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C, N, P, S content of the Chlorophyta *Chaetomorpha linum* (Müller) Kützing in a vast high density mat of a Mediterranean non-tidal lagoon

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Abstract – In a *Chaetomorpha linum* high density mat extending over 300 hectares, between 2017 and 2019 samples were collected for C, N, P, S tissue content determination, biomass (b) was estimated, sediment samples collected for labile organic matter (LOM) determination, and water chemical-physical variables measured. The latter showed extreme conditions with a wide range of values and with zero oxygen for long periods. N-NO_3:SRP atomic ratio showed extreme P-limitation. Tissue macronutrients showed very variable values, highlighting a strong P-limitation and relatively high level of S. With exception of nitrogen, no significant differences were detected for each macronutrient between the months and between the stations, neither was any correlation found between macronutrients and LOM and b data-set. The growth and survival of the mat occurred despite the scarcity of P, which probably reached with very low frequency the surface layer of the mat, the one capable of performing photosynthesis, where it was quickly re-assimilated and utilised.

Keywords: *Chaetomorpha linum* / nutrient / carbon / nitrogen / phosphorus / sulphur / macroalgal mat / lagoon / eutrophication

Résumé – Teneur en C, N, P, S de la chlorophyte *Chaetomorpha linum* (Müller) Kützing dans un vaste mat de haute densité d'une lagune méditerranéenne sans marée. Dans un tapis à haute densité de *Chaetomorpha linum* s'étendant sur 300 hectares, entre 2017 et 2019, des échantillons ont été prélevés pour la détermination de la teneur en C, N, P, S des tissus, la biomasse (b) a été estimée, des échantillons de sédiments ont été prélevés pour la détermination de la matière organique labile (LOM), et les variables physico-chimiques de l'eau ont été mesurées. Ces dernières ont montré des conditions extrêmes avec une large gamme de valeurs et avec un taux d'oxygène nul pendant de longues périodes. Le rapport atomique N-NO3:SRP a montré une limitation extrême du P. Les macronutriments tissulaires ont montré des valeurs très variables, mettant en évidence une forte limitation de P et un niveau relativement élevé de S. À l'exception de l'azote, aucune différence significative n'a été détectée pour chaque macronutriment entre les mois et entre les stations, et aucune corrélation n'a été trouvée entre les macronutriments et la LOM et l'ensemble de données de biomasse. La croissance et la survie du tapis se sont produites malgré la rareté du P, qui a probablement atteint avec une très faible fréquence la couche superficielle du tapis, celle capable d'effectuer la photosynthèse, où il a été rapidement réassimilé et utilisé.

Mots-clés : *Chaetomorpha linum* / nutriment / carbone / azote / phosphore / soufre / tapis de macroalgues / lagune / eutrophisation

1 Introduction

In the past forty years, nutrient supply resulted in the excessive growth of opportunistic macroalgae in many coastal ecosystems of the world (Morand and Briand, 1996; Raffaelli *et al.*, 1998; Smetacek and Zingone, 2013), which can uptake, assimilate and store nitrogen (N) and phosphorus (P) in large amounts. Bottom-floating macroalgal masses often accumulate along the coasts, bays and estuaries and in lagoon coasts, representing a significant biomass, and can produce high

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density mats, causing severe ecological and economic problems (Bombelli and Lenzi, 1996; Hauxwell and Valiela, 2004; Liu *et al.*, 2009).

Many coastal lagoons have been affected by these phenomena, due to the strong human impact and their structural and morphological characteristics (Sfriso *et al.*, 1987; Viaroli *et al.*, 1996; Lenzi *et al.*, 2011; Duck and Figueiredo da Silva, 2012). Macroalgal decay decreases water quality and enriches the sediment in labile organic matter, which results in widespread anoxic conditions and stimulates sulfate-reducing bacterial activity (Holmer *et al.*, 2004) that can lead to increasingly recurrent dystrophic crises (Amanieu *et al.*, 1975; Hargrave *et al.*, 2008; Lenzi *et al.*, 2011; Lenzi, 2019).

Non-tidal lagoon environments suffer even more from these phenomena, due to the scarce water exchange, shallow water, and, very often, morphology. For all these factors they have a structural tendency toward eutrophication, and are therefore particularly subject to opportunistic and galenophilic development of algal masses. Dense algal mats, sometimes well above 50 cm thick, also worsen the already scarce hydrodynamics, since they create areas of stagnation and modify the quality of the water significantly (Hargrave *et al.*, 2008; Lenzi *et al.*, 2013a; Sorce *et al.*, 2017). Inside a macroalgal mat, Lenzi *et al.* (2013a) observed stratification in the water quality, and a different nutrient uptake between the under-layer and the over-layer of the mat. Krause-Jensen *et al.* (1996) observed a sharp gradient of light penetration towards the inside of the mat, extinguishing itself a few centimeters below the mat surface.

The success of opportunistic macroalgae, also in high density mat, is attributed to their physiological responses to abiotic factors (Cohen and Fong, 2004; Sorce *et al.*, 2017). Since these dense mats continue to survive and grow for a long time, it remains to be understood how the over-layer, the one that can use light for photosynthesis, can thrive in conditions sometimes very limited for some nutrients.

In this study, we investigated the changes in the macronutrients C, N, P, S content in a high density mat of the Chlorophyta *Chaetomorpha linum*, which has persisted in a lagoon over an area of 300 hectares, for over 12 years to date. We sought to establish (1) if there are evident changes in relation to seasons and mat densities, and (2) if there are significant relationships between the quantity of labile organic matter present in the sediments covered by the mat and the mat itself. We believe that these elements are preliminarily necessary to understand how a bottom-floating mat can last so long at very high densities, demonstrating considerable resistance to environmental adversity, and to develop better environmental management of these critical lagoon environments, since not solving the problem of macroalgal blooms and their persistence, will make it difficult to achieve environmental remediation. Furthermore, we included among the objectives of the study to know (3) the percentage content of sulfur in the algal thalli since, in this regard, little information is available in the literature for the genus *Chaetomorpha*, and *C. Linum* in particular, even though some studies have found the presence of sulfated polysaccharides.
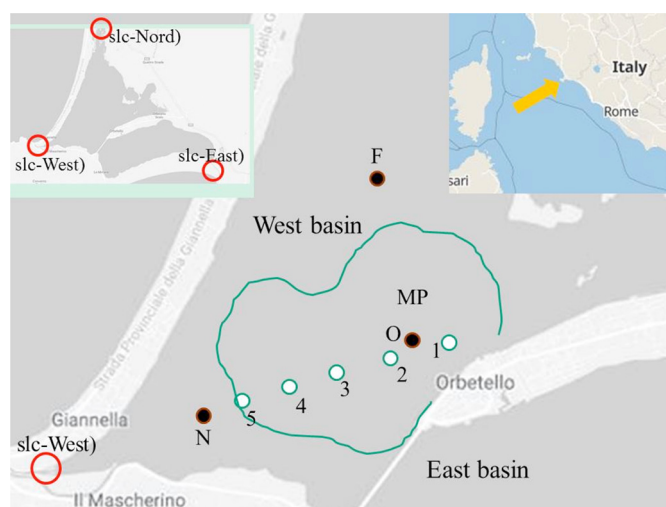

Fig. 1. Orbetello lagoon (Italy west coast). Area delineated in green, high density mat of *C. linum*; white points (1, 2, 3, 4, 5), sampling stations for the analysis of algal tissue; black points (N, O, F), water sampling stations; MP, multiparameter probe on fixed location; red circles, sea-lagoon canals and marine mouths.

2 Materials and methods

2.1 Study area

The Orbetello lagoon is a shallow, eutrophic coastal water body of about 25.25 km^2 in the southern Tuscan coast of Italy ($42°25'-42°29'$ N, $11°10'-11°17'$ E). This lagoon has two communicating basins known as "West" and "East", with an area of 15.25 km^2 and 10.00 km^2 respectively, and with a 1 m average depth (Fig. 1). Three artificial sea-lagoon canals, 0.5–3 km long and 10–15 m wide, two in the West (slc-Nord; slc-West) and one in the East basin (slc-East), connect the lagoon to the sea (Fig. 1). Because of the modest tidal excursions of the Tyrrhenian Sea and for the length and modest width of the three sea-lagoon canals, lagoon water turnover is poor and depends mainly on wind force and direction. Lagoon salinity ranges from 28 to 43 (practical salinity scale) changing with rainfall and evaporation.

The environment is eutrophic due to fish-farm wastewaters discharged in the East basin, intermittent streams containing agricultural run-off and civil effluent coming from the Albegna river, and historical input stored in the sediment (Lenzi *et al.*, 2003). Due to high nutrient availability, morphology and low-water-turnover, this lagoon is subject to severe macroalgal proliferation, which can cause dystrophic crises with consequential die-offs (Lenzi *et al.*, 2011).

Macroalgal harvesting and sea-water pumping were the first management actions taken to counteract the eutrophication effects. Macroalgal harvesting took place for 6 months per year and removed 4000–6000 tonnes per year of wet algal mass. It was estimated that 6000 tonnes of macroalgae removed from the ecosystem involved the resuspension of over 15,000 tonnes of sediment (Lenzi *et al.*, 2013b). From 2014, the main action to counter eutrophication has mainly been based only on direct resuspension of the sediment anoxic top layer (Lenzi *et al.*, 2017). Sea-water pumping is still practiced
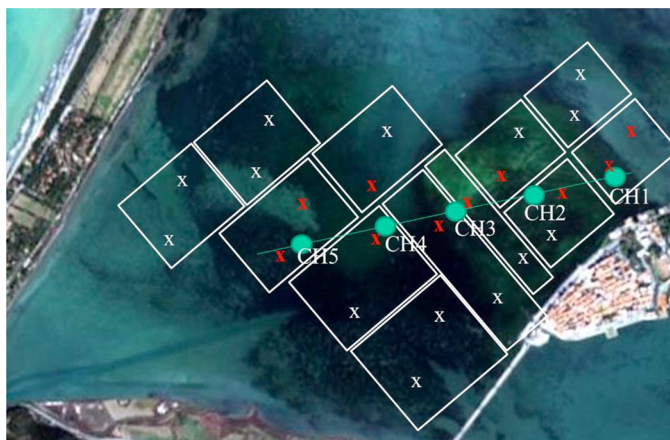
Fig. 2. Extension of the mat in January 2018 in the west basin of the Orbetello lagoon. Blank squares, areas of division of the mat for sampling of LOM (x) and biomass; green spheres, sampling stations for the tissue content of algae; x in red, LOM sampling stations close to those of the algal thalli, used for correlation analysis.

every year, between June and August, to promote water turnover, with the input from two western pumping stations placed at the mouth of the two western sea-lagoon canals, and with the output by the eastern sea-lagoon canal (Fig. 1).

This study was conducted on a high density mat of *C. linum* extending over 300 hectares located in the south-western area of the West basin (Figs. 1 and 2).

2.2 Conceptual models of the high density macroalgal mat growth and decay

Chaetomorpha linum (Müller) Kütz. is a thin, filamentous unbranched structured Chlorophyta widely distributed in bays, lagoons and estuaries all over the world. It is bottom-floating and can proliferate considerably to form dense and thick mats (Lavery *et al.*, 1991; McGlathery *et al.*, 1997; Menéndez *et al.*, 2002; Sorce *et al.*, 2017).

In the Orbetello lagoon, *C. linum* growth was estimated within about the first 20 cm of the mat, while the underlying part did not carry out photosynthesis (Sorce *et al.*, 2017). When the macroalga cannot perform photosynthesis, it can be subjected to bacterial attacks (Krause-Jensen *et al.*, 1996), to the point that the last decimeter of the mat thickness decays and produces organic mud on the bottom, enriching the sediment in carbon and nitrogen (Corzo *et al.*, 2009) and, in turn, affecting the microbial community (García-Robledo *et al.*, 2008). The anaerobic mineralization that occurs at the sediment–water interface releases inorganic nutrients toward the water column (McGlathery *et al.*, 2001), such as ammonium (NH_4^+) (Marty *et al.*, 1990), carbon dioxide (CO_2) and orthophosphate (PO_4^{3-}) (Peckol and Rivers, 1995a). Phosphorus release occurs mainly in the anoxic degradation of organic detritus (Peckol and Rivers, 1995b). Oxygen and pH decreases lead to the reduction of the Fe^{3+} which involves the breaking of the bond of PO_4^{3-} with the ferric oxides-hydroxides (Holmer, 1999), and subsequently its release into the water column (Gao *et al.*, 2013). This can support growth of the mat, but also liberates soluble and acid volatile sulfides (H_2S, HS^-, S^{2-}; AVS) by the sulfate-reduction bacterial activity, which can

accelerate the under-layer mat decay (Nedergaard *et al.*, 2002). In these dynamics, therefore, macroalgal mats can constitute both a sinking and a source of nutrients (Sfriso *et al.*, 1987; Hanisak, 1993). The mat is thus self-sustaining in a continuum, and can increase or decrease in density in relation to the prevalence of the activity of its over-layer or that of the bacteria on the under-layer. Zou *et al.* (2020) emphasized a positive feedback between macroalgal blooms and benthic P flux, and, due to the impacts of internal nutrient cycling (macroalgae and sediment), affirmed that lake (and lagoon, we say) ecosystems often showed vain recovery efforts precisely for this reason.

In the mat over-layer, where macroalgal growth is highest, N and P availability from upward diffusion of benthic flux could be low due to under-layer macroalgal uptake. In such a case, nutrients should be taken from the water column, causing a downward flux of nutrients from surface into the mat over-layer. Under these conditions, paradoxically, dissolved nutrients analyses provide very often an oligotrophic framework, and are not reliable to determine the actual trophic state of the basin and nutritional bioavailability (Peckol *et al.*, 1994; McGlathery *et al.*, 1997). In this regard, in a high density mat of *C. linum*, Lenzi *et al.* (2013a) observed dissolved nutrient stratifications with relatively higher values on the bottom of $N-NH_4$, dissolved organic nitrogen (DIN), and soluble reactive phosphorus (SRP), while DIN:SRP atomic ratio was decreasing downward. Moreover, they observed significant tissue nutrient stratification: a greater accumulation of N and P in the under-layer thalli than in over-layer.

2.3 Water quality detemination

Physico-chemistry values of the water column were obtained by a multiparameter probe (MP) installed on a fixed location placed in the western basin inside the *C. linum* mat (Fig. 1). The MP was immersed at 50 cm on 160 cm of the water column, about 10 cm from the surface of the mat, and sent the readings every 15 min by GSM frequencies. Variables measured were dissolved oxygen (DO, mg L^{-1}), pH and temperature (T, °C). Salinity (s, practical salinity scale) was measured directly *in situ* during the monthly sampling trials by an ATAGO S/Mill refractometer.

For the water column dissolved nutrient, three sampling stations were positioned: one (N) on the southwestern edge of the mat, towards the SW sea-mouth; the second (F) at the northern edge of the mat, towards the North sea-mouth; the third (O) near MP, in the heart of the higher density part of the mat. Water samples were collected monthly in March, June and October 2017, February, May, July and October 2018, and April 2019. Samples were refrigerated and quickly brought to the laboratory for analysis, where they were filtered at 0.40 µm. Nitrates ($N-NO_3^-$), total dissolved nitrogen (TDN), soluble reactive phosphorus (SRP) and total dissolved phosphorus (TDP) were determined. The analyses were conducted according to APAT IRSA-CNR (2003). $N-NO_3$: SRP molar ratio was them computed.

2.4 Mat density estimates

West basin *C. linum* mat examined (Fig. 1), characterized by high biomass (>5 kg m^{-2}), has persisted in this area stably

since 2008 (Lenzi *et al.*, 2011, 2013a), even during severe dystrophic crises (Sorce *et al.*, 2017), occupying a bottom surface-area between 450 and 350 hectares and moving between east and west by no more than 500–600 m.

Trials to measure the *C. linum* biomass (b) were conducted in June and October 2017, February, May, July and October 2018 and April 2019. The mat had previously been divided into 12 areas (Fig. 2), marked with flags and georeferenced, within which 3 samples were collected along a diagonal transect of each area, by avoiding the few and not very large bare bottom holes. Therefore, 36 samples for the whole mat were weighed for each trial. Samples were collected inside a square metal frame of 3600 cm^2 (60×60 cm), drained for 3 min and weighed directly on board with a portable electronic balance with sensitivity ± 0.5 g. The values were then expressed in kg wet weight per square meter ($kg_{WW} \, m^{-2}$), using conversion factors, according to equation (1):

$$b_x \, kg_{WW} \, 3600 \, cm^{-2*} 10^{-4*} 2.778 = b_y \, kg_{WW} \, m^{-2} \quad (1)$$

where b_x is the *in situ* weighted value.

Mat standing crops were then calculated according to equation (2):

$$SC = b * CT * 10^{-3} \quad (2)$$

where b results from the average of all the estimated values (b_y); CT is the total cover of the substrate by the algal mats estimated through Sentinel-2 satellite images, obtained from the Land-Viewer site (EOS DATA ANALYTICS, USGS/NASA), and calculated through the Fiji-Image software (Schindelin *et al.*, 2012); 10^{-3} is the conversion factor for kg into tonnes, as standing crop was expressed in tonnes wet weight (T_{WW}).

2.5 Sediment labile organic matter estimates

Trials to measure the labile organic matter (LOM) in the top sediment of the substrate covered by the mat were conducted in November 2016, May, July, September and November 2017, April, July, September and November 2018, January, March and June 2019. In each trial, two sediment samples were collected from each of the 12 areas into which the mat was divided (Fig. 2), for a total of 24 samples.

After having displaced the algal layer, 3–4 cm sediment top-layer samples were collected with a 60 mL syringe, lowered from the boat and guided by an appropriate device (Lenzi and Renzi, 2011). The samples were frozen until analysis. All samples were wet sieved with fresh water (1 mm mesh), dried at 75 °C for 48 h and then weighed. Labile organic matter (LOM) was determined as loss of weight after combustion in a muffle furnace for 6 h at 250 °C (Loh, 2005).

2.6 CNPS content in *Chaetomorpha linum*

The macronutrients C, N, P can constitute a limiting factor in the physiology and growth of aquatic vegetation. C can limit growth in important bloom conditions, through an intense hoarding of CO_2, so many species have developed the strategy of using bicarbonates. N and P can constitute important limiting factors and cause stunting. On the contrary, they can abound in eutrophic conditions and produce high

accumulations in algal thalli. In sulfate-rich marine environments, it is not known if S is a limiting factor. Conversely, in eutrophic environments subject to intense dystrophic phenomena, S could accumulate in the thalli as sulfides, as observed in seagrass (Holmer and Nielsen, 2007), and constitute a source for the production of hydrogen sulfide during the anaerobic decay of the plant mass. For this last reason, we also wanted to take this macronutrient into account, of which there is little information in the literature as an elementary component of this opportunistic macroalgae.

The sampling of the *C. linum* thalli was carried out in five stations (CH1–CH5) arranged along a transect that proceeded from the edge of the mat close to the urban center, towards the marine mouth of SW (slc-West; Figs. 1 and 2). Eight sampling trials were conducted between 2017 and 2019 (March, June and October 2017, February, May, July and October 2018, and April 2019). At each station, two samples of thalli (1 kg each) at the surface of the mat (in the first 20 cm), about 50 m apart from each other, were taken and immediately refrigerated. Subsequently, in the lab, the samples were quickly washed with fresh water to remove the salt and debris, dried with paper towels to remove the water in excess, and then dried with an air jet fan. The samples were carefully cleaned of concretions, animals and impurities, using silicone gloves to avoid contaminating the material, and the result obtained was dried at constant weight at 75 °C, and used for analytical determinations.

Total tissue C was determined with the Walkley-Black method (Soltner, 1988) and N with the Kjeldahl method (Egli, 2008). P and S were determined using an inductively coupled plasma − optical emission spectrometry instrument, ICP-OES Agilent 5110, U.S. (Boss and Fredeen, 2004). Using the percentages of the estimated macronutrients, the molar ratios C:P, N:P, C:N and C:S were subsequently calculated. We have introduced the atomic ratio N:S to compare the dynamics of S with respect to the relationship that exists between the two elements in proteins (tissue proteins, nuclear proteins, hormones, enzymes, etc.), whereby N is present for 16% and S varies between 0.5% and 3.5%, with an atomic ratio N:S between 89 and 13.

2.7 Statistical analysis

Data of macroalgal thalli macronutrient content (C, N, P, S) were processed by one way ANOVA, respectively with month (8 levels) and station (5 levels) as fixed and orthogonal factors for each analysis, in order to detect significant differences among sampling trials and stations, respectively.

Cochran's *C*-test was used before each analysis to check for homogeneity of variance and data were ln (x) transformed when necessary (Underwood, 1997). The *post-hoc* Tukey's test was used to perform *a posteriori* multiple comparisons of means. Significant effects were considered for $p < 0.05$.

Linear regressions analyses were performed to test the relationship between thalli macronutrient contents and LOM values obtained in the same stations or in very close point-stations (Fig. 2), at about the same time of the thalli sampling (LOM1), and at 2–3 months earlier than the thalli sampling (LOM2). The degree of correlation between each macronutrient and LOM was calculated and reported as squared correlation coefficient (determination coefficient, r^2). The relationship with macronutrients was also tested for

macroalgal biomass (*b*) following the same procedure. Significance of regressions was tested by the Fisher's exact test (*F*).

3 Results

3.1 Water quality

The monthly ranges of the chemical-physical variables monitored during the period examined by the MP placed in the

Table 1. Physico-chemistry variables of the water column obtained by a multiparameter probe placed in the western basin inside the *C. linum* mat. DO, dissolved oxygen; pH; T, temperature; s, salinity (practical salinity scale).

	DO mg L^{-1}	pH	T °C	s
March-17	4.5–12.0	8.16–8.90	11.4–17.7	29
June-17	0.0–11.4	7.15–8.41	24.9–31.6	34
Oct-17	4.0–17.5	8.20–8.82	16.2–22.9	42
Febr-18	5.6–9.2	8.14–8.68	7.7–12.5	30
May-18	0.0–20.0	8.10–9.10	17.4–27.0	32
July-18	0.0–10.5	7.33–8.70	23.8–31.1	37
Oct-18	3.0–12.7	8.25–8.65	19.0–22.9	40
Apr-19	3.3–9.2	8.30–8.47	12.9–20.2	32

center of the *C. linum* mat, are shown in Table 1. DO, pH and T showed wide variability within the same month. In the warmer months, DO values dropped to zero at night and in the early morning, while in the hours of intense illumination the values often increased in over-saturation. Temperature varied widely over the same month, even 10 °C, as a consequence of meteorological events, due to the low inertia of a laminar water column to atmospheric events. Salinity was more stable within the same month, varying between one month and another in relation to meteorological events and summer evaporation.

Dissolved nutrient concentration, estimated between April 2018 and April 2019 in the water column of the stations O (inside the mat) and N and F (at the western and north margins of the mat, respectively), are shown in Figure 3. Means (±SD) of N-NO$_3$, TDN, SRP and TDP for the entire period examined, for each station and for all records, are reported in Table 2. SRP values presented the widest variability, those of TDP were a little less variable. Nitrate values proved less variable overall and, above all, within each month, and highlighted the northern station as a probable major source, while TDN, although the variability of the data was relatively important, presented values greater within the mat.

Calculating the molar ratio N-NO$_3$:SRP, omitting the components of ammonia and nitrous nitrogen, which have never been higher overall on average than 4 μM, high P-limitation values were obtained, with 835 ± 423, 782 ± 479,
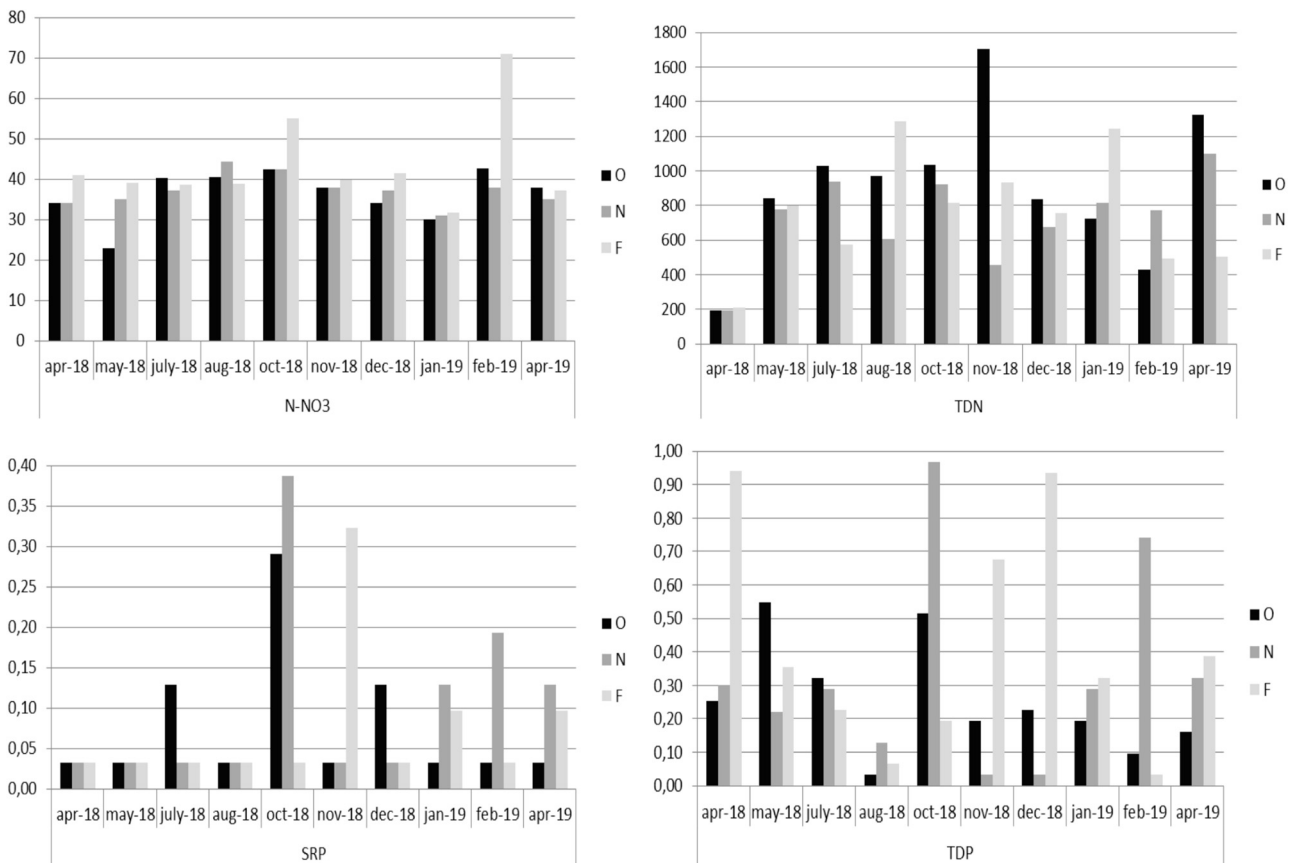

Fig. 3. Nitric nitrogen (N–NO$_3$), total dissolved nitrogen (TDN), soluble reactive phosphorus (SRP) and total dissolve phosphorus (TDP) trends, in μM, estimated between April 2018 and April 2019 at stations-O, central in *C. linum* mat, station-N, at the end of the SW boundary of the mat, and station-F, at the end North boundary of the mat, in the west basin of the Orbetello lagoon.

Table 2. Dissolved nutrient in µM (mean ± SD) estimated in the sampling stations O, N and F, placed inside and on the edges of the *C. linum* mat. N-NO$_3$, nitric nitrogen; SRP, soluble reactive phosphorus; TDN, total dissolved nitrogen; TDP, total dissolved phosphorus. TM, total mean (±SD) among all the station records.

	O	N	F	TM
N-NO$_3$	36.25 ± 5.89	37.17 ± 3.72	43.31 ± 10.75	38.91 ± 8.03
SRP	0.08 ± 0.08	0.10 ± 0.11	0.07 ± 0.09	0.08 ± 0.09
TDN	907.97 ± 403.11	726.42 ± 247.69	761.19 ± 320.03	798.53 ± 339.04
TDP	0.25 ± 0.16	0.33 ± 0.28	0.41 ± 0.31	0.33 ± 0.27

Table 3. Means (±SD) among the 36 biomass stations (b) of the entire *C. linum* mat, and related monthly standing crop (SC), estimated between June-17 and April-19.

	b, kg$_{ww}$/m^2	SC, tonnes ww
June-17	11.80 ± 7.68	35837
Oct-17	7.86 ± 4.92	26690
Feb-18	8.85 ± 4.67	25850
May-18	8.10 ± 5.62	28480
July-18	7.05 ± 5.54	24787
Oct-18	8.23 ± 4.04	28885
Apr-19	7.82 ± 5.115	27348

Table 4. Means (±SD) among all 24 sampling stations, of the percentage of labile organic matter per month (M-LOM) present in the sediments covered by the *C. linum* mat, between November-2016 and June-2019.

	M-LOM
Nov-16	7.91 ± 2.68
May-17	9.58 ± 2.71
July-17	11.63 ± 3.74
Sept-17	12.68 ± 5.01
Nov-17	10.15 ± 3.83
Apr-18	11.47 ± 4.91
July-18	9.30 ± 3.48
Sept-18	11.49 ± 3.00
Nov-18	9.39 ± 2.30
Jan-19	8.58 ± 2.26
Mar-19	8.54 ± 1.71
June-19	8.94 ± 4.19

1090 ± 611 for O, N, F, respectively, for a range between all the stations of 109–2199.

3.2 Mat density estimates

The extension of *C. linum* mat between June 2017 and April 2019 fluctuated between 366 and 340 hectares (Fig. 2). The monthly means (±SD) of biomass for the entire mat and the estimates of SC are shown in Table 3. Among all available records (*n* = 252), the mean was 9.07 ± 5.40 kg$_{ww}$ m^{-2} and the median 8.55 kg$_{ww}$ m^{-2}. The mean mat density was highest in the first monthly estimate; subsequent estimates were lower by 25–40% compared to the initial month. The mat was almost surfacing in the part close to the city of Orbetello, and deepened going west, the water column height varying between 100 and 170 cm.

3.3 Sediment labile organic matter

For the entire mat sediment area, the LOM mean of all records (T-LOM), estimated for the sediments immediately below the mat, was 9.99 ± 3.79%, the median 9.02%, in a range of 4.27–25.48%. Monthly LOM means (±SD) for the entire mat sediment area (M-LOM) are shown in Table 4. On average, the highest organic load values in sediments below the mat occurred between July 2017 and September 2018.

3.4 CNPS content and atomic ratios

A total of 80 records were obtained for the 8 trials, for each macronutrient present in the *C. linum* thalli, from which C resulted on average 2205 ± 1003 µmol % (median 2017 µmol %),

N 102 ± 49 µmol % (median 102 µmol %), P 2.188 ± 1.412 µmol % (median 1.745 µmol %) and S 51.00 ± 0.33 µmol % (median 52 µmol %), with range of 1525-3804 µmol %, 42.60–177.80 µmol %, 0.329–5.677 µmol %, 36.56–72.43 µmol %, respectively.

In Table 5, means (±SD) and medians of each macronutrient between all the records of each trial (March 2017–April 2019) and among all the records of each station (CH1–CH5) are reported.

Results of one-way ANOVA analysis indicated that values of macroalgal thalli macronutrient content significantly varied with factor *Month*, with the exception of P (Tab. 6). However, high variability of the data for the C, P and S values was highlighted by the non-homogeneity of the variances at the Cochran test. The post-hoc Tukey's test comparison showed significantly higher values of N content in March-17 compared to values observed in both June and October-17 ($p < 0.01$) while values of thalli collected in June-17 resulted significantly lower than those collected in July-18 ($p < 0.05$). Similarly, thalli macronutrient content did not vary significantly among the stations with the exception of N, although high variability of data for this variable was highlighted by the Cochran's test (Tab. 6).

Linear regressions analysis showed no significant correlation between P, N and S with LOM1 or LOM2; in the same way, C values were not significantly correlated with LOM2

Table 5. Monthly means (±SD) and medians of C, N, P, S percentages, using the records of all 5 stations, and means (±SD) and medians of each of the stations using all the records available between March-17 and April-19.

	C		N		P		S	
	Mean	Median	Mean	Median	Mean	Median	Mean	Median
Mar-17	24.40 ± 0.55	24.40	2.03 ± 0.75	1.55	0.040 ± 0.020	0.039	1.79 ± 0.15	1.78
June-17	19.16 ± 3.05	18.90	0.99 ± 0.29	0.99	0.030 ± 0.010	0.036	1.02 ± 0.12	1.02
Oct-17	19.07 ± 2.03	19.15	1.05 ± 0.50	1.07	0.080 ± 0.040	0.082	1.97 ± 0.17	2.00
Feb-18	22.42 ± 1.77	23.15	1.71 ± 0.72	1.55	0.110 ± 0.050	0.114	1.58 ± 0.14	1.57
May-18	21.94 ± 2.21	21.65	1.52 ± 0.48	1.62	0.040 ± 0.020	0.047	1.63 ± 0.32	1.55
July-18	38.43 ± 3.77	36.88	1.53 ± 0.41	1.78	0.070 ± 0.040	0.081	1.65 ± 0.07	1.64
Oct-18	32.92 ± 7.37	30.78	1.29 ± 0.29	1.39	0.057 ± 0.042	0.046	1.72 ± 0.13	1.64
Apr-19	32.88 ± 2.56	32.65	1.42 ± 0.40	1.35	0.074 ± 0.028	0.077	1.89 ± 0.18	1.87
CH 1	28.02 ± 10.41	24.20	1.23 ± 0.48	1.32	0.070 ± 0.038	0.072	1.66 ± 0.35	1.74
CH 2	25.59 ± 8.21	22.20	1.31 ± 0.44	1.45	0.074 ± 0.041	0.059	1.61 ± 0.38	1.64
CH 3	26.37 ± 6.52	25.10	1.39 ± 0.41	1.43	0.059 ± 0.026	0.052	1.62 ± 0.26	1.66
CH 4	26.21 ± 7.65	23.50	1.41 ± 0.47	1.40	0.062 ± 0.040	0.046	1.62 ± 0.30	1.67
CH 5	26.13 ± 5.35	24.40	1.83 ± 0.83	1.82	0.074 ± 0.054	0.056	1.67 ± 0.33	1.61

Table 6. Results of one way ANOVA performed on macroalgal thalli macronutrient content for the *Month* and *Station* source of variability. Significant *P* values are reported in bold type; ns, not significant.

		Month				Stations			
	Source	df	MS	F	P	df	MS	F	P
C	RES	7	516.30	35.86	**0.0000**	4	12.06	0.20	0.9394
		72	14.40			75	61.37		
	TOT	79				79			
Cochran's test			C = 0.5243 (*P* < 0.01)				C = 0.3557 (ns)		
N	RES	7	1.28	4.34	**0.0005**	4	1.13	3.31	**0.0148**
		72	0.29			75	0.34		
	TOT	79				79			
Cochran's test			C = 0.2628 (ns)				C = 0.4830 (*P* < 0.01)		
P	RES	7	0.02	1.87	0.0865	4	0.1105	0.19	0.9413
		72	0.01			75	0.5722		
	TOT	79				79			
Cochran's test			C = 0.9035 (*P* < 0.01)				C = 0.3612 (ns)		
S	RES	7	0.83	24.52	**0.0000**	4	0.0111	0.10	0.9818
		72	0.03			75	0.1097		
	TOT	79				79			
Cochran's test			C = 0.4169 (*P* < 0.01)				C = 0.2636 (ns)		

data-set, while significant correlation, albeit modest, was observed with LOM1 data-set (Tab. 7). An equally modest significant correlation between thalli macronutrient content and macroalgal biomass was observed only for nitrogen (Tab. 7).

C:P, N:P, C:N, N:S atomic ratios are reported in Table 8 as mean (±SD) and median between all the stations for each trial, and between all the trials for each station. Means (±SD), medians and ranges between all records for the four atomic ratios are shown in Table 9.

4 Discussion

DO, pH and T values (Tab. 1) highlighted extreme environmental conditions periodically arose in the mat area. In particular, as early as March, DO was depleted between

Table 7. Values of the squared correlation coefficient (r^2) and Fisher's test (*F*). Significant *F* values were reported in bold.

	r^2	F
C − LOM1	0.087	**0.0130**
N − LOM1	0.018	0.2693
P − LOM1	0.010	0.4112
S − LOM1	0.051	0.0591
C − LOM2	0.013	0.3440
N − LOM2	0.000	0.9526
P − LOM2	0.012	0.3786
S − LOM2	0.012	0.3615
C − b	0.038	0.1060
N − b	0.179	**0.0003**
P − b	0.012	0.3767
S − b	0.034	0.1268

Table 8. Monthly means (±SD) and medians of the C:P, N:P, C:N, N:S atomic ratios, using the records of all 5 stations, and means (±SD) and medians of each of the stations using all the records available between March-17 and April-19.

	C:P		N:P		C:N		N:S	
	Mean	Median	Mean	Median	Mean	Median	Mean	Median
March-17	2357 ± 1346	1613	146 ± 55	155	16 ± 5	19	2.7 ± 1.2	2.1
June-17	1691 ± 921	1298	65 ± 11	68	25 ± 12	21	2.2 ± 0.6	2.6
Oct-17	810 ± 419	525	36 ± 9	34	22 ± 10	21	1.2 ± 0.6	1.1
Febr-18	510 ± 150	487	36 ± 12	35	15 ± 5	17	2.5 ± 1.0	2.2
May-18	1699 ± 1349	1157	103 ± 100	85	19 ± 7	17	2.2 ± 0.8	2.3
July-18	1798 ± 1417	1295	59 ± 23	53	29 ± 15	26	2.4 ± 0.7	2.4
Oct-18	1818 ± 843	1845	62 ± 32	58	31 ± 10	29	1.8 ± 0.4	1.8
Apr-19	1466 ± 885	1204	56 ± 42	36	29 ± 9	27	1.7 ± 0.4	1.8
CH1	1417 ± 1033	1254	59 ± 51	46	27 ± 12	22	1.8 ± 0.7	1.9
CH2	1159 ± 669	900	49 ± 31	38	24 ± 8	23	2.0 ± 0.7	2.1
CH3	1516 ± 1207	1301	60 ± 25	67	25 ± 14	20	2.0 ± 0.7	2.0
CH4	1537 ± 991	1295	71 ± 49	61	24 ± 9	24	2.0 ± 0.6	2.0
CH5	1909 ± 1091	1603	97 ± 93	66	22 ± 12	16	2.5 ± 1.1	2.3

Table 9. Means (±SD) and of the C:P, N:P, C:N, N:S atomic ratios, using all available records.

	C:P	N:P	C:N	N:S
Mean ± SD	1498 ± 1124	67 ± 55	24 ± 11	2.1 ± 0.8
Median	1189	50	22	2.0
Range	5623–327	395–18	69–9	0.7–4.7

the night and the early hours of the morning, due to intense respiratory activity, while in the hours of insolation the values went up to supersaturate the water column. With the first increase in temperature, then, the values dropped to zero, at night, even for several hours, to recover in the daytime phase. Similarly, pH increased strongly, due to alkalinization produced by photosynthesis, but it fell well below the more stable marine values, when respiration dominated, due to CO_2 increase. Acidification of the mat substrate and part of the water column could be a stressor, although Young and Gobler (2016) observed, for mats of *Gracilaria* sp. and *Ulva* sp., an increase in growth due to CO_2 partial pressure rise. CO_2 increase induced a shift from the use of bicarbonate to that of CO_2 itself as a source of C (bicarbonate mainly used consequently to the diurnal limitation of CO_2 to high density of the mat). This increase in growth occurred despite the relative acidification of the environmental conditions of the mat.

In the sampling stations for dissolved nutrients O, and F, on a few occasions, SRP values were higher than the analytical detection limit, and this happened just a little more in the station N. Zou *et al.* (2020) observed a positive feedback between macroalgal blooms and benthic P flux, and this was certainly true also in our case; but highlighting this in the field depends on many factors, not least the thickness of the mat. In fact, our results, for a mat between 50 and 100 cm thick, confirmed the difficulty of SRP to go up along the water column and fully affect the over-layer mat, as already noted by Lenzi *et al.* (2013a), despite the over-layer mat being the only

part of the mat that can grow. The lesser variability of TDP was probably due to a greater quantity of organic component (dissolved organic phosphorus), as already observed in this lagoon (Lenzi *et al.*, 2015). Nitrates, on the other hand, were quite stable, maintaining more or less the same order of magnitude of values in the three stations, with slightly higher values in station F (Tab. 2). This station is 3 km from the lagoon-mouth of the North sea-lagoon canal (slc-North), which at the opposite end, in turn, flows near the sea-mouth of Albegna river. The significant annual contribution of nitrogen by the river, ascertained by DICEA (2018), might reach the northern margins of the mat (Fig. 1).

N-NO_3:SRP molar ratio expressed an extreme P-limitation in the first 20–30 cm of the water column. If we consider that the optimal Redfield N:P ratio for phytoplankton is 16 and macroalgae have values around 30 (Atkinson and Smith, 1982), the water column did not bring nutrients in a physiologically balanced ratio for *C. linum*. Hence, P availability might have constituted a limiting factor for achieving the physiologically necessary tissue ratio. However, *C. linum*, although it is not a nitrophilous species and prefers to accumulate P, is rarely considered to be nutrient-limited even for the latter, unless subject to prolonged nutrient scarcity (Lavery *et al.*, 1991). This may mean the times in which the orthophosphates managed to reach the surface of the water column were sufficient to allow adequate storage of P in the over-layer mat thalli by a very efficient uptake.

The biomass showed consistently high values (Tab. 3), which, in some areas, as a consequence of the wind pressure on the mat, exceeded 30 kg$_{WW}$ m^{-2}. Biomass also varied seasonally with winter minimums and spring maximums, to which must be added the effects of anthropic interventions that aimed to mitigate the problem (Lenzi *et al.*, 2017), and more or less extensive summer dystrophic events. Consequently, LOM values were consistently very high (Tab. 4), with peaks greater than 20%. High mat biomass and high LOM established constant anaerobic condition in the sediments which in the spring-summer period led to the release of AVS up to 30–40 cm from the bottom (detected in the field by injecting a few drops

of FeCl₂ solution at different depths). The high density created a permanent extreme condition, which did not lead to the collapse of the mat, but allowed its self-regeneration and probably freed it from competition with other species.

C content showed a large fluctuation with periods of probable limitation, between June and October 2017, which could have led consequence of physiological stress, and with values of relative abundance, between July 2018 and April 2019. We have no data to establish the reasons for these fluctuations, however we must take into account that, in addition to the high densities, between 2014 and 2016 the mat was subjected to intense disturbance through the resuspension of the top layer sediment by boats (Lenzi *et al.*, 2017), and to a heavy dystrophic event (Sorce *et al.*, 2017), which may have contributed to the mat decline. Starting from 2017, the mat was restored, as soon as the anthropogenic pressure was reduced, showing a high resilience.

C. linum thalli, even in a context of wide variability both between stations and between months, showed a tendency towards P-limitation, the tissue molar ratio N:P very often being greater than 30 (Atkinson and Smith, 1983; Wheeler and Bjornsater, 1992), with an average of 67 ± 57 and a median of 49. These results agree with those of a previous study conducted in another *C. linum* high density mat of the same lagoon (Lenzi *et al.*, 2013a), in which tissue P-limitation was found, with N:P mean values of about 170, and more storage of N and P in the mat under-layer than in the over-layer. This indicates that the under-layer was a filter of the nutrients released from the sediment. That study and the present study suggest that the over-layer mat adapted to long periods of limitation alternating with periods of P availability, during which the storage that the thalli managed to carry out was probably sufficient for growth and maintenance.

Sulfur content was relatively high, much greater than that required for formation of proteins containing cysteine and methionine. The N:S atomic ratio was about 2, compared to an expected range of 89–13 for proteins only. Sulfur, in fact, is also present in sulfolipids of the cell membranes, as a constituent of vitamins, regulatory compounds, and in a number of sulfur-metabolites, such as dimethylsulfonionpro-pionate (van Bergeijk, 2000; Gambaro *et al.*, 2007). The latter is a tertiary sulfonic compound present in the cells of various species of macroalgae and microalgae, functioning as an osmoregulator, cryoprotectant and antibacterial. Moreover, some Chlorophyta can synthesize sulfated polysaccharides, such as ulvans in the cell wall of Ulvaceae, which are known to be inducers of plant immunity (Jaulneau *et al.*, 2010; Li *et al.*, 2018). Having strong antioxidant activity, they can neutralise reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Qi and Sun, 2015). According to Bischof *et al.* (2006), *C. linum* is highly tolerant to UV and PAR radiation. If we consider that the over-mat can reach the surface of the water column, it can increase production of antioxidant molecules and super-oxide dismutase activity as a physiological acclimation strategy to persistent and high light exposure. In addition, Freile-Pelegrín *et al.* (2020) extracted water-soluble sulfated polysaccharides with gelling capacity and antioxidant activity from *Chaetomorpha gracilis*. Pierre *et al.* (2011) obtained a water-soluble extract from *Chaetomorpha aerea*, consisting of a sulfated (6.3%) galactan and dextran sulfate or fucoidan polysaccharides that showed selective antibacterial activities. A similar result was obtained for *C. linum* by Rosaline *et al.* (2017), and Venkata Rao and Sri Ramana (1991) detected the presence of sulfated arabinoga-lactans in *Chaetomorpha anteninna*.

Our results showed that the quantity of sulfur is more stable compared to the other three macronutrients, as evidenced by the N:S atomic ratio (Tab. 8). Moreover *C. linum* never manifested S-limitation conditions. In fact, since the sulfur demands of algae are met mainly by the sulfate, sulfur is unlikely to be a growth limiting factor for algae in marine and lagoon environments (Hawkesford, 2008).

Using the records of all 5 stations, monthly means and medians of C, N, P, S were found to vary from each other, more than the means and medians of each station varied from each other, using all the records available between March-17 and April-19. This means greater seasonal variability, rather than between one area and another on the mat (Tab. 5). Moreover, the high data dispersion did not allow to establish significant variations with due reliability among both stations and months for C, P, S; only N showed significant differences for a few months (Tab. 6). This last result could be a seasonal effect, when the months that differed from each other were June and October 2017 from March 2017, where this last month had higher N values, and the months of the intermediate seasons the lower values, probably due to intense mat growth; while the significant difference between June 2017 and July 2018, with the latter month having highest values due to macroalgal decay, was due to local and meteorological factors.

No correlations were found between the macronutrients neither with LOM nor with biomass. The modest correlations observed, $C - LOM1$ and $N - b$, have a r^2 decidedly too low to take them into account (Tab. 7). Since labile organic matter is a source of nutrients and the algal mass creates sedimentary anoxia capable of releasing orthophosphates, it is conceivable that the over-layer mat captured the nutrients during the brief period in which they were available in the upper layer of the water column, storing and using them quickly in view of their scarcity, so that the cycles of biomass, organic matter in the sediments, and the macronutrients contained in the thalli, were disconnected and apparently random.

C:N and, above all, N:S showed greater data homogeneity, compared to C:P and N:P (Tabs. 8 and 9). This depended on the percentage values of N and S in the thalli, the latter in particular resulted fairly stable in the various samples monthly and over the entire period examined, and on the wide variability of P.

5 Conclusions

The responses to the goals we set, *i.e.* any changes in the macronutrients C, N, P, S content in a high density mat of *C. linum*, and any significant relationships between the quantity of LOM present in the sediments covered by the mat, and the density of the mat itself, are summarized in the following points: the seasons and, above all, the extreme summer environmental conditions can affect the content of the macronutrients, but significant changes were not observed between the different sampling points along the mat in each of the trials; significant relationships between mat density and LOM were not highlighted. As for the content in S of the thalli,

as a third objective, it was relatively high. The variability of the data, however, does not reveal a synchronous behavior of the mat.

Over a long period, we observed that when a mat of *C. linum* "takes root" in an area, it remains for a long time, unless there are important environmental events. We hypothesize that the accumulations of bottom-floating macro-algal masses in a given area, can be random, often due to the wind, therefore normally this occurs in relatively low-energy areas where what is pushed and accumulated no longer moves; but then the high density of the mat and the accumulation of high quantities of LOM in the sediment impose peculiar environmental conditions, in a sort of process of colonization of the territory. And it is precisely in these conditions, chaotic, variable and tendentially extreme, that *C. linum* finds advantage because it re-assimilates nutrients of its own degradation, collects atmospheric carbon in addition to that deriving from its degradation. This prevents C-limitation that could occur at very high densities, and seems to help it tolerate bacterial aggressions. This tolerance could be due to the presence of antibacterial sulfur-metabolites, which could explain, at least in part, the considerable resilience to environmental adversity of *C. linum* high density mat; the quantity of S in the thalli allows us to consider this possibility as an important element to be investigated in further research.

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