been related to reduced light intensities for some species (Ferrat et al., 2003), itsn't a general reaction of seagrasses. For example Cerny et.al. (1995) found that after an 87-90% decrease in surface irradiance *Halophila wrightii* had higher chlorophyll concentrations, while *Thalassia testudinum* didn't change its chlorophyll content at all.

Increasing chlorophyll content under low light isn't preferred by most seagrass species since previous studies proved that large variations of chlorophyll content in leaves only resulted in relatively small variations on leaf absorbance that were still not enough to withstand low light stress (Enriquez et al., 1992; Cummings et al., 2003; Enríquez, 2005). This phenomenon has been ascribed to the package effect, namely the self-shading among thylakoid membranes, chloroplasts and cell layers that doesn't allow all pigments to contribute in the light harvesting process (Major et al., 2002).

Physiological responses to light limitation are always faster to occur after a stress incident than morphological, however, morphological changes always follow to complete the first in order for the plant to fully adapt (Abal et al., 1994). Various morphological adaptations to light limitation have been published, with some species increasing their leaf length and width in order to increase the light capturing area (Longstaff et al., 1999a), and others decreasing leaf size in order to reduce the respiratory demand (Campbell et al., 2002).

Leaf length was significantly higher in Nea Karvali meadow in this study a result that has been also indicated earlier (Orfanidis et al. 2010). Leaf elongation could be attributed to the plants effort to increase the photosynthetic area in order to capture more photons and increase its photosynthetic efficiency. Low shoot density in the same meadow (unpublished data) in combination with longer leaves also counters the auto shade effect within the meadow, allowing light to penetrate through the patches and to be used uniformly by all leaves for photosynthesis. Moreover, longer leaves means increased canopy height, which in turn allows the plants to reach closer to the surface and receive higher light intensities. Shoots from Brasidas on the other hand, couldn’t afford increased leaf growth, both because of low nutrient concentrations, as well as high light intensities that require even more nutrients for continual photosynthesis. The same phenological adaptations to low nutrient environment was found by Short (1983) for *Zostera marina*.

Leaf elongation is also attributed to higher nutrient concentrations in Nea Karvali. Most seagrasses under moderate nutrient concentrations have increased leaf growth (Agawin et al., 1996; Ferdie et al., 2004), canopy height (Bulthuis et al., 1992) and cover rate (Kenworthy et al., 1992), however decreases of shoot size due to high nutrient concentrations have also been reported (Leoni et al., 2006). Nutrient and light are two strongly related parameters, since increase nutrients allow the plant to grow faster and further, while increased leaf growth and coverage create a more efficient light harvesting mechanism that can sustain constant nutrient assimilation. When nutrients are abundant, the plant shifts from being nutrient to light limited (Alcoverro et al., 2001a).

The use of leaf length as a bioindicator of an ecosystems health needs caution as other factors could be responsible for increased shoot size, such as hydrodynamism or genetic adaptations. Indeed, *C. nodosa* with large shoots has been reported from other sites in the Aegean Sea that are considered pristine with no anthropogenic impacts. While leaf length by itself needs caution at its use as a bioindicator, Orfanidis et. al. (2009b) used the skewness index on the ln transformed relative frequencies of length values and created the Cymoskew indicators that successfully used the morphological features of *C. nodosa* to characterize the water quality of a coastal area. The index identified light as the main factor affecting the population.

Leaf length distribution may also increase with reduced light (Orfanidis et al. 2010). Increased turbidity leading to reduced light levels may result in increased seagrass competition for light, thus increasing size distribution asymmetry of the plant canopy (Schwinning et al., 1998), i.e. larger individuals obtain a disproportionate share of light because they shade smaller ones (a pre-emptable resource). As a result they may show plastic growth responses, i.e. increase in length growth to enhance the amount of captured light and to decrease shading (Weiner et al., 1992).

Increase of leaf width and decrease of number of leaves per shoot has been also reported as an adaptive respond to low light conditions (Ralph et al., 2007). A basic trend with wider leaves in Nea Karvali meadow can be seen in Figure 30, however no statistically significant difference was located. Leaves in this meadow were 2.3 times longer and this significant increase in length could be more than enough in order for the plant to increase its photosynthetic area. By investing nutrients and energy to lengthening its leaves, the plant succeeds at increasing leaf canopy at the same time.

Ruiz et. al. (2001) suggested that number of leaves per shoot declines with low light availability as a means to counter the self shading effect and allow light to penetrate the canopy. Such a response could indeed make a difference in seagrasses that form dense meadows, such as *P. oceanica*. However this reaction wasn’t witnessed in *C. nodosa* meadows and both meadows had similar number of leaves per shoot, since patches are already relatively sparse and longer leaves in Nea Karvali already minimized the self-shading effect. A steady number of leaves per shoot between different sites of *C. nodosa* was recorded by Cancemi et. al. (2002) as well, implying that for *C. nodosa* this parameter isn’t well correlated to environmental factor, whilst its a species specific feature.

Another response to high nutrient concentrations that was also observed for *C. nodosa* in this study, is increased nutrient tissue content (Burkholder et al., 2007). Both N and P in leaf tissue were significantly higher in Nea Karvali, reflecting the increased concentrations of both nutrients in that meadow. Since seagrasses lack an uptake feedback mechanism an increase in nutrient availability increases nutrient acquisition, a response that has been documented for both nitrogen and phosphorus (Perez-Llorens et al., 1995; Pedersen et al., 1997). N content was measured in different parts (leaves, stems and rhizomes), however it was significantly higher in Nea Karvali only in leaves (Table 25). Leaves are the preferred uptake route for nutrients when they are available in the water column (Romero et al., 2006), while Kraemer et. al. (1999) found that N is first assimilated in leaves and then moves to other tissues.

Lower N and P content in leaves at Brasidas meadow, reflected the low nutrient concentrations in the water column, as well as the high light conditions that have a high demand on nutrients to sustain photosynthesis, and often lead to internal nutrient supply depletion (Romero et al., 2006). Even though nutrient concentrations in Brasidas meadow didn’t imply limitation, C/N values portrayed increased usage of nutrients for photosynthesis.

Carbon content is fairly constant between seagrass species (Duarte, 1990), but fluctuations that have been linked to anthropogenic stress have been documented for some species. For example Ruiz et. al. (2001) showed that *P. oceanica* had 60% less carbohydrates in tissues under moderate shading for 90 days, while Touchette (2002) showed how nutrient enrichment caused a decrease in C content in *Z. marina*. No such differences were found in this study between the two meadows that had similar C content in all tissues (Tables 25, 28 and 31).

Significant differences were found between C/N ratio in leaves (Table 25). C/N ratio is a function of nutrient availability as well as light, since high photosynthetic rates that occur under high light lead to depletion of nutrient supplies that are in turn depended in nutrient availability in the environment (Grice et al., 1996). Nea Karvali had significantly lower C/N leaf content than Brasidas. However, since C content didn’t vary significant between the two meadows, this variability is explained by the N stored in tissues and not by the light regime.

Differences between the two periods

Seagrasses are highly influenced by seasonality in numerous ways, such as growth rates, photosynthetic rates, nutrient assimilation etc. (Vermaat et al., 1996; Sfriso et al., 1998). All measured parameters had significant variation between the two periods, except P content in leaves, C content in roots and stems and C/N in leaves.

Light and temperature are considered key factors controlling photosynthesis and growth of seagrasses (Drew, 1978). It's not always easy to separate these two parameters, however, most studied seagrasses were able to quickly adapt their photosynthetic rate and efficiency between changing light intensities, thus leading many researchers to the conclusion that temperature is the primary factor controlling photosynthesis (Dawes et al., 1980; Phillips et al., 1983; Bulthuis, 1987; Lee et al., 1996) because of its significant effect on the biochemical process involved in photosynthesis and tissue metabolism (Bulthuis, 1987).

Seasonal variation of photosynthesis with suppressed values during winter months has been reported for *C. nodosa* in previous studies, using P-I curves (Drew, 1978; Perez et al., 1992; Enriquez et al., 2004). In general, as temperature increases so does photosynthesis and in particular the rate of net photosynthesis at light saturation (Pmax), the respiration rate and the compensation irradiance (Ic) (Bulthuis, 1987).

In this study all PAM parameters measured (ΔF/Fm', Fv/Fm, Fm) were significantly lower during the cold period (January-March) than in the hot (June-July), suggesting thermally induced photoinhibition. Low Fm and Fv/Fm values indicated the closure of PSII reaction centers and chloroplast damage and dysfunction as a result to the inactivation of temperature dependent enzymes (Havaux, 1994). As a result PSII wasn't as efficient in capturing and using photons during these months, thus the lower ΔF/Fm' values measured. One mechanism to counter this deficiency was increased chlorophyll a content measured in leaves, in an effort to exploit more efficiently the light captured.

In laboratory experimentation Ralph (1998) found that even though moderate increase of temperature didn’t affect significantly fluorescence parameters, a decrease had a significantly negative effect on ΔF/Fm’, Fv/Fm and Fm of *Halophila ovalis*. The result was linked to chronic inhibition of photosynthesis since both Fo and Fm were completely quenched during extreme low temperatures and could also explain the low values measured during cold temperatures.

Morphological parameters all had higher values during the hot period (Figure 28). These findings are consistent with previous studies on *C. nodosa* (Perez et al., 1992; Terrados et al., 1992; Perez et al., 1994a; Vidondo et al., 1997; Cancemi et al., 2002). Longer and wider leaves, as well as higher number of leaves per shoot reflect the increased growth and photosynthetic rate that is generally measured during summer and is mainly related to increased temperature and irradiance (Terrados et al., 1992). At that time, increased photosynthesis allows the utilization of available and stored nutrients in order to produce new leaves and rhizomes in order to increase coverage.

Increased photosynthetic and growth rates during the hot period leads to the depletion of stored nutrients in plant tissues and as a result nitrogen content measured in all tissues was lower at that time (Figure 33). This reaction reflected the seasonal variation of nutrient concentration in the environment. High nutrient availability during winter and early spring, resulted in higher tissue N content. However, with the coming of summer and the increasing temperature, all nutrients from the water column were consumed by faster growing macrophytes and phytoplanktonic organisms, so the plant resolved to stored N in tissues for photosynthesis, that were significantly lower in the leaf compartment. This procedure can be witnessed in most seagrass ecosystems (Lee et al., 2007a).

Carbon content was lower during the cold period (Figure 33), as it has been documented for most seagrass species (Mateo et al., 2006). As in *P. oceanica* (Alcoverro et al., 2001b) an asynchrony was observed in *C. nodosa* between carbon fixation and carbon use. During summer months when there is more light than winter, C content wasn't spent in photosynthesis and was higher, due to lower N concentrations in the environment that couldn't back up high photosynthetic rates. On the other hand, during winter months when nutrients were ample, the plant couldn't fully utilize them to produce organic matter due to low light and temperature. A common strategy of seagrasses is to store C during cold months, and as soon as temperature and light become optimal, usually at late spring- early summer to raise their growth rates and invest in leaf elongation, density and biomass increases.

Most species store excess C in belowground tissues, such as rhizomes and roots, in order for it to be protected from grazing and extreme weather conditions (Touchette et al., 2000a). However this was not the case with *C. nodosa* since significant difference between the two periods existed only between carbon stored in leaf tissues, meaning that the species stores C in leaves preferably. Since N content was lower during the hot period and C higher a decrease of C/N in winter was also noted (Table 25).

Suitable parameters for bioindicators

Since seagrasses were acknowledged as key ecosystem components, highly sensitive to anthropogenic impact, several European [Water Framework Directive (WFD, 2000/60/EC)] and international (United States Environmental Protection Agency, 2013) policies have encompassed their use as indicators of ecological quality. The use of PAM fluorometry as an indicator of stress has been widely used to this end, however most studies have focused on responses to single stressors such as heavy metals, temperature, light intensity and salinity, in controlled conditions (Ralph et al., 1995; Ralph, 1998; Ralph et al., 1998a; Ralph, 1999; Macinnis-Ng et al., 2002; Campbell et al., 2003; Macinnis-Ng et al., 2003; Macinnis-Ng et al., 2004a; Macinnis-Ng et al., 2004c; Malta et al., 2006; Bite et al., 2007; Pages et al., 2010).

A key finding of this study is the potential use of PAM fluorometry to identify the overall stress on a population. More specifically, effective quantum yield (ΔF/Fm’) and maximum fluorescence (Fm) were negatively correlated with anthropogenic stress. Nea Karvali, an area that is subjected to many sources of pollution, mainly naming the Phosphoric Fertilizer Industry (PFI), effluents of Kavala City waste water treatment plant and the Kavala Oil land facilities, as well as the agricultural land uses, had higher ΔF/Fm’ values, than Brasidas, a relatively non-impacted site.

The changes of these two parameters were attributed to differences in light availability and intensity between the two meadows. While Brasidas is characterized by clear water (light attenuation coefficient=0.174; un-published data), that allows high intensities to reach the meadow, Nea Karvali (light attenuation coefficient=0.31; un-published data) is the opposite due to high algae biomass in the water column that is sustained by increased nutrient effluents, as well as due to suspended materials.

When trying to interpret chlorophyll fluorescence parameters, special care is needed as they are highly affected by recent history of local factors. However, in this study, measurements were taken prior to acclimatization of the samples to laboratory conditions, at a steady intermediate for the local conditions temperature (21^{o}C). Also, much data was analyzed in order to produce these results [n=180 – 400 per site (cold-hot period) in contrast to previous similar efforts (i.e. n=4 per site in Gera et. al., 2012)], so variability of the parameter was limited. Still, from the two parameters, ΔF/Fm’ showed higher variability between the two sampling periods and secondly between quadrats (Table 9). Fm on the other hand proved to be a more reliable parameter, since its variability was located mainly among the stress gradient (Table 9) and therefore we suggest that is better suitable as a bioindicator.

Although studies focused on specific natural and toxic stressors conclude that seagrasses respond by a decrease in both ΔF/Fm’ and Fm under high stress, we found that in the field they both tend to increase with increasing anthropogenic pressures. In one of the very few similar efforts so far, measuring photosynthetic responses of *P. oceanica* across an anthropogenic stress, Gera et. al. (2012) reached to a similar surprising conclusion. In their study, Fm and Fv/Fm were consistently responsive to differences between locations in pressures, yielding higher values in sites of high anthropogenic impact. This contradiction can be attributed to the fact that the anthropogenic gradient between Brasidas and Nea Karvali wasn’t caused by a single well-identified stressor, or that a single stressor had a significant effect on the plants photosynthesis masking all others and causing an increase of photosynthetic efficiency. As in most cases in the field, a meadow experiences stress due to a number of concurrent influences, such as nutrients, heavy metals, water transparency, the combined action of which we don’t yet fully understand. The synergistic or antagonistic effect of these parameters needs to be further studied as to be able to explain these controversial results. At the same time, the effect of these factors alone needs to be tested on *C. nodosa* from these sites in order to distinguish the effect and its intensity on photosynthesis.

Leaf length was significantly different between the two meadows, with Nea Karvali having much longer leaves (Table 15). Morphological responses of some seagrass species have been studied for major stress factors, such as light availability and intensity (van Lent et al., 1995; Longstaff et al., 1999a; Ruiz et al., 2001), nutrient concentrations (Orth, 1977; Leoni et al., 2006) and heavy metals (Marin-Guirao et al., 2005). However, their response under multiple stressors that reflect the environmental conditions has but barely been tested. Lee et. al. (2004) in such an effort found that leaf length of *Z. marina* varied only with depth and not along an anthropogenic gradient, while the ratio of leaf mass (mg dry weight cm^{-2}) to leaf nitrogen content was strongly correlated to it, in three sites in the United Kingdom.

In this study leaf length responded with an increase to the gradient and the parameter’s variability was located between meadows (Table 16). Even so, we should be careful in its use as a bioindicator, as the phenotypic plasticity and genetic adaptations of seagrasses isn’t yet fully understood, and it could play an important role as to why *C. nodosa* populations have so diverse morphological features. Previous studies have focused on the evolutionary path, disperse and the importance of sexual reproduction of seagrasses (Alberte et al., 1994; Waycott, 1998; Alberto et al., 2005; Procaccini et al., 2007; Alberto et al., 2008), however a significant effect of genetic variability to certain responses of seagrasses to stressors has been identified, without a clear insight on the underlying mechanisms (Waycott et al., 2006). A further genetic study is needed in order to test whether genetic diversity of stress factors is responsible for morphological variations.

The last sensitive parameter to the anthropogenic gradient was nutrient content in leaves. Both N and P were significantly higher in leaves from Nea Karvali meadow (Table 24 and 33), reflecting the higher nutrient concentrations in both the water column and sediment (Sylaios et al., 2005; Papastergios et al., 2011). Higher nutrient tissue content in sites with high nutrient concentrations has been found for many seagrass species from different biogeographic regions (Perez et al., 1994a; Ceccherelli et al., 1997; Udy et al., 1997a; Lee et al., 2004; Mvungi et al., 2012), implying that its a common response to increased nutrient availability. Since they lack a feedback mechanism for nutrient uptake (Duarte, 1990), they keep absorbing available nutrients from their leaves and roots. As a result they increase photosynthetic and growth rates, while at the same time they store excess concentrations in compartments for future use (Burkholder et al., 2007).

Seagrasses can uptake nutrients from either roots or leaves, however when its possible they prefer the second (Romero et al., 2006). Such a preference hasn’t been found for the storage compartments of nutrients, however in the populations studied here leaves were the preferable tissue, as they had higher N concentrations (Figure 33) than roots or stems. A similar behavior of *C. nodosa* was witnessed in populations from the northeastern coast of Spain by Perez et. al. (1994a). In the same research they also found that shoot size also increased with increasing nutrient availability.

The use of leaf N content as an indicator of anthropogenic nutrient inputs has been suggested for *C. nodosa* (Oliva et al., 2011) as well as *P. oceanica* (Romero et al., 2007). However, in some previous studies, even though a correlation between N content and N concentration was established its use as an indicator was discarded due to high variability in small scales (Lee et al., 2004; Martinez-Crego et al., 2008). This wasn’t the case in the populations studied in N. Aegean Sea, as there was no variability of the parameter between sites of areas (Table 26). Even though C/N was significantly lower to the anthropogenic stress, the variation was caused by differences in the N content and couldn’t be linked to the stress gradient.

Brasidas meadow as a reference site

The morphology and physiology of plants from Brasidas meadow were also compared with those from Thasos meadow, in order to test whether Brasidas population had been affected by the increasing anthropogenic activity in the broader area. Nutrient analysis of the water column showed that Brasidas and Thasos had similar concentrations of macronutrients, which were much lower than those found at Nea Karvali (Table 5). The results presented in Table 37 show that plants from the two meadows did not differ significantly in terms of their morphology, physiology and biochemistry.

One of the main concerns in Brasidas meadow was the observed shift in local patches from deeper to shallower depths (from 2-3m to 1-2). In order to examine how such a change might have affected the interpretation of the results in this study, a third site (Site E), close to Brasidas , where *C. nodosa* was found deeper, was sampled. As shown in Table 36, results obtained from site E did not differ significantly from the other sites (A and B) within Brasidas meadow, and we concluded that small changes in depth distribution didn’t alter the comparisons between Brasidas and Nea Karvali, while concerns that the Brasidas population had started to show signs of anthropogenic stress were dismissed.

CONCLUSIONS

The rising concern about seagrass meadows and their conservation, as well as their sensitive nature to environmental conditions has led many scientists to use them as bioindicators of anthropogenic stress or environmental status in general in order to produce widely applied, easy and cheap monitoring protocols (Marba et al. 2012). However, this effort has proved difficult. The wide distribution of seagrasses to cover different type of habitats, their high phenotypic plasticity as well as their genetic diversity has been the main obstacles, since results obtained from one species in a specific region don’t always apply for other species (Lee et al., 2007a).

Seagrasses are sensitive to natural stress and *C. nodosa* is no exception. Almost all parameters studied (except C/N content in leaves and C content in stems) were significantly impacted by natural stress during cold months (January- March), mainly the low light and temperature and the increased nutrient concentrations. In order to distinguish anthropogenic and natural stress, measurement should be better taken during summer months, when the plant has made use of all environmental factors to its best use.

The variability of seagrass responses among species and regions has highlighted the need to study these responses in the field, covering different environmental conditions. This study has come to add to this goal by describing two populations of *C. nodosa* in the N. Aegean Sea, one that receives serious anthropogenic inputs and one non-impacted. From all described parameters, five were able to identify the anthropogenic gradient and could be good indicators of stress. These were ΔF/Fm', Fm, Leaf length and N and P content in leaves.

The idea that PAM can be used as a bioindicator of ecological quality is a very attractive one, since it's application is quick, easy and handles results immediately (Beer et al., 1998). Many approaches have been proposed on how this goal could be achieved. The most appealing one is direct measurements of the effective quantum yield and the maximum quantum yield (Horn, 2006). The use of Rapid Light Curves has also been proposed (Ralph et al., 2005) as indicators of seagrass photosynthetic activity, however they don't seem to provide satisfying data due to considerable small-scale variability (Gera et al., 2012). Concerning *C. nodosa* meadows in the North Aegean Sea, ΔF/Fm' and Fm were linked to the stress gradient and could be used as bioindicators. One of the reasons for this positive result is the high amount of measurements taken in contrast to previous studies and the hierarchical nested sampling design that allowed for within meadow variability to be identified.

In conclusion, PAM fluorescence parameters, and nutrient leaf content could be used together in order to monitor the ecological health of a meadow. This approach isn't able to identify the underlying stress sources, however it provides an overall picture of the meadows condition based on a fast and easy methodology, that could be followed by secondary indicators that can identify specific stressors, if the need arises.

Chapter 4: Experimental response of *Cymodocea nodosa* to nutrients, light and copper

SUMMARY

Excess of nutrients, heavy metal contamination and light limitation are the most common anthropogenic stressors in coastal waters worldwide and their effects on numerous seagrass species has been studied in different regions. In this study, the effect of three stressors (nutrients-N, P, copper and irradiance) was investigated on *Cymodocea nodosa* shoots, collected from three differently impacted meadows in the N. Aegean Sea. Single shoots were exposed to stressors and monitored for 8 days. The effective quantum yield (ΔF/Fm’), an indicator of photosynthetic efficiency, was measured every second day. Experiments were carried out with samples from two pristine meadows (Thasos, Brasidas) and one under significant anthropogenic stress (Nea Karvali), so that the role of the plants’ acclimation history on a stress incident could be studied. Results showed that *C. nodosa* was highly tolerant to copper enrichment, with only high copper concentrations having a significant ($p<0.05$) negative effect on ΔF/Fm’. Shoots from the undisturbed meadows showed increased tolerance to copper in comparison to shoots from a degraded area. High nutrient concentrations increased ΔF/Fm’ values but showed significant differences only in shoots collected from the pristine meadow. Light had a significant effect on photosynthesis, with higher ΔF/Fm’ values under low light conditions and it was identified as the main pathway through which eutrophication leads to a meadow’s decline.

INTRODUCTION

Seagrasses are a unique group of plants that have adapted to submersion in seawater. They evolved through a single monocotyledonous lineage through three independent lineages (Hydrocharitaceae, Cymodoceaceae and Zosteraceae) between 70 and 100 million years ago (Les et al., 1997). Since then they have successfully managed to colonize all but the polar seas, thriving in a variety of climatic conditions. They form meadows with high biodiversity and productivity (Hemminga et al., 2000), that act as sources of carbon (Suchanek et al., 1985) and nursery grounds for other marine biota (Heck et al., 2003). In addition, they trap particles from the water column while at the same time stabilizing the sediment (Fonseca, 1989) and they influence the hydrodynamic regime (Fonseca et al., 1992).

Seagrass meadows are sensitive ecosystems that show variations on a seasonal and spatial basis (Boudouresque et al., 2009). Natural occurring changes in seagrass distribution have been recorded and attributed to storms, creation of gaps during winter storms, disease and grazing by herbivores (Walker et al., 2006). The impact of human population growth on coastal environments has introduced new sources of environmental stressors for seagrass beds that have led to the complete loss of meadows in several cases (Orth et al., 2006). Anthropogenic stress can be linked to a variety of processes, but the ones most affecting seagrasses are those that alter the water quality and clarity such as nutrient and sediment loading from runoff and sewage disposal, dredging, chemical pollution, upland development, and certain fishing practices (Short et al., 1996.).

In order to develop plans for the conservation of these valuable ecosystems, a clear understanding of the response mechanisms to anthropogenic stressors needs to be established. Research so far has shown that seagrasses are moderately tolerant to anthropogenic contamination with recovery being either slow and taking from decades to centuries (Kirkman et al., 1990; Meehan et al., 2000), or relatively fast taking from 2 to 3 decades (Kendrick et al., 1999; Kendrick et al., 2000). However, there is still a lot to be learned on the impacts of environmental stressors on seagrasses and how they recover from such events.

Towards this end, many studies have focused on the effect of different stressors on specific seagrass species, with varying results. A problem occurs when trying to extrapolate these findings to general seagrass ecology, as Romero et. al. (2006) pointed out, since different responses have been observed not only between different species but also within the same species in different biogeographical regions. Moreover, little is known about the interactive effects of anthropogenic stressors and natural factors (temperature, salinity etc.); do they act synergistically, enhancing mortality, or does one nullify the other?

The need to identify the tolerance limits of different species in different regions will help construct management plans and predict future incidents and distribution patterns, as well as allowing us to fully interpret results from field studies. In this research the effects of three major stressors, nutrients (N and P), irradiance and metals (Cu) were investigated on *Cymodocea nodosa* that grows in the N. Aegean Sea, through a series of experiments. At the same time the effect of the acclimation history of shoots on their response to stressors was tested.

Nutrients

Nutrients play an important role in a plant’s life, promoting growth, and providing energy. Equation 1 has been often used to demonstrate the importance of nutrients, and especially that of carbon dioxide, nitrate and phosphate, in the creation of organic matter.

$$435CO_2+395H_2O+20NO_3^-+PO_4^{3-}\rightarrow C_{435}H_{790}O_{305}N_{20}P+522O_2 \quad (1)$$

Seagrasses have the ability to take up nutrients through roots and leaves, from both water column and interstitial spaces of sediment, a mechanism that allows them to thrive in low nutrient environments (Lee et al., 1999b). Nutrient uptake is achieved with the utilization of membrane pumps (Grossman et al., 2001), with a variety of plasma membrane transporters, such as H^+ATPases and ABC transport proteins.

Seagrasses have a preference for ammonium (NH^+_4), since its uptake is less demanding of energy compared with e.g. nitrate that needs to be transformed to ammonium before assimilation, a process of high energy demand (Lepoint et al., 2002). Nitrate is regarded as “new” nitrogen in the ecosystem, as its most often linked to anthropogenic sources (Touchette et al., 2000b). Phosphate is present in much lower concentrations (0-0.4μM) because it’s easily absorbed to particles, but is still the main source of P for seagrasses (Lee et al., 2007a). While there are a number of other elements (e.g. Fe, Mo, Mn, and Cu), required in small concentrations for seagrass metabolism, its the availability of N and P in the water column and sediment pore water, and the balance between these two, that mostly determines seagrass growth (Udy et al., 1999).

While only a few species have been tested, differences in the responses of seagrasses to nutrients have been reported from both *in situ* (Ceccherelli et al., 1997; Udy et al., 1997a; Udy et al., 1999) and laboratory-based studies (Ceccherelli et al., 1997; Udy et al., 1997a; Udy et al., 1999; Lee et al., 2004). For example *Zostera marina*

Variation in response to nutrient enrichment (i.e. positive, negative and none) has been found between and within (between biogeographical regions) species (Ceccherelli et al., 1997; Udy et al., 1997a; Udy et al., 1999; Walker et al., 2004; Lee et al., 2007a). For instance *Thalassia testudinum* and *Syringodium filiforme* increased their shoot size with nutrient addition (Ferdie et al., 2004), when *Z. marina* decreased it (Short et al., 1995). The range of results obtained from these studies suggests that one can’t extrapolate findings to all species or regions.

Generally, low to medium nutrient enrichment (3.5-35μM) is known to lead to increases in biomass, productivity and shoot size (Udy et al., 1997a) although, absence or negative physiological and morphological response to nutrients have also been reported (Burkholder et al., 1992; Erftemeijer et al., 1994; Worm et al., 2000). Therefore its important to identify the effects that nutrient enrichment has on individual species within a specific region in order to be able to determine the responses of seagrass local populations to eutrophication. Towards this goal, shoots of *C. nodosa*, collected from Thasos Island (a pristine site) and Nea Karvali (a highly impacted area) during the summer, were exposed to low to high nutrient enrichment under laboratory conditions, while NO_3^- was the preferred form of N in order to study the effect of anthropogenic impact on the species.

Light

Light is a natural form of stress that through the day causes a direct response on seagrasses (Campbell et al., 2008). As irradiance rises from the early morning so does photosynthesis, reaching an optimal rate around noon. Seagrasses are shade-adapted plants, and by the afternoon when irradiance peaks, photoinhibition occurs, through the destruction of D1 protein in Photosystem II (PSII). In order to cope with light stress, seagrasses increase non photochemical quenching (NPQ) at the tips of their leaves, since the upper canopy receives the highest quantities of light (Zimmerman et al., 2006). NPQ refers to the process of returning singlet excited state chlorophylls (Chl) to the ground state through internal conversion. In this way they consume excess energy via molecular vibrations and utilize it as heat. Sharon and Beer (2007) also described another strategy that helps seagrasses to withstand high irradiance. They showed, using light and confocal microscopy, that chloroplasts tend to clump, in order to increase shading and thus lower direct irradiance (Sharon et al., 2008). However, prolonged exposure to high irradiance can be harmful, thus maximum growth rates are achieved at intermediate light irradiance (Lee et al., 2007a).

Low irradiance on the other hand, affects negatively seagrasses by reducing their growth and limiting their vertical distribution to deeper waters (Longstaff et al., 1999b). Abal et al. (1994) showed that if *Zostera capricorni* receives at its midday peak, less than 100-500 μmol photons $m^{-2}s^{-1}$, its respiration demand exceeds the rate of carbon fixation, resulting in reduced growth rates. When under low light conditions, seagrasses tend to maintain a large number of inactive PSII centers that can act as energy quenchers of trapped light energy. This mechanism leads to a reduction in effective quantum yield (Ralph, 1999) and allows them to withstand light deprivation for a certain period of time. Meadows under prolonged low light conditions will eventually decline and may even disappear (Orth et al., 2006).

Even though the correlation of nutrients and irradiance in the field has been studied by a few researchers (Leoni et al., 2008), the experimental correlation of these two factors under laboratory conditions hasn’t. Its important to isolate the two stressors in the laboratory in order to study how they affect PSII, without other abiotic factors confounding the results. As seen in the previous chapter nutrients and light are the most important stressors in the coast of Kavala Gulf. An experimental study on the interaction between these two factors was carried out using plants collected from meadows at Thasos and Nea Karvali.

Copper

Another major anthropogenic contaminant in estuaries and coastal waters is metals (Macinnis-Ng and Ralph 2002). The process of metal uptake by seagrasses, and their physiological and biochemical responses, has been previously described (Lyngby, Brix et al. 1982; Clijsters and Assche 1985). Results indicate that metals act on CO_2 fixation, thus affecting photosynthesis and, in particular, PSII (Prasad and Strzalka 1999). More recent studies have successfully made use of chlorophyll-*a* fluorescence to measure the efficiency of PSII under metal exposure (Ralph and Burchett 1998; Macinnis-Ng and Ralph 2002). The method has not previously been used for *Cymodocea nodosa*.

Copper (Cu) is an important trace nutrient for seagrasses, as its used in the synthesis of plastocyanins, proteins that play a role in transporting electrons from PSI to PSII (Macinnis-Ng et al., 2002), and is taken up actively (Ambo-Rappe et al., 2011). At high concentrations, Cu inhibits the enzymic activity that control pigment biosynthesis and leads to chlorophyll degradation, depletes iron or substitutes for magnesium in the chlorophyll atom (Prasad et al., 1999), alters the permeability of the thylakoid membrane and modifies the chloroplast ultrastructure (Ouzounidou, 1993). Since it inhibits electron transport at both donor and acceptor sites of PSII, exposure of shoots to copper should lead to a decrease in effective quantum yield.

The response of *C. nodosa* to copper exposure was studied by measuring chlorophyll-*a* fluorescence in shoots from three sampled meadows, Brasidas and Thasos that are considered to be pristine and Nea Karvali, a meadow that is exposed to anthropogenic-derived environmental stress. The aims of these experiments were to study the photosynthetic behavior of *C. nodosa* under a toxic event caused by heavy metal, specifically Cu since it’s known to be one of the most toxic heavy metals for seagrasses, as well as to determine how the three populations naturally growing under different nutrient conditions responded to metal exposure.

MATERIALS AND METHODS

Sample handling and study area. Samples were collected using SCUBA, from three meadows of known ecological status in the Kavala Gulf, N. Aegean Sea. Brasidas meadow is located in the inner part of Cape Brasidas on the Eleutheron Gulf and is one of the least impacted areas on the mainland of the Kavala Gulf. The closest town is that of Nea Peramos, with mainly touristic, fishing and port activities. Thasos island is at the eastern part of Kavala Gulf, approximately 10 km from the mainland. The island's main income comes from tourism, while the agricultural activities do not influence significantly the coastal ecological quality, because of the increased hydro-dynamism of the area. These two areas where chosen as reference sites, while a meadow at Nea Karvali was chosen as a highly impacted area. Nea Karvali is a small town to the east of Kavala city. A phosphorus fertilizer plant and a crude oil de-sulphurization complex exist, while a wastewater treatment facility for Kavala city and the agriculture in the surrounding area of Chrisoupolis further impacts the coastal zone.

All samples were collected during the summer, at ca. 10:00am, from 2-3m and transported, inside a portable cooler, to the laboratory within 30 minutes. A total number of 20 shoots was sampled in order to distinguish 12 healthy ones for experimentation. Once in the laboratory, shoots were separated, so that each shoot was attached to 2cm of rhizome and a single 1cm root and cleaned of epiphytes using a microscope slide. Samples were then left to acclimate fully submerged in plastic aquaria tanks with artificial sea water and no nutrient addition for one hour at 21^0C. The solution, Münster sea salt (Meersalz) was used for producing the artificial seawater at a salinity of 35psu. PAR measured at the bottom of the tanks was 67μmol photons*m^{-2}*s^{-1} and a 14h period of light and 10 of dark was set for all experiments.

Experiments were carried out in a control temperature (CT) room, maintained at 21^oC, for a period of eight days. Samples were placed in 1l plastic containers containing oxygenated artificial seawater of 35psu**.** Every container was covered using a watchglass, in order to prevent evaporation. Each treatment had six replicates. At the start of the experiment and on every second day the effective quantum yield (ΔF/Fm') was measured using the DIVING PAM by Walz, at the second leaf, 2cm above the stem. This protocol was kept steady since ΔF/Fm’ is known to vary along the leaf (Durako et al., 2002). Daily measurements were avoided, since plants, especially under high stress conditions, became very fragile and made handling difficult. The medium was renewed after the measurements were taken to avoid nutrient depletion with time.

At the start of the experiments a hole was produced with a needle at the basis of each shoot, just above the stem. At the end of the experiment the distance between the hole and the stem was measured using a ruler in order to gain information on leaf elongation for the experimentation time (Short, 1987). All experiments were conducted twice, once with shoots sampled from a reference and once from the impacted site, so that the effect of the shoot’s acclimation history on its response to certain stressors could be studied. All experiments couldn’t be run together due to lack of space, while leaf elongation wasn’t measured in the P enrichment experiment.

Experimental conditions: The hypothesis that light limitation, nutrient enrichment, and copper exposure affect photosynthetic efficiency of *C. nodosa* and leaf elongation was tested using the following three experimental set-ups.

1. *Effect of N and P enrichment*

The effect of different combinations of N and P concentrations was tested by exposing single shoots collected from Nea Karvali and Thasos to four nutrient regimes:

Solution A: 0.3μM N-NO_3^- 0.02μM P- PO_4^{3-}

Solution B: 10μM N-NO_3^- 0.5μM P-PO_4^{3-}

Solution C: 20μM N-NO_3^- 1μM P-PO_4^{3-}

Solution D: 30μM N-NO_3^- 2μM P-PO_4^{3-}

2. *Effect of* PO_4^{3-} *enrichment under different light conditions*

The effect of P-PO_4^{3-} load under two different light levels (37 and 65 μmol photons m^{-2} s^{-1}) was tested by exposing shoots from Nea Karvali and Thasos to one of four concentrations (0.02, 0.5, 1.0 and 2 μM) while maintaining N-NO_3 at a concentration of 30μM. PAR values given were measured at the bottom of water filled aquaria using a Li- COR Li-250 light meter.

3. *Effect of copper enrichment*

Shoots collected from all three meadows were exposed to 4 different concentrations of copper (0, 1.6, 4.7 and 7.9 μM) while maintain constant levels of N and P (0.3μM N-NO_3 and 0.02μM P-PO_4, respectively) reflecting a low nutrient environment of the study area.

Statistical analysis: Data analysis was conducted using the R environment, provided by the R Foundation**.** Leaf elongation was analyzed using a one-way ANOVA, while physiological responses with a repeated measures mixed model, using the “lme” function from the “nlme” package. In all cases ΔF/Fm’ was treated as the dependent variable, treatments (nutrient solution, phosphorus, copper, light) as fixed variables and day and replicate as random variables. Models were accepted only when they had significance difference from the null model.

The model used was lme(ΔF/Fm’~1+(*Cu or Nutrients or* PO_4^{3-}**Light*), random=~Day|Samples, control = lmeControl(maxIter = 10000, msMaxIter = 5000, niterEM = 500, msMaxEval = 500, msVerbose = TRUE, opt = "optim"), data=Data, method="ML") ->model. The weighting variable varExp was used in the analysis of Thasos data in both copper and phosphorus experiments and varPower in the analysis of data from Nea Karvali in the nutrient and phosphorus experiments.

Figure 37. Experimental set up of plastic containers with *Cymodocea nodosa* shoots, oxygenated and kept in a controlled temperature room.

RESULTS

Effect of N and P enrichment

Different concentrations of nutrients didn't affect ΔF/Fm' of *C. nodosa* shoots collected from Nea Karvali, after an eight day exposure period (Table 41), even though a small increase in mean values was observed by the end of the experiment (Figure 38). Low nutrient treatments experienced a decrease in ΔF/Fm' in the first two days, from 0.753±0.004 on day 0 to 0.709±0.022, only to rise again to 0.744±0.011 by day 8. Shoots grown in solutions B and C showed a small increase of effective quantum yield. In solution B the mean increased to 0.755±0.013 by day 8 from an initial value of 0.731±0.009, while the mean value of those in solution C increased to 0.771±0.002 from 0.728±0.012. A smaller increase was measured in shoots grown under treatment D from 0.754±0.006 in day 0 to 0.765±0.004 by day 8. However, a despite these trends, no significant differences were documented.

All nutrient treatments resulted in an initial increase of ΔF/Fm' in shoots from Thasos meadow (Figure 38), but this was followed by a decrease after the 2nd day in all treatments. Shoots grown in solutions A and B reached their minimum mean values by day 6 (0.760±0.002 and 0.784±0.004, respectively), while those in solutions C and D in day 8 (0.772±0.005 and 0.761±0.012 respectively).

Statistically significant difference was noted between solutions A and D for shoots from Thasos meadow (Table 41). High nutrient concentrations yielded higher ΔF/Fm' values ($p=0.021$). At the end of the experiment mean ΔF/Fm' of plants under low nutrients was 0.753±0.006, while under the high nutrient treatment 0.761±0.012.

Leaf elongation showed no significant variation between the different nutrient solutions (Table 42), even though an increasing trend can be seen in Figure 39. Leaves from Nea Karvali grew 1.33±0.41cm under the low nutrient solution and 2.11±0.54cm under the highest nutrient solution after 8 days. Leaves from Thasos meadow grew less in the same period; under low nutrients, elongation was 0.84±0.20cm, while under the highest nutrient solution it was 1.01±0.21cm. Shoots from Nea Karvali had a significantly different response to nutrient enrichment than those from Thasos, both in their leaf elongation and ΔF/Fm', as shown in the analysis of Table 43.

Meadow	Factor	Value	Std.Error	df	t-value	p-value
Nea Karvali	**Intercept**	0.746	0.006	96	116.385	<0.001*
	Solution B	0.007	0.008	20	0.979	0.339
	Solution C	0.004	0.008	20	0.529	0.602
	Solution D	0.013	0.007	20	1.868	0.077
Thasos	**Intercept**	0.758	0.004	96	186.787	<0.001*
	Solution B	0.000	0.006	20	-0.032	0.975
	Solution C	0.011	0.006	20	1.933	0.068
	Solution D	0.014	0.006	20	2.511	0.021

Table 41. Mixed model analysis of effective quantum yield (ΔF/Fm') measurements of *Cymodocea nodosa* leaves from two meadows in the N. Aegean Sea, after an eight day exposure in four nutrient solutions (Solution A: 0.3μM N-NO_3^- 0.02μM P-PO_4^{3-}, Solution B: 10μM N-NO_3^- 0.5μM P-PO_4^{3-}, Solution C: 20μM N-NO_3^- 1μM P-PO_4^{3-} and Solution D: 30μM N-NO_3^- 2μM P-PO_4^{3-}).* $p<0.001$

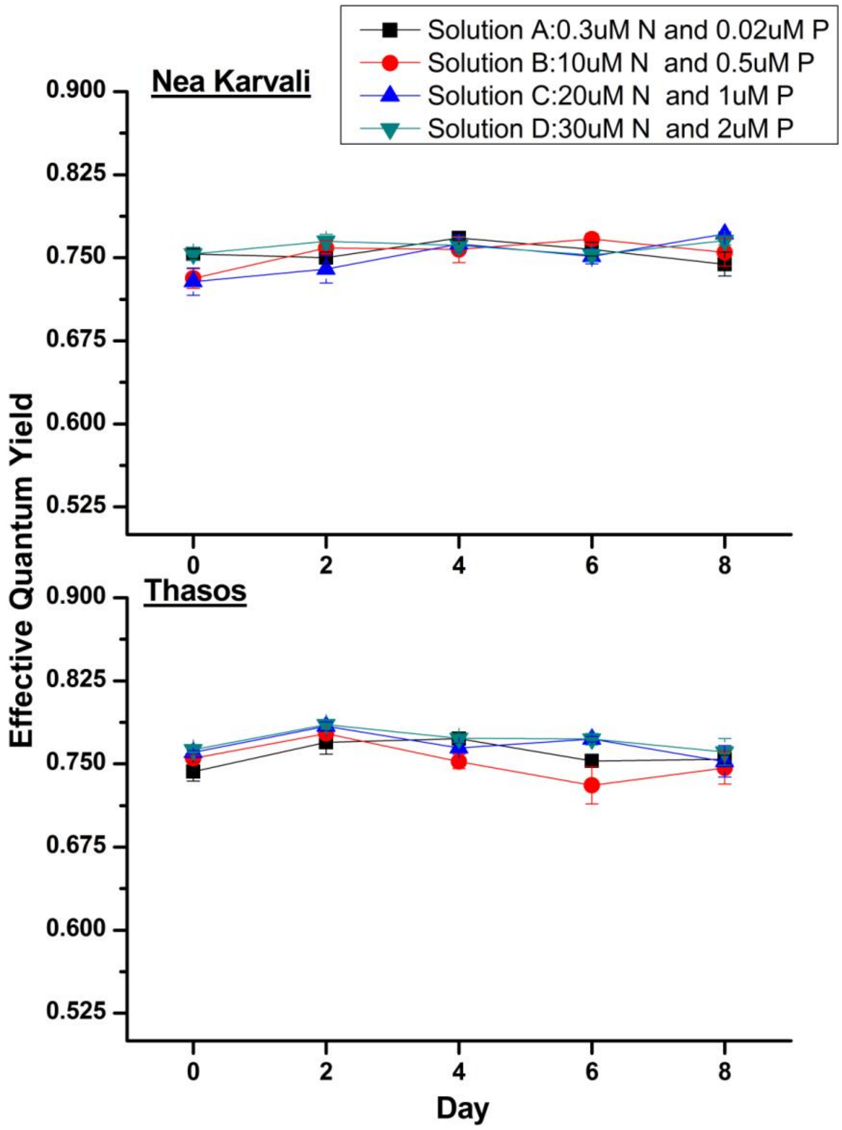

Figure 38. Mean values of effective quantum yield **(**ΔF/Fm' ± Standard Error; n=6) of *Cymodocea nodosa* shoots collected from two meadows, after an 8 day exposure to four nutrients solutions (Solution A: 0.3μM N-NO_3^- 0.02μM P-PO_4^{3-}, Solution B: 10μM N-NO_3^- 0.5μM P-PO_4^{3-}, Solution C: 20μM N-NO_3^- 1μM P-PO_4^{3-} and Solution D: 30μM N-NO_3^- 2μM P-PO_4^{3-}). Standard errors that are not visible in the graph were too small.

Meadow	F	p
Nea Karvali	0.478	0.700
Thasos	0.641	0.593

Table 42. One way ANOVA of *Cymodocea nodosa* leaf elongation from two meadows (Thasos and Nea Karvali) in the N. Aegean Sea, after an 8 day exposure to four nutrients solutions (Solution A: 0.3μM N-NO_3^- 0.02μM P-PO_4^{3-}, Solution B: 10μM N-NO_3^- 0.5μM P-PO_4^{3-}, Solution C: 20μM N-NO_3^- 1μM P-PO_4^{3-} and Solution D: 30μM N-NO_3^- 2μM P-PO_4^{3-}).

Parameter	*Factor*	Value	Std.Error	df	t-value	p-value
ΔF/Fm'	Intercept	0.753	0.002	192	308.407	0.000*
	Thasos	0.010	0.003	46	2.775	0.008
Leaf elongation	Intercept	1.755	0.169	137	10.36	0.000*
	Thasos	-0.785	0.22	137	-3.564	0.001

Table 43. Statistical comparison of *Cymodocea nodosa* leaf elongation and effective quantum yield after an 8 day exposure to four nutrients solutions (Solution A: 0.3μM N-NO_3^- 0.02μM P-PO_4^{3-}, Solution B: 10μM N-NO_3^- 0.5μM P-PO_4^{3-}, Solution C: 20μM N-NO_3^- 1μM P-PO_4^{3-} and Solution D: 30μM N-NO_3^- 2μM P-PO_4^{3-}) between two meadows, Nea Karvali and Thasos in the N. Aegean Sea. * p<0.001

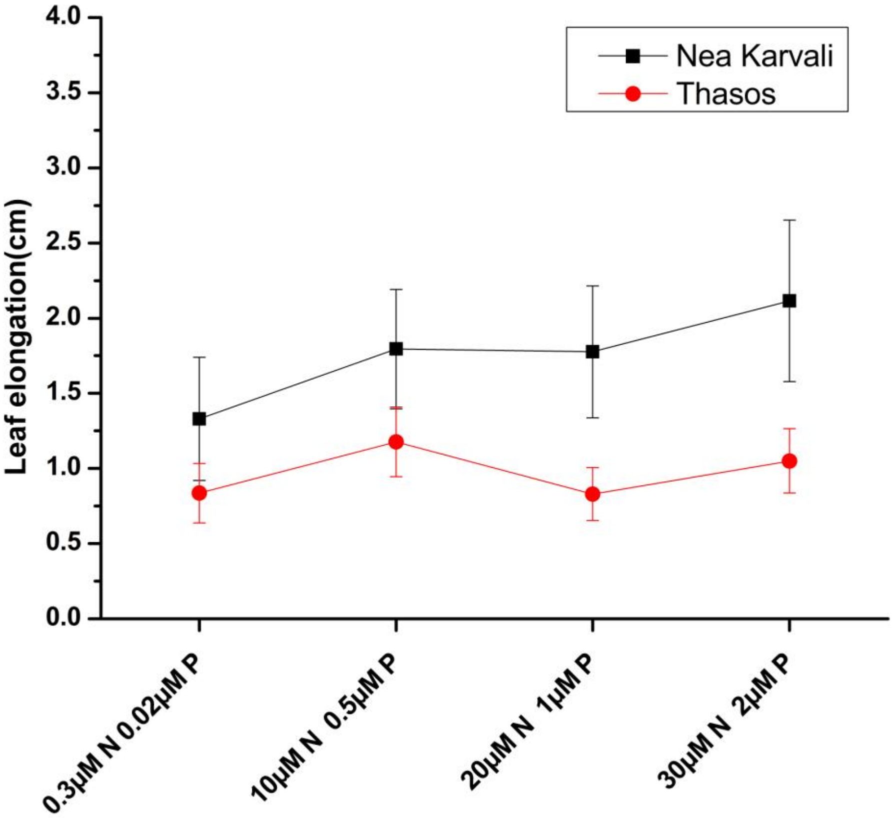

Figure 39. Mean values of leaf elongation (cm) ± standard error (n=6) of *Cymodocea nodosa* leaves after an 8 day exposure to four nutrients solutions (Solution A: 0.3μM N-NO_3^- 0.02μM P-PO_4^{3-}, Solution B: 10μM N-NO_3^- 0.5μM P-PO_4^{3-}, Solution C: 20μM N-NO_3^- 1μM P-PO_4^{3-} and Solution D: 30μM N-NO_3^- 2μM P-PO_4^{3-}) sampled from two meadows (Nea Karvali and Thasos) in the N. Aegean Sea.

Effect of P-PO_4^{3-} *exposure under different light conditions.*

There was no significant variation in ΔF/Fm' between the control and any P-PO_4^{3-} treatment for shoots from both meadows or between the interaction of light and P-PO_4^{3-} (Table 44). Light was the only significant factor affecting the photosynthetic efficiency of the plants, and higher ΔF/Fm’ values were measured in low than in high light treatments (Figures 40 and 41).

In both experiments low light conditions resulted in an increase of ΔF/Fm’. Shoots from Thasos after 8 days had a mean effective yield of 0.769±0.004 while at the experiment’s start 0.757±0.002 under low light, while the high light treatments started from 0.754±0.003 and at the 8th day had a mean value of 0.752±0.005.

A similar pattern was observed for shoots from Nea Karvali meadow. Shoots from the low light treatments had a mean value of 0.763±0.004 at day 0 and 0.769±0.002 at day 8 of the experiment, while under high light 0.761±0.004 at day 0 that dropped to 0.752±0.006 at day 8. Shoot acclimation history had no significant effect on its reaction to light treatment as is shown by the ANOVA in Table 45.

Meadow	Factor	Value	Std.Error	df	t-value	p-value
Thasos	**Intercept**	0.760	0.004	191	200.486	0.000*
	Low Light	0.013	0.004	191	2.910	0.004
	B	-0.002	0.006	41	-0.266	0.792
	C	0.001	0.006	41	0.245	0.808
	D	-0.006	0.006	41	-1.086	0.284
	B:Low Light	0.003	0.007	41	0.426	0.672
	C:Low Light	0.005	0.007	41	0.782	0.439
	D:Low Light	0.009	0.007	41	1.250	0.218
Nea Karvali	**Intercept**	0.747	0.007	192	101.957	0.000*
	B	0.009	0.009	40	0.986	0.330
	C	-0.001	0.010	40	-0.060	0.952
	D	0.001	0.010	40	0.125	0.901
	Low Light	0.024	0.008	40	3.074	0.004
	B:Low Light	-0.006	0.010	40	-0.556	0.581
	C:Low Light	0.001	0.011	40	0.071	0.944
	D:Low Light	0.000	0.011	40	0.027	0.978

Table 44. Mixed model analysis of effective quantum yield measurements of *Cymodocea nodosa* leaves from two meadows in the N. Aegean Sea (Thasos and Nea Karvali), after an eight day exposure in four P-PO_4^{3-} concentrations (0.02, 0.5, 1 and 2μM), under two different light conditions (37 and 65μmol photons*m^2*s^{-1}).* $p<0.001$

Factor	Value	Std.Error	df	t-value	p-value
Intercept	0.762	0.004	384	184.808	0.000*
Thasos	-0.007	0.006	91	-1.125	0.264

Table 45. Mixed model analysis of effective quantum yield measurements of *Cymodocea nodosa* shoots between two meadows (Nea Karvali and Thasos) in N. Aegean Sea to P-PO_4^{3-} exposure under different light conditions (37 and 65μmol photons*m^2*s^{-1}). *, $p<0.001$

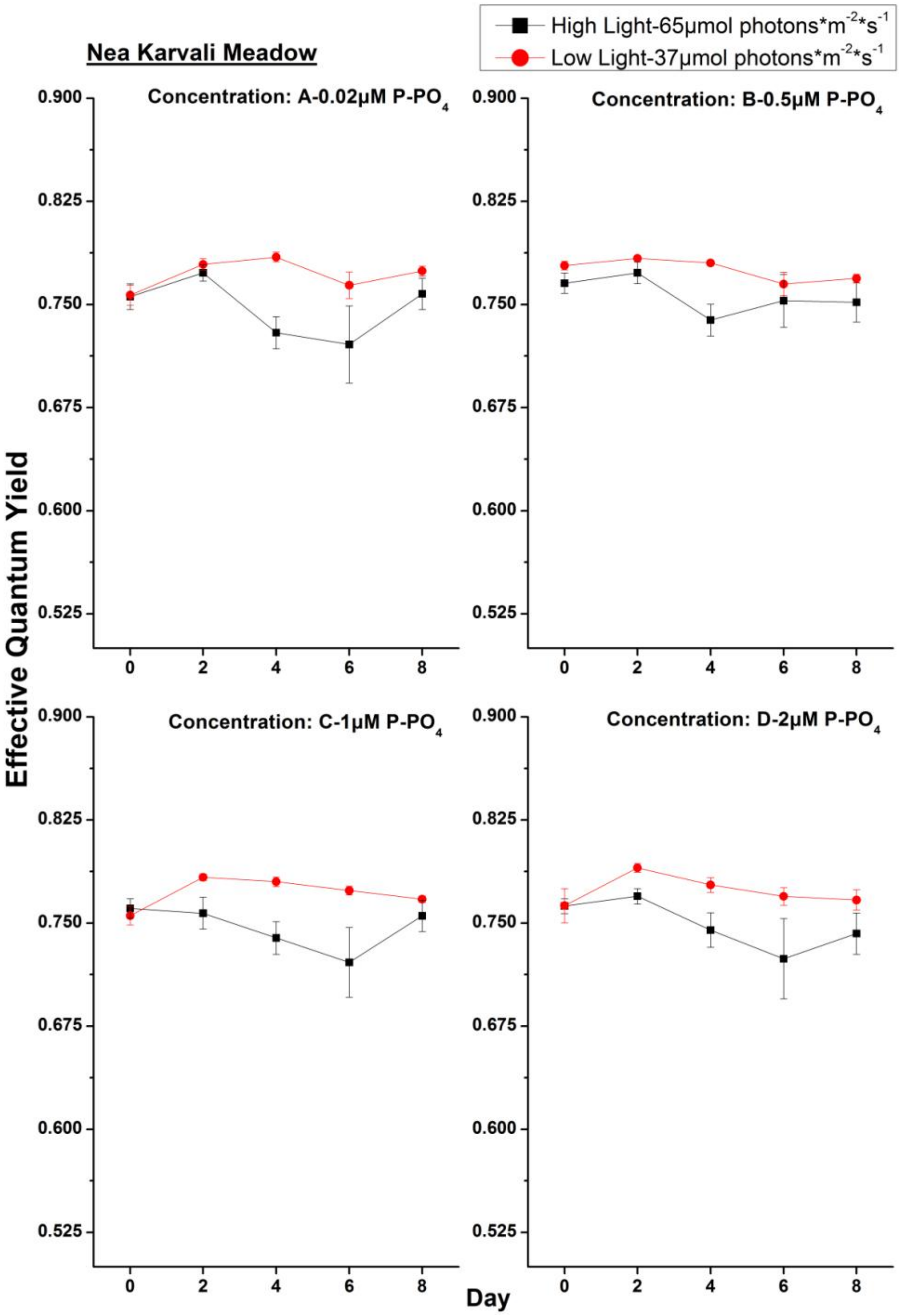

Figure 40. Mean values of ΔF/Fm' ± Standard Error (n=6) of *Cymodocea nodosa* leaves collected from Nea Karvali meadow during an eight day exposure in four P-PO_4^{3-} concentrations (0.02, 0.5, 1 and 2μM), under two different light conditions (37 and 65μmol photons*m^2*s^{-1}). Standard errors are too small were not shown.

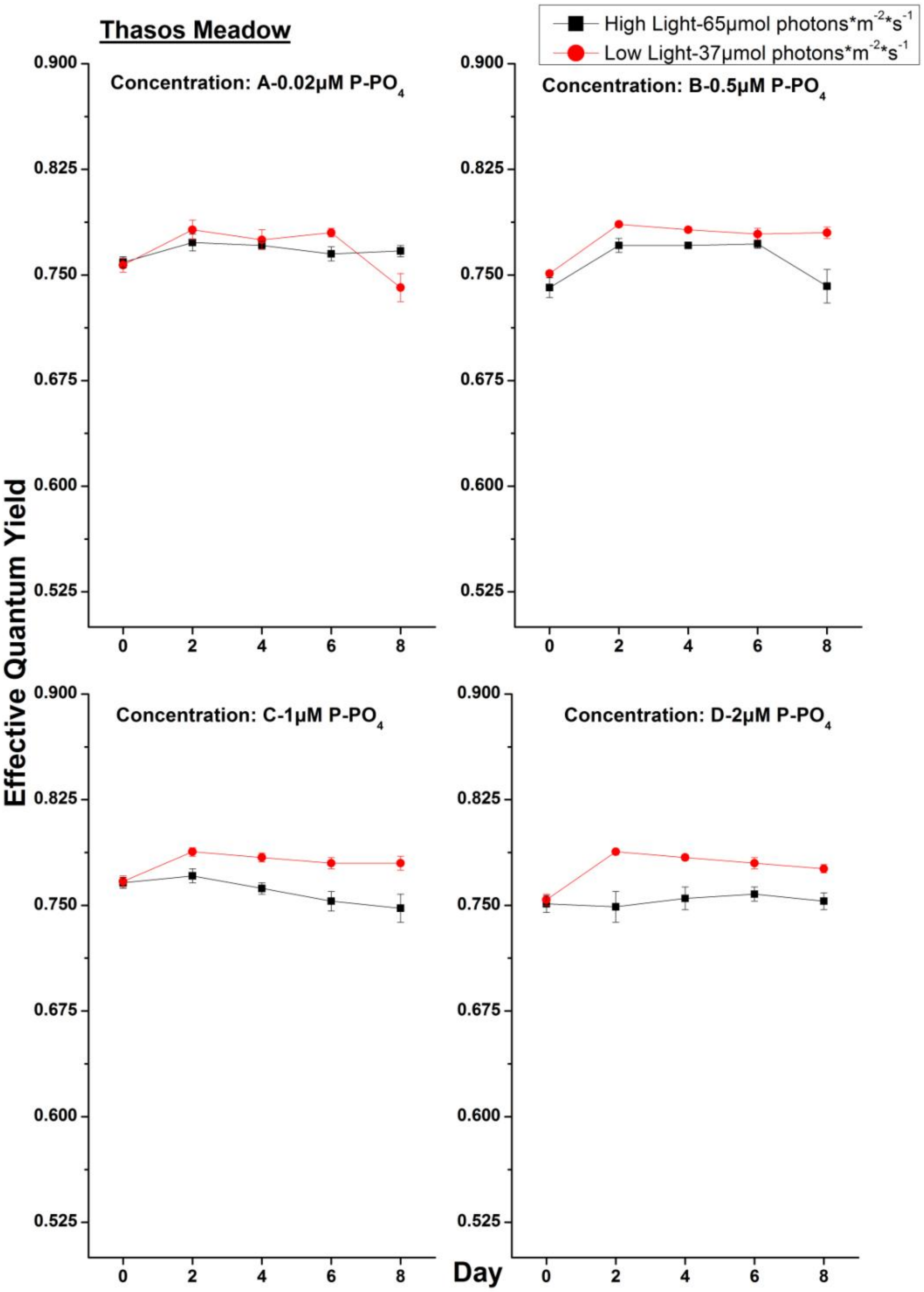

Figure 41. Mean values of ΔF/Fm' ± Standard Error (n=6) of *Cymodocea nodosa* leaves collected from Thasos meadow during an eight day exposure in four P-PO_4^{3-} concentrations (0.02, 0.5, 1 and 2μM), under two different light conditions (37 and 65μmol photons*m^2*s^{-1}). Standard errors are too small were not shown.

Copper exposure

Different concentrations of cooper affected significantly ΔF/Fm’ of *C. nodosa* shoots collected from Brasidas, Thasos and Nea Karvali, after an eight day exposure period (Table 46). The analysis showed that photosynthetic efficiency of shoots collected from the two non-impacted sites decreased as copper concentration increased above 1.6μM (Figure 42). Leaves from Brasidas had an effective quantum yield of 0.763±0.006 at the start of the experiment, which decreased significantly to 0.702±0.024 ($P= 0.010$) at 4.7μM and 0.593±0.019 ($P<0.001$) at 7.9 μM of Cu after 8 days exposure. The effective quantum yield of leaves from Thasos decreased from 0.752±0.009 to 0.605±0.022 ($P = 0.01$) and 0.522±0.037 ($P =0.001$) at 4.7 and 7.9 μM, respectively after 8 days exposure.

Copper also had a negative effect on photosynthetic efficiency of leaves in plants collected from the impacted meadow at Nea Karvali. The trend was similar to that reported for the two non-impacted sites (Table 46) but the severity of the damage at the three exposure concentrations was greater in leaves from Nea Karvali, where high senescence was witnessed, especially under the high concentrations (Figure 42). While mean ΔF/Fm’ was 0.745±0.003 at the control treatment at day 8, under 7.9 μM of Cu it decreased to 0.547±0.028.

Cu had a significantly negative effect on leaf elongation only for shoots from Nea Karvali (Table 47). Figure 43 shows the mean values of leaf elongation between all Cu treatments. Only the two higher conditions had a significant effect ($p<0.001$; Figure 47) and they were significantly different to the control (Figure 44). Leaves grew 2.926±0.612cm under the control treatment, while under 4.7μM of Cu they grew by 1.15±0.35cm and under 7.9μM of copper by only 0.976±0.154cm.

In contrast, there was no significant effect of Cu on the elongation of leaves from Thasos. A negative trend can be seen in Figure 47. Under 1.6μM of copper, leaves grew more (1.264±0.380cm) than leaves from the control that grew by 1.184±0.265cm, while under 7.9μM of Cu growth was 0.722±0.146cm, however no significant difference was found.

Meadow	**Concentration**	**Value**	**Std.Error**	**df**	**t-value**	**p-value**
Brasidas	**Intercept**	0.758	0.005	96	167.306	0.000*
	B	0.013	0.006	20	2.057	0.053
	C	-0.018	0.006	20	-2.849	0.010
	D	-0.039	0.006	20	-6.047	0.000*
Nea Karvali	**Intercept**	0.746	0.011	93	70.192	0.000*
	B	0.011	0.015	20	0.750	0.462
	C	-0.045	0.015	20	-2.963	0.008
	D	-0.077	0.015	20	-5.115	0.000*
Thasos	**Intercept**	0.764	0.004	96	198.661	0.000*
	B	0.004	0.005	20	0.761	0.456
	C	-0.061	0.012	20	-5.193	0.000*
	D	-0.114	0.028	20	-4.047	0.001

Table 46. Analysis of effective quantum yield measurements of *Cymodocea nodosa* leaves from three meadows (Brasidas, Thasos and Nea Karvali) in the N. Aegean Sea, after an eight day exposure in four Cu concentrations: A:0, B:1.6, C:4.7 and D:7.9 μM, under 0.3μM N and 0.02μM P.* $p<0.001$

Meadow	***Factor***	**Estimate**	**Std. Error**	**t value**	**p**
Nea Karvali	**Intercept**	2.926	0.443	6.606	0.000*
	CuB	-1.039	0.599	-1.736	0.086
	CuC	-1.776	0.619	-2.871	0.005
	CuD	-1.950	0.611	-3.190	0.002
Thasos	**Intercept**	1.184	0.256	4.634	0.000*
	CuB	0.080	0.392	0.204	0.839
	CuC	-0.106	0.392	-0.269	0.789
	CuD	-0.463	0.361	-1.280	0.205

Table 47. One way ANOVA of *Cymodocea nodosa* leaf elongation under four Cu concentrations (A:0, B:1.6, C:4.7 and D:7.9 μM) and 0.3μM N - 0.02μM P.*, for shoots from two meadows (Thasos and Nea Karvali) in the N. Aegean Sea. *, $p<0.001$

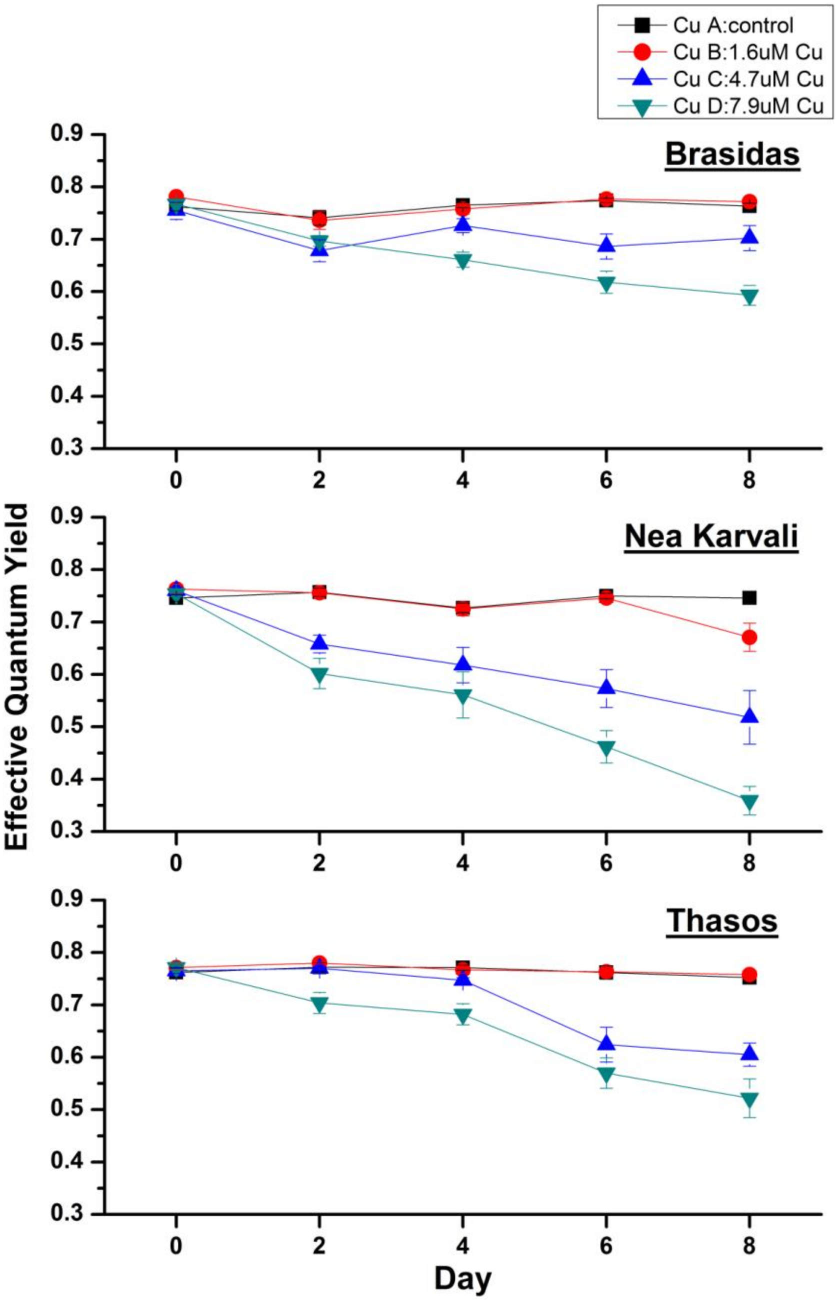

Figure 42. Mean values of ΔF/Fm’ ± Standard Error (n=6) of *Cymodocea nodosa* leaves after an 8 day exposure to four Cu concentrations: A:0, B:1.6, C:4.7 and D:7.9 μM, with the addition of 0.3μM N and 0.02μM P, for samples collected from three meadows (Brasidas, Thasos and Nea Karvali) in N. Aegean Sea. Standard errors are too small were not shown.

a) ΔF/Fm’	Value	Std.Error	df	t-value	p-value
Intercept	0.767	0.004	351	209.854	0.000*
Nea Karvali	-0.029	0.005	351	-6.166	0.000*
Thasos	0.006	0.004	351	1.532	0.126

b) Leaf elongation	F	df	p
Nea Karvali	3.926	3	0.016
Thasos	1.233	3	0.315

Table 48. Statistical analysis of *Cymodocea nodosa* a)ΔF/Fm’ and b) leaf elongation response to 4 copper concentrations (A:0, B:1.6, C:4.7 and D:7.9 μM) and 0.3μM N - 0.02μM P exposure for 8 days, on samples from three meadows (Brasidas, Nea Karvali and Thasos) in N. Aegean Sea of known ecological status.*p<0.001

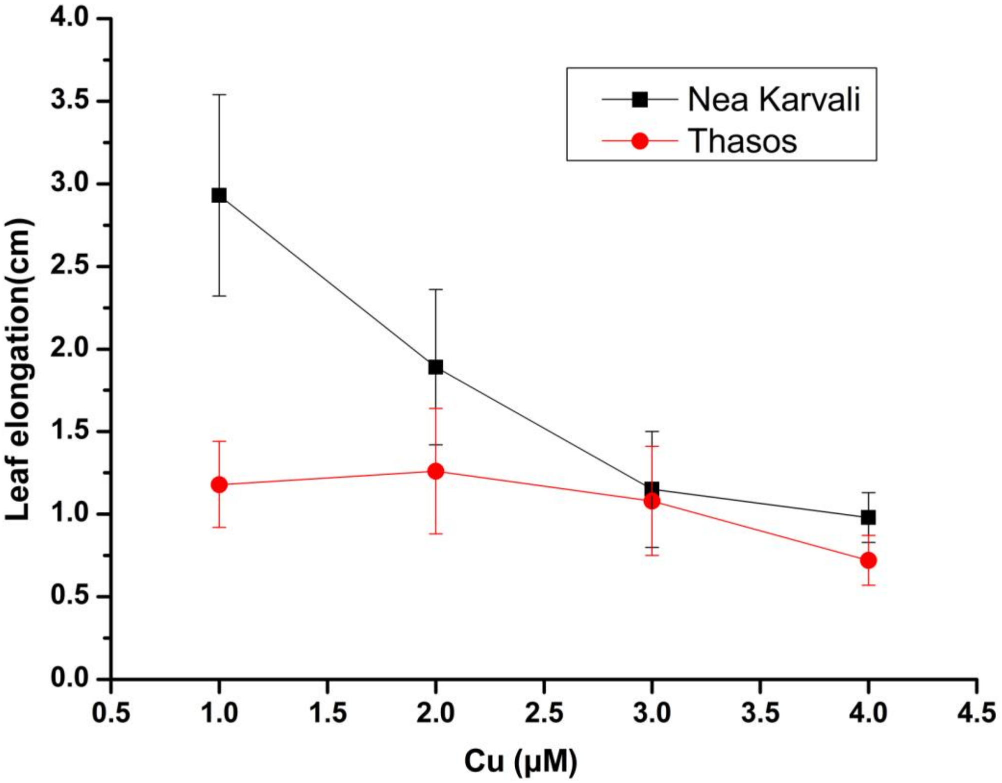

Figure 43. Mean values of *Cymodocea nodosa* leaf elongation (cm)±standard error (n=6) from two meadows (Nea Karvali and Thasos) in N. Aegean Sea, after exposure to 4 Cu concentrations (A:0, B:1.6, C:4.7 and D:7.9 μM).

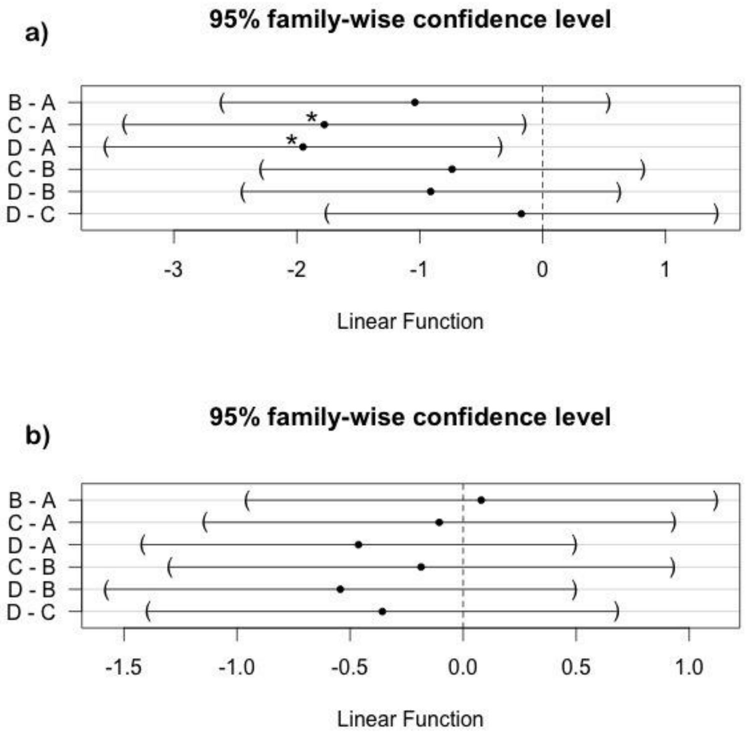

Figure 44. Post hoc pairwise comparisons of *Cymodocea nodosa* leaf elongation after an 8 day exposure to four copper concentrations (A:0, B:1.6, C:4.7 and D:7.9 μM) for shoots from two meadows in the N. Aegean sea a) Nea Karvali and b) Thasos with 95% confidence intervals using the Tukey test. * denotes statistical significant difference.

DISCUSSION

Nutrient exposure

A significant effect of nutrients on plant photosynthetic performance (ΔF/Fm’) in this study was documented but only in shoots from an oligotrophic site (Thasos; Table 41), while nutrients had no effect on leaf elongation (Table 42). Nutrient concentration had a positive effect on photosynthetic efficiency of shoots from Thasos meadow. The late summer period, when these experiments were conducted, is characterized by low nutrient availability, since phytoplankton and fast growing epiphytes and macroalgae have depleted them (Orfanidis et al., 2005a; Sylaios et al., 2005) and it marks the end of high growth season for seagrasses (Leoni et al., 2006). These conditions can be seen in the significantly lower nitrogen and phosphorus content in plant tissues and nutrient concentrations measured during the summer period (Chapter 3). The generally low growth rate and the ability to utilize stored nutrients allowed seagrasses to continue growing in the low nutrient treatments for the short period of the experiment so no response was noted for shoots from both meadows.

Shoots from Nea Karvali had higher nutrient content due to the high nutrient concentrations in the water column (Sylaios et al., 2005), and had already higher photosynthetic efficiency (Chapter3), so further nutrient enrichment didn't have any effect on $\Delta F/Fm'$. Even though seagrass decline under nutrient enrichment is most commonly linked to indirect ecological processes, mainly through the stimulation of high biomass algal overgrowth that causes light limitation (Shepherd et al., 1989), there is evidence linking negative responses to direct physiological mechanisms, the most common being shoot die-off due to internal carbon limitation. Though it hasn't been tested for all seagrass species, most of those that have been tested show limited or negligible nutrient feedback inhibition and continue nutrient uptake at high rates for as long as they are available in the environment (Burkholder et al., 2007). The fact that they evolved in oligotrophic habitats led to the development of a sustained nutrient uptake and assimilation process in order to take advantage of temporary enrichment that would eventually stop. Since nitrate assimilation and reduction is energetically costly, continuous exposure to high N concentrations would eventually lead to significant declines in plant growth, by consuming carbohydrate reserves for synthesizing amino acids and causing internal carbon limitation (Leoni et al., 2008). This mechanism was responsible for the 75-95% *Z. marina* shoot die off relative to controls after low level nitrate enrichment (3.5 and 7μM) for 7 weeks according to Buckholder et. al. (1992). The short time (8 days) didn't allow studying any such effects on *C. nodosa*, however short period nutrient enrichments had no effect on the plants photosynthesis.

A different reaction was witnessed for shoots from Thasos meadow. Those shoots should have lower N content, coming from an oligotrophic environment. Since our plants were sampled during late summer, internal nutrient pools were almost depleted and since light was abundant, nutrients were the limiting growth factor (Lee et al., 2007a). The plants were quick to use the nutrients available in order to increase their photosynthetic efficiency, a response that was significant only in the high nutrient solution. A similar response was reported by Alcoverro et. al. (2001a) for *P. oceanica*, that had twice the photosynthetic capacity at a slightly eutrophic site in comparison to an oligotrophic one, as well as other seagrass species (Agawin et al., 1996; Lee et al., 1999a). The photosynthetic efficiency increase under higher nutrient concentrations is in agreement with the results of chapter 3 that showed higher ΔF/Fm’ values in plants from a eutrophic meadow (Nea Karvali) opposite to plants from an oligotrophic one (Brasidas).

Leaf elongation showed no significant response to nutrient concentrations (Thasos p=0.593 and Nea Karvali p=0.700), even though an increasing trend could be seen in Figure 42. What is most common in seagrasses under intermediate concentrations of nutrients is the increase of uptake and assimilation rates and the utilization of the excess nutrients in carbon fixation, through increased photosynthetic activity, resulting in a biomass increase, when there is no light limitation (Lee et al., 2007a). However the absence of growth response to nutrient enrichment has been documented for other species (Kenworthy et al., 1992; Udy et al., 1997a; Ramirez Garcia et al., 2002), suggesting that other mechanisms are of influence like seasonality or temperature.

Effect of PO_4^{3-} exposure under different light conditions

Increasing concentrations of PO_4^{3-} had no significant effect on ΔF/Fm’, while light was the only significant variable for differences between effective quantum yield (p=0.004; Table 44). In all experiments conducted, low light irradiance (37μmol photons*m^{-2}*s^{-1}) resulted in higher ΔF/Fm' values than a higher light irradiance (65μmol photons*m^{-2}*s^{-1}), while the acclimation history of shoots didn't have any significant effect on their reaction (Table X). These findings are consistent with studies of other seagrass species (Beer et al., 1982; Dennison, 1987; Abal et al., 1994; Masini et al., 1995; Ralph et al., 1995; Lee et al., 1997; Longstaff et al., 1999b; Ralph, 1999; Kraemer et al., 2000; Bite et al., 2007).

The increased ΔF/Fm' values in low light treatments was the result of the plants effort to increase its photosynthetic efficiency, in order to capture more photons and sustain growth. In order for that to happen, the plant must reduce the minimum quantum requirements for photosynthesis ($1/\Phi_{max}$) to approach the theoretical minimum of 8 photons (Govindjee, 1999) and enhance its leaf absorptance (Ralph et al., 2007), usually by increasing its chlorophyll content (Longstaff et al., 1999a). Other reported photoadaptative responses include increased maximum effective yield (Fv/Fm), photosynthetic efficiency (a), reduced maximum electron transport rate ($rETR_{max}$) and a reduction in the saturating irradiance (E_k), data that can be collected from rapid light curves (RLC) (Ralph et al., 2005). Various morphological adaptations to light limitation have been also published, with some species increasing their leaf length and width in order to increase the light capture area (Longstaff et al., 1999a), while others decrease leaf size in order to reduce the respiratory demand (Campbell et al., 2002).

C. nodosa follows the first strategy, as seen in chapter 3, where leaf length and width were significantly higher in shoots from Nea Karvali, a habitat with increased turbidity. Increase of ΔF/Fm' was noted as fast as the first two days of the experiments and it was more intense in shoots from Thasos meadow, probably due to the higher irradiances that those shoots were receiving in the field. After 8 days of exposure mean ΔF/Fm' values were similar for each light condition, regardless the shoots acclimation history.

Copper exposure

Shoots from all three studied meadows confirmed the negative effects of copper to *C. nodosa* ΔF/Fm’. The lowest copper concentration (1.6μM) didn’t have any significant effect on PSII, however, higher concentrations (C=4.7μM and D=7.9μM) caused significant decline of ΔF/Fm’ (Table 46). The negative effect of 4.7μM of Cu was observed in shoots from Thasos and Brasidas only after the 4th day of the experiment, while in shoots from Nea Karvali after the 2nd. The highest cooper values had the most intense effect on PSII, as it lowered effective quantum yield immediately and most drastically (Figure 42). A reduction of Ft and Fm’ values was observed, indicating a reduction of the available reaction center pools (Macinnis-Ng et al., 2004a). Even though Cu is needed for plastocyanin production, a protein utilized in the electron transport from cytochrome *f* to PSII, in high concentrations it works negatively, blocking the electron transport between PSI and PSII (Govindjee, 1995). At the same time Cu causes chlorosis and damage to plasma membrane permeability that eventually leads to ion leakage (Ouzounidou et al., 1992). More specifically Cu attacks sulphydryl groups thus damaging permeable layers and allowing ion diffusion into the chloroplast leading to its degradation (Ouzounidou, 1994).

Significant leaf growth inhibition took place only in shoots from Nea Karvali, and only for the two highest Cu concentrations (Figure 42). A similar reaction wasn’t observed at shoots from Thasos. Growth was slightly sustained with the addition of Cu, however no significant trends were identified. There aren’t many studies focusing on leaf growth under metal stress; however, Ambo-Rappe et. al. (2011) showed that leaf growth of *Halophila ovalis* was significantly reduced after the addition of 0.5mg/L Cu. Older studies also suggested that many seagrass species have smaller and narrower leaves in more stressful conditions (McMillan, 1978; McMillan et al., 1979; Phillips, 1980).

Leaf senescence due to increased copper concentration has also been reported for many seagrass species (Malea et al., 1995b; Ambo-Rappe et al., 2011) and was also witnessed in this study, especially in shoots from Nea Karvali. This phenomenon is associated with the stimulation of phytochrome activity, which leads to increased abscisic acid and ethylene production, that in turn signal the leaf abscission and loosening of cell walls (Malea et al., 1995b).

Negative effect of heavy metal concentration on ΔF/Fm' has been reported for other seagrass species as well (Ralph et al., 1998a; Macinnis-Ng et al., 2002; Macinnis-Ng et al., 2004a). Like most plants, they can cope with heavy metals with one of three ways. They can isolate the metals to a neutral function, fixate the toxic particle, or metabolize it by biotransformation enzymes, increasing their water solubility (Pergent-Martini et al., 2000). Considering that Cu has a number of impact sites on the plant, itsn't always easy to predict the endpoint of the toxic event and the ability to withstand metal stress finally rests to the individual's ability to undergo physiological adaptations, or genetically based resistances (Klerks et al., 1987).

Ambo-Rappe et. al. (2011) commented on the unpredictable nature of heavy metal exposure, presenting conflicting data on the effect of heavy metal exposure on the morphology of *Halophila ovalis*. Increasing metal concentration resulted in reduced morphological traits in laboratory experiments, while it had the opposite effect in the field. One explanation is that concentrations used in laboratory experiments are significantly higher than those usually met in the field, in order to yield a response. Also heavy metal absorption and bioavailability are influenced by many environmental factors, such as pH, sediment particle size, temperature, salinity, season as well as biological ones like species, type and age of tissue, that most of the times reduce the availability of a toxicant and its effect on a population.

Looking at Table 43, plants from the two least impacted meadows (Brasidas and Thasos) had a similar response to copper exposure (p statistic=0.126), while those from Nea Karvali responded in a significantly different way (p statistic<0.001). Shoots from the pristine meadows demonstrated a higher tolerance to copper, as they were affected less by a medium copper concentration (4.7μM) and even under a higher concentration (7.9μM) still maintained an increased photosynthetic efficiency.

Jepson and Sherratt (1996) suggested that assessment of ecotoxicological risks can be highly site specific, while Foy et. al. (1978) discussed the evolution of metal tolerant ecotypes within plant species. Even so there aren't many studies focusing on metal toxicity on plants with different acclimation histories. This study shows that plants from an undisturbed habitat could cope better with Cu stress than plants from a eutrophic site. These results comply to the previous research by Macinnis-Ng and Ralph (2004b) that studied metal exposure on *Zostera capriconi* from three isolated populations. Even though they hypothesized that previously exposed to metals plants would be more tolerant than naïve ones, they found a significant decrease of chlorophyll-*a* fluorescence under additions of 0.5 and 1mg l^{-1} of Cu only in plants from a polluted site, while the mechanisms responsible couldn't be identified.

In this study, there are two basic differences between plants from the two pristine sites and plants from the impacted one. Plants from Nea Karvali are acclimated to a high nutrient environment and have larger and wider leaves. Since copper uptake is a surface-area dependent process (Malea et al., 1995b), wider leaves from Nea Karvali could uptake more Cu. This explanation however, doesn't cover the fact that the copper concentrations used were significantly high and kept high throughout the experiment with medium change every 2 days, as well as the fact that the experimental time (8 days) was much longer than that used in previous studies (10h to 96h). Even under these extreme conditions plants from the undisturbed meadows maintained high ΔF/Fm’ values until the end of the experiment and contrary to plants from Nea Karvali, senescence was limited. A further study of the physiochemical parameters, as well as the genetics of the populations involved could help determine the influence of genetic variation on resistance.

CONCLUSIONS

According to Waycott et. al. (2009) since 1987 seagrass meadows have been in decline in all areas of the globe, where data exist. Many researchers and managers have highlighted the importance of seagrass ecosystems, both from a local and a global perspective, while anthropogenic activity has been identified as the main reason for their decline (Orth et al., 2006). Rapidly increasing human population density on coastlands contributes high nutrient loading to the coastal zone and nutrient over-enrichment (especially nitrogen and phosphorus) has been identified as one of the major causes for seagrass disappearance (Burkholder et al., 2007).

Many studies have focused on describing the effect of eutrophication on seagrasses, so that managers can take the necessary steps towards seagrass recovery. Experiments have shown that responses to eutrophication are variable and highly dependent on the species as well as the region, making it difficult to construct a general management strategy. Moreover, most seagrass studies focus on field experiments, that even though are more suited in acquiring ecologically relevant results, cannot always provide clear connections between stressors and effects due to the complex nature of the environment.

In this study, an effort was undertaken to identify the effects of nutrient, irradiance and copper on the photosynthetic efficiency of *C. nodosa* that grows in the N. Aegean

Sea under controlled environmental conditions. Nutrients in small to moderate concentrations can enhance growth and photosynthesis, while under high concentrations have been shown to negatively affect seagrass health due to photosynthetic high carbon requirements that eventually lead to internal carbon limitation (Lee et al., 2007a).

Copper affected the species photosynthesis and higher concentrations lead to lower effective yield and sustained growth. This result was expected, as copper is one of the most toxic metals, however a reaction was clear only under significant concentrations that are very rarely met in the environment, indicating that *C. nodosa* is very tolerant to it in the field. More interestingly, plants from an undisturbed meadow were shown capable to sustain growth and photosynthesis, under much higher copper concentrations.

Light was the most important factor, affecting photosynthesis. High irradiance resulted in lowering ΔF/Fm', while low irradiance had the opposite effect. This reaction is known for other seagrass species as well. Together with the fact that nutrients didn't have any direct effect on *C. nodosa*, while copper needed increased concentrations in order to do so, light was the main factor controlling the physiological responses of the seagrass. In the field, low irradiances can be caused by coastal land disturbance in development, dredge and fill operations, aquaculture and fishing activities, as well as by stimulation of high biomass of phytoplankton, epiphytes and macroalgae due to nutrient over enrichment. Under these conditions, *C. nodosa* has higher photosynthetic efficiency, which can be measured effectively with the PAM instrument.

This study showed that the fast growing seagrass *C. nodosa* has a significant tolerance to Cu, as well as nutrient concentrations. These parameters can't be directly responsible for meadow loss in the field, while nutrients can be responsible for increased biomass of photosynthetic organisms in the water column that in turn lower light irradiances. In order to link irradiance to the anthropogenic stressors and study the underlying mechanisms that lead to seagrass degradation, further experimental studies are needed on the combined action of stressors. Since rarely meadow degradation is linked to a single parameter, the further understanding on the combined action of stressors will allow us to better manage and conserve these biotopes.

Chapter 5: General Discussion

This research set up to study the physiological and morphological changes of the seagrass *Cymodocea nodosa* at a gradient of anthropogenic stress and to understand the mechanistic basis of these changes with the application of factorial experiments testing the effects of key stressors to the species under constant laboratory conditions.

Seagrasses have a high phenotypic plasticity i.e. variations in morphology and physiology, that allows them to adapt in different environmental conditions and maintain a positive growth rate. Knowledge on the way that anthropogenic stress forces these populations to adapt could allow us to work backwards and use key features as bioindicators of the water quality of coastal or transitional waters. Such an approach has been strongly encouraged in the last decade by the implementation of European Water Framework Directive (WFD).

Key biotic features of meadows growing in locations of contrasting ecological status in the N. Aegean Sea, Greece, were sampled and analysed applying a hierarchical designed approach at three spatial ranging from tens of meters (area) to hundreds of meters (site) to kilometers (meadow) and one temporal (period) scales.

Since the development of PAM fluorescence, it has been widely used to identify and quantify the effect of specific stressors on seagrass physiology such as heavy metals, irradiance, salinity and thermal stress (Ralph et al., 1995; Ralph, 1998; Prange et al., 2000; Macinnis-Ng et al., 2002; Bite et al., 2007; Sandoval-Gil et al., 2012). Few researchers have tried to link it to the overall effect of anthropogenic stress on the seagrasses with limited success. The difficulty in such an effort lies with the high variability of PAM parameters in low spatial scales and the effect of other abiotic factors on them (Gera et al., 2012).

These issues were addressed in this study with two ways: (1) A large amount of data was collected, as photosynthetic efficiency was measured in a total of 1113 plants, in order to minimize variability, (2) samples were taken to the laboratory where they were left for an hour under constant temperature and irradiance conditions, in order to take the PAM measurements under similar conditions. This methodology even though destructive, allowed us to minimize the effect of weather conditions on the photosynthetic efficiency estimations.

The species showed significant physiological differences between the two meadows of Nea Karvali (impacted) and Brasidas (non-impacted). PAM fluorescence parameters showed that plants from the Nea Karvali meadow had significant higher ΔF/Fm’ and Fm values than those from Brasidas, proving them as useful indicators of anthropogenic stress. Both of these parameters have already been used to quantify stress (Ralph et al., 1995; Prange et al., 2000) indicating that the exposure to certain stressors like heavy metals or high irradiance leads to a decrease of photosynthetic efficiency. Surprisingly, we measured an increase of these parameters in plants growing from less (Brasidas) to highly degraded meadows (Nea Karvali). This contradiction could be attributed to a single stress factor that masked all others or to the fact that the anthropogenic gradient wasn’t caused by a discrete stressor, but by a number of concurrent influences, such as nutrient load, increased turbidity and higher heavy metal concentrations. Even though each of these stressors alone causes a putative disruption in the degree of electron transport thus leading to an overall decline of the photochemical efficiency (Ralph et al., 1995), their combined effect hasn’t been studied. For instance high nutrient concentrations allow macroalgae to better cope with heavy metal stress (Orfanidis et al., 2009a), so the possibility of a similar mechanism on seagrasses should be tested. In general, a quantitative model of the relationship between the severity of different kind of stress e.g. physical, chemical, or their combinations and the seagrass stress responses is lacking.

In order to gain some insight on how the main stress factors affected the photosynthetic efficiency on plants from the studied meadows (nutrients-N, P, irradiance and Cu, as a representative heavy metal) a series of laboratory experiments were carried out. As a result light and nutrients were identified as the main factors affecting the photosynthetic efficiency of *C. nodosa*. Nutrient (N-NO_3^- and P-PO_4^{3-}) concentration had significant effect on ΔF/Fm’ only on shoots from the oligotrophic site, while Cu resulted in significant decline but only under high concentrations. According to these results, higher nutrient availability and low irradiance both lead to increased ΔF/Fm’ values. Nutrients are consumed in the metabolic pathway enhancing photosynthesis, in order to achieve higher growth rates. At the same time the plant invests in increasing its photosynthetic efficiency in order to make better use of low irradiance.

Based on the above results one could hypothesize that the higher PAM measurements taken in the meadow of Nea Karvali were caused by chronic exposure of the plants to lower light irradiances in comparison to reference. Light attenuation coefficient (k) values estimated in Nea Karvali (k=0.31) and in Brasidas (k=0.21) in June 2010, (un-published data) were very similar to those measured during the period 2004-2005 (Orfanidis et al. 2010). This low light availability in Nea Karvali is connected to increased nutrient and concentrations that sustain a relative high phytoplankton biomass (Sylaios et al. 2005) during spring and summer, as well to organic loadings of nearby aquaculture activities (un-published data). Moore et. al. (2000) reached to the same conclusion after studying the combined effect of nutrient enrichment and light availability on *Zostera marina* microcosms. While nutrient concentration affected the epiphytic biomass only, it was light that governed seagrass growth.

It has been found for other species that low light availability triggers an increase of chlorophyll-*a*, in order to more effectively facilitate captured photons (Lee et al., 2007a), however such a mechanism wasn’t found for *C. nodosa*. It has been argued that such a response isn’t preferred by many species since large variations of chlorophyll content in leaves only result in relatively small variations on leaf absorbance that are still not enough to withstand low light stress (Enriquez et al., 1992; Cummings et al., 2003; Enríquez, 2005).

Morphological adaptations that could be explained by nutrient availability and the plants effort to capture more light in the Nea Karvali meadow include longer leaves, that increase the photosynthetic area as well as the canopy height bringing it closer to the surface and higher irradiances. Even though leaf length in this study was significantly higher in the degraded meadow than the meadow in reference, its use as a bioindicator of water quality needs caution. Shoot morphology might also be influenced by other factors such as wave exposure and therefore meadows with long leaves have been found in protected pristine meadows as well (personal observation). In an earlier extensive study of *C. nodosa* leaves morphology in Kavala Gulf two meadows (Nea Karvali, Erateino) exposed at different water quality conditions showed very similar total leaf length values (Orfanidis et al. 2010). Based on this and other relevant results Orfanidis et. al. (2009b) proposed CymoSkew index, a quantitative expression of leaf length asymmetry as an early warning indicator of anthropogenic stress as quantified by means of light and nutrients and of *Cymodocea* habitat degradation, in general.

Nitrogen content in leaves is an indicator of nutrient availability in the environment (Marba et al., 2012). As it has been reported for other species, nitrogen was significantly higher in plants from the meadow with high nutrient concentrations (Perez et al., 1994b; Udy et al., 1997b; Mvungi et al., 2012) but only in leaf tissue, reflecting the preferred uptake and storage path. Seagrasses use their roots or leaves as the main uptake pathway depending whether high nutrient concentrations are in the water column or pore water (Touchette et al., 2000b). They store nutrients at periods with low light and hight nutrient availability (usually during winter and early spring), when photosynthetic and growth rates are low and they resolve to them when there is light but not enough nutrients (during late summer and autumn).

Significant differences between a cold (January- March) and a warm (June- July) period were found for almost all parameters measured. Its well known that *C. nodosa* in the Mediterranean shows a strong seasonality, with maximum growth during summer and lowest growth during winter (Caye and Meinesz, 1985; Peduzzi and Vukovie, 1990; Pe´rez and Romero, 1994; Agostini et al., 2003). Seasonality affects the meadows since during winter natural stress (weather, low temperatures, increased turbidity and nutrients and low irradiance) further stresses the meadows. For this reason the early summer period is suggested as the better period for taking fluorescence measurements. Since light has a significant effect on PAM parameters taking measurements from the same depth is crucial. Comparison of plants from two sites of different depth (1-2m and 2-3m) showed that small depth changes (1m) didn't affect PAM measurements. However a more intense difference in depth would be expected to significantly change ΔF/Fm’ and Fm, since shoots from the lower meadow limit are known to have higher photosynthetic efficiency (Lee et al., 2007a).

From the significant *C. nodosa* responses measured in the field and under laboratory conditions long with the proposed explanation of their causes its inferred that the effective quantum yield and maximum fluorescence parameters can be applied as useful indicators of anthropogenic stress in the Aegean coasts. The relative easy application of the method as well as its low cost makes it valuable for monitoring programs and can provide results with significant speed. However further research is needed in order to understand the effect of combined stressors on the plants physiology so that we can clearly understand the overall increase in photosynthetic efficiency in shoots from an impacted meadow, in order for the final development of a fluorescence based indicator that could at the same time provide more detailed assessment on water quality.

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