ORIGINAL ARTICLE

Phytoplankton RNA/DNA and 18S rRNA/rDNA ratios in a coastal marine ecosystem

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The RNA/DNA ratio is used as indicator of growth in various marine organisms and to assess physiological status at species or community level. To evaluate the utility of the RNA/DNA ratio as a proxy of phytoplankton primary production, the relationships between phytoplankton RNA/DNA, taxon-specific diatom and dinoflagellate 18S rRNA/rDNA ratios and autotrophic phytoplankton biomass were investigated as a first step. Significant correlations between all phytoplankton ratios and total phytoplankton, diatom and dinoflagellate biomass as chlorophyll a (chl a) and carbon content were found. Diatoms showed higher correlation than dinoflagellates (18S rRNA/rDNA vs. chl a, $r_s = 0.74$ and 0.64, $P < 0.001$; 18S rRNA/rDNA vs. carbon, $r_s = 0.66$ and 0.53, $P < 0.001$, respectively), because they represented the most abundant and frequent group within sampled assemblages. Further, phytoplankton biomass production is known to be linked to protein biosynthesis and significant relationships between RNA/DNA ratios and protein content of phytoplankton assemblage were found ($r_s = 0.62$ and 0.52, $P < 0.001$ for diatom and dinoflagellates, respectively). As taxon-specific RNA/DNA ratios were correlated with biomass and protein content, our results can be regarded as the first step toward further studies on the applicability of RNA/DNA ratios as indicators of growth rate and primary production in phytoplankton assemblages.

KEYWORDS: biomass; growth; 18S rRNA/rDNA ratio; phytoplankton; primary production

INTRODUCTION

Marine phytoplankton includes a wide set of different phylogenetic taxa from eukaryotes to prokaryotes and accounts for ∼50% of global primary production (Falkowski et al., 1998; Falkowski et al., 2007; Sommer et al., 2017). Through the sedimentation of fixed organic matter, phytoplakton primary production acts as a biological pump that removes carbon from the surface ocean thus playing a major role in global carbon cycle and climate regulation (Legendre et al., 2015; Polimene et al., 2017; Tréguer et al., 2018). As primary production is directly related to the global carbon cycle, its estimation at phytoplankton assemblage level has received much attention and, accounting for phytoplankton community structure, has crucial importance to improve our understanding of oceanic biogeochemical cycles (Uitz et al., 2010; Toseland et al., 2013; Jensen et al., 2019).

Primary productivity may be defined as the product of phytoplankton biomass times phytoplankton growth rate (Cloerns et al., 2014) and primary productivity or different metabolic parameters (e.g. growth rate, organic carbon, DNA, ATP) can provide direct or indirect measurements of autotrophic activity in the marine ecosystems, respectively (Valiela, 1995). Primary productivity and metabolic activity rates of phytoplankton vary in relation to abiotic (e.g. water column stability, light, temperature, nutrients) and biotic (e.g. cell size and shape, colony formation, grazing pressure) factors and constraints (Valiela, 1995). Large-scale variability of phytoplankton growth rates depends primarily on resource supply and a consistent phytoplankton growth rate gradient from oligotrophic to coastal and productive waters has been described (Cermeño et al., 2006; Marañón et al., 2014).

The northern western (NW) Adriatic Sea (Mediterranean Sea) is characterized by a continental shelf that receives important freshwater riverine input originating mainly from rivers. One of the main riverine inputs is from the Po River; it contributes about 50% of external nutrient inputs (Penna et al., 2004; Giani et al., 2012; Djakovac et al., 2015; Grilli et al., 2020). In the Adriatic Sea, coastal trophic conditions, characterized by nutrient availability and riverine input variability, generally sustains seasonal winter–spring and autumn blooms dominated by numerous frequently occurring diatom taxa of high biomass, including *Chaetoceros* spp., *Pseudo-nitzschia* spp. and *Skeletonema marinoi* (Bernardi Aubry et al., 2004; Mangoni et al., 2008; Socal et al., 2008; Mangoni et al., 2013; Penna et al., 2013; Totti et al., 2019).

Autotrophic activity can be estimated by both direct and indirect estimations. Phytoplankton primary productivity is directly estimated by ^{14}C seawater microorganism incorporation measurements or the increase in the concentration of soluble oxygen (Steemann-Nielsen, 1952; Platt and Sathyendranath, 1993; Antal et al., 2001; Marra, 2009). Even though these methods are extremely sensitive, they involve numerous artifacts, such as physiological stress on phytoplankton during sampling, manipulation and enclosure (Eppley, 1980) and uncertainties about what photosynthetic processes are being measured (Williams, 1993). Moreover, the radioactive incorporation method is labor-consuming and requires handling radioactive isotopes, adequate facilities and equipment that is not always available (Marra, 2009). Indirect measure of the photosynthetic activity can be provided by pump-and-probe fluorometer methods giving fluorescence-based productivity estimates (Kolber and Falkowski, 1993; Schreiber et al., 1995; Garrido et al., 2013).

Given the difficulties of directly estimating primary production, many primary production models have been developed. These models are based on field measurements and remotely sensed data. Different models have been compared and variability between them attributed to various sources of error and inaccuracy (Longhurst et al., 1995; Behrenfeld and Falkowski, 1997; Goetz et al., 1999; Saba et al., 2010; Saba et al., 2011). It seems that the most accurate measurements of community production are those based on field measurements of metabolic activities including growth rates and biomass variation.

Photosynthetic primary production generates various biochemical components, such as nucleic acids, proteins, carbohydrates and lipids within the phytoplankton biomass. For decades, DNA, RNA, RNA/DNA ratio, DNA/dry weight ratio, proteins and RNA/protein ratio have been used as indicator of living biomass predicting quality plankton growth (Nejstgaard et al., 2003; Berdalet et al., 2005; Ikeda et al., 2007; Finkel et al., 2016). These indicators have been used to assess the biomass and growth rate of planktonic species and community providing useful information on real living individuals in high activity within a population assuming the growth rate as a function of RNA/DNA ratio (Dortch et al., 1983). Further, the RNA/DNA ratio has been used as a proxy for short-term growth potential in dominant zooplankters, copepods and rotifers in the Baltic Sea (Gorokhova et al., 2014) and as a growth indicator for the periphyton in freshwater ecosystems (Mewes et al., 2017). However, the rRNA use as a reliable indicator of metabolic state in microbial assemblages may have limitations due to the variability between rRNA concentration and life strategies of microbial communities or single microbial taxon (Blazewicz et al., 2013).

However, rRNA can be used as indicator of protein synthesis rather than metabolic activity with the purpose of understanding the complex dynamics in microbial communities. Proteins are known to be always a major contributor to the total biomass, followed by lipids and carbohydrates, providing a higher range of values (12–35%) than lipids and carbohydrates (Brown, 1991; Fabiano *et al.*, 1993). Further, proteins retain highest efficiency in carbon transfer to herbivores through food web (Bleakley and Hayes, 2017; Campanyà-Llovet *et al.*, 2017; Ruess and Müller-Navarra, 2019).

While RNA/DNA ratio has been used for evaluating potential growth in various organisms, from microbes to mammals (Berdalet and Estrada, 1993; Kemp *et al.*, 1993; Elser *et al.*, 2003; Chícharo and Chícharo, 2008; Lin *et al.*, 2013; Lankiewicz *et al.*, 2016; Fu and Gong, 2017), few data are available for marine eukaryotic phytoplankton (Dortch *et al.*, 1983; Berdalet *et al.*, 2005). Consequently, the effectiveness of using rRNA/rDNA to infer growth rate in photosynthetic organisms has not been clearly demonstrated (Lee *et al.*, 2009; Nicklisch and Steinberg 2009; Lin *et al.*, 2018). Furthermore, rRNA/rDNA ratio data from field studies in relation also to taxon-specific metabolic activity in phytoplankton assemblages are still lacking. Instead, ribosomal rRNA and rDNA have been used to quantify target phytoplankton species and to characterize genotypes and ecotypes in marine ecosystems (Medlin *et al.*, 2000; Godhe *et al.*, 2008; Penna and Galluzzi, 2013; Casabianca *et al.*, 2014; Casabianca *et al.*, 2017; Limardo *et al.*, 2017; Pugliese *et al.*, 2017; Rengefors *et al.*, 2017; Sefbom *et al.*, 2018).

In the present study, our working hypothesis was that there is a relationship between phytoplankton biomass and total phytoplankton RNA/DNA ratio. We also examined taxon-specific phytoplankton 18S rRNA/rDNA ratios, quantitatively determined by real-time polymerase chain reaction (PCR), and protein content within the assemblages. Although a significant correlation between RNA/DNA ratio and phytoplankton biomass would not be a direct proof that these molecular variables can be regarded as a proxy for phytoplankton primary production, biomass is certainly a main driver of primary production. Therefore, a significant correlation of phytoplankton assemblage RNA/DNA ratios and biomass is a necessary condition for further exploring the relationships between phytoplankton RNA/DNA and 18S rRNA/rDNA ratios and primary production through direct measurements in marine coastal ecosystems. Therefore, we tested several bivariate hypotheses of independence by analyzing phytoplankton RNA/DNA and diatom and dinoflagellate 18S rRNA/rDNA ratios, proteins and biomass, which was estimated through

chlorophyll *a* (chl *a*) and phytoplankton carbon content based on monthly surveys.

MATERIALS AND METHODS

Study sites and seawater sampling

A total of 40 surface seawater samples (0.5 m depth) were collected monthly by Niskin bottle from May 2018 to December 2019 along transects of Foglia (43°56′.55 N; 12°56′.18E) and Metauro (43°50′.54 N; 13°05′.9 E) rivers at 3000 m from coastland in the NW Adriatic Sea during the monitoring activities. At each station, a 5 L sample was obtained and, after carefully mixing, various subsamples were fractionated for different analyses. Aliquots of 250 mL subsamples for phytoplankton analysis were dispensed into polyethylene bottles and preserved by adding 0.2% Lugol's solution and stored at +4°C until analysis. Amounts of 4 L were filtered on 3-µm pore size polycarbonate membrane filters (47 mm, TSTP, Merck Millipore, MA) under gentle vacuum. The phytoplankton fraction (mainly picoeukaryotes and prokaryotes) below the pore size of 3 µm was excluded from molecular analyses. Filters were carefully washed using sterile artificial seawater, and phytoplankton cells retained by filters were gently scraped away from the membrane by sterile loops. The recovered cells were harvested by centrifugation at 4000 rpm for 15 min at room temperature and pellets stored at −80°C until nucleic acid extractions. Finally, seawater subsamples were also collected for chl *a* determination. Samples were filtered on Millipore nitrocellulose filters with 0.45-µm pore size (Millipore, Bedford, MA) and immediately analyzed.

Phytoplankton analyses

Identification and counting was carried out using an inverted microscope (Zeiss Axiovert 40 CFL) equipped with phase contrast according to the Utermöhl method (Hasle, 1978). Counting was carried out at ×200 or ×400 magnification on entire Utermöhl chamber. Identified taxa were reported per major groups such as diatoms, dinoflagellates and other flagellates. The latter group included phytoplankton taxa mostly <10 µm of uncertain taxonomic identification under light microscopy. Dinoflagellates were considered as an entire taxonomical group including both autotrophic and heterotrophic species in counting. Total phytoplankton through the manuscript is considered as taxa detectable by light microscopy excluding, therefore, prokaryotes and picoeukyotes. Phytoplankton biomass was estimated

through cell biovolume: it was estimated by measuring single cells and assigning cell shape approximately to geometrical figures (Hillebrand *et al.*, 1999). Then, total carbon content per each taxon was calculated from mean biovolume using the formula introduced by Menden-Deuer and Lessard (2000) and biomass was expressed as µg C L^{-1}.

DNA, RNA and protein extraction, qPCR assay and 18S rRNA/rDNA ratio calculation

The genomic DNA and total RNA from seawater samples were extracted using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) and TRIzol Reagent (Invitrogen, Carlsbard, CA), respectively, according to the manufacturer's instructions with DNA and RNA being eluted in a final volume of 50 µL RNase-free water. An amount of 50 ng of exogenous human DNA and RNA standards were added immediately after cell disruption in order to improve assay accuracy, as nucleic acids were extracted by different procedures and as RNA required a reverse-transcription step to obtain cDNA. A purification step for total RNA was performed using the RNeasy mini kit (Qiagen, Hilden, Germany) including on-column DNase digestion with the RNase-Free DNase Set (Qiagen, Hilden, Germany) and a final elution in 50 µL RNase-free water.

The cDNA was prepared from equivalent amounts of DNase-treated RNA using SuperScript III First-Strand Synthesis SuperMix (Invitrogen, Life Technologies, Carlsbad, CA) in a 2 720 Thermal Cycler (Applied Biosystems, Foster City, CA) following manufacturer's instructions.

In each sample, proteins were isolated from the phenol-ethanol supernatant kept from total RNA extraction following the manufacturer's instructions. DNA, RNA and proteins were quantified using a Qubit fluorometer with a Qubit dsDNA HS Assay Kit, Qubit RNA HS Assay Kit and Qubit Protein Assay Kit (Invitrogen, Carlsbard, CA), respectively. Considering the quantification of total extracted DNA and RNA, RNA/DNA ratios in each sample were calculated. Extracted nucleic acids were stored at $-80°C$ until quantitative PCR (qPCR) analysis. Proteins from representative samples were visualized on 8% polyacrylamide gel (data not shown).

The qPCR reactions were performed in a final volume of 25 µL using the Hot-Rescue Real-time PCR Kit SG (Diatheva, Fano, Italy). Class-specific primers targeting diatoms and dinoflagellates were used as described in Casabianca *et al.* (2020). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers (Galluzzi *et al.*, 2012) were used for the amplification of the human exogenous control with some modifications: MgCl$_2$ and primer final concentration were 2.5 mM and 100 nM, respectively.

DNA and cDNA were used as templates in separate reactions. All amplification reactions were carried out in a StepOne Real-time PCR System (Applied Biosystems, Foster City, CA). Thermal cycling conditions were 10 min at 95°C, followed by 40 cycles at 95°C for 15 s and 52, 60 and 62°C for 1 min for diatoms, dinoflagellates and GAPDH, respectively. For diatoms and GAPDH, an extension step at 72°C for 30 s was added. All samples were run with three biological replicates, each of which was run with three technical replicates. In all experiments, negative controls containing MilliQ water were tested. At the end of each run, a melting curve analysis was performed to exclude the presence of primer dimers or non-specific amplified products.

Standard curves of 18S rDNA of diatoms and dinoflagellates were constructed following Casabianca *et al.* (2019). Standard curves of diatom and dinoflagellate 18S rRNA were generated from six 10-fold dilution series of cDNA obtained from reverse transcription of RNA extracted from diatom *S. marinoi* CBA4 and dinoflagellate *Alexandrium minutum* CBA57 cultures. Finally, standard curves were also generated from six 10-fold dilution series of GAPDH target using human DNA and cDNA. Acquisition of the qPCR data and subsequent analyses were carried out using StepOne Software ver. 2.3. Standard curves were created automatically and accepted when the slopes were between -3.55 and -3.32 (91–100% efficiency) and the determination coefficient (r^2) was at least 0.99. Amplification efficiency was calculated as $(10^{(-1/\text{slope})} - 1) \times 100$. The 18S rRNA/rDNA ratio of each class of diatoms and dinoflagellates in each sample was calculated on the basis of a conceptual model described in Pfaffl (2001) using cycle threshold obtained by qPCR of diatom and dinoflagellate 18S and GAPDH cDNAs and DNAs, respectively. Relationship between taxon-specific 18S rRNA/rDNA ratio and diatom and dinoflagellate biomass or other molecular variable variations was investigated.

Chl *a* analyses

Chl *a* was determined in 90% acetone homogenates of particulate matter collected on nitrocellulose filters. The Chl *a* was analyzed spectrophotometrically (mod. UV-1700, Shimadzu, Japan) according to the method described by APHA-AWWA-WPCF (1985).

Statistical analyses

Spearman's rank correlation was computed in place of linear correlation because our goal was to detect monotonic relationships, not only strictly linear ones. All statistical analyses were performed with PAST ver. 4.01 (Hammer *et al.*, 2001).
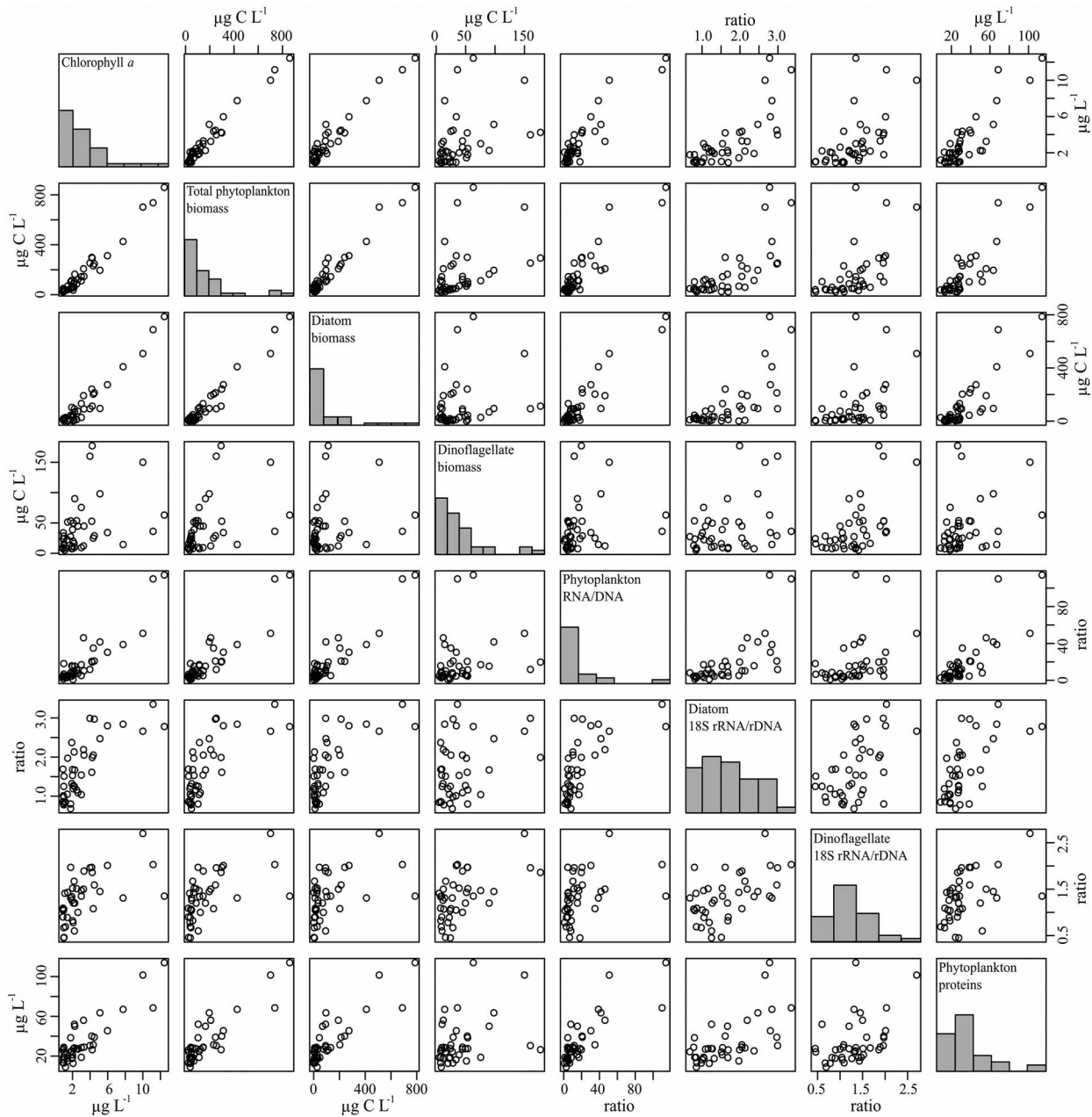
Fig. 1. Matrix plot showing data distributions as histograms in the main diagonal and bivariate relationships among all the studied variables as scatterplots. Names of variables in each row and column are shown above the corresponding histogram.

RESULTS

Standard curve characterization

Different standard curves were generated using DNA or cDNA, which was obtained from reverse transcription of extracted RNAs. In particular, when DNA was used as templates, the mean standard curves for diatoms, dinoflagellates and GAPDH showed a PCR efficiency ranging from of 91 and 100% (mean standard curves: $y = -3.55x + 33.84$; $y = -3.32x + 28.60$ and $y = -3.31x + 28.52$ for diatoms, dinoflagellates and GAPDH, respectively) and a linear relationship over six orders of magnitude ($r^2 = 0.99$). When cDNA was used as templates, the mean standard curves for diatoms, dinoflagellates and GAPDH showed a PCR efficiency ranging from 96% and 98%, respectively (mean standard curves: $y = -3.42x + 14.88$; $y = -3.37x + 19.81$ and $y = -3.40x + 26.83$ for diatoms, dinoflagellates

Table I: Phytoplankton (total phytoplankton, diatoms and dinoflagellates) biomass (µg C L⁻¹), chl a (µg L⁻¹), phytoplankton RNA/DNA ratio, diatom and dinoflagellate 18S rRNA/rDNA ratio and phytoplankton proteins in the present study

	Average	Minimum	Maximum
Total phytoplankton (µg C L^{-1})	168.7 ± 31.4	23.0 ± 1.7	859.8 ± 39.7
Diatoms (µg C L^{-1})	119.3 ± 28.9	0.7 ± 0.05	786.8 ± 51.9
Dinoflagellates (µg C L^{-1})	41.4 ± 6.6	5.0 ± 0.3	177.2 ± 7.8
Chlorophyll a (µg L^{-1})	3.2 ± 0.4	0.9 ± 0.1	12.4 ± 0.6
Phytoplankton RNA/DNA ratio[a]	18.9 ± 4.0	1.0 ± 0.1	114.2 ± 6.1
Diatom 18S rRNA/rDNA ratio[a]	1.7 ± 0.1	0.7 ± 0.1	3.4 ± 0.1
Dinoflagellate 18S rRNA/rDNA ratio[a]	1.3 ± 0.1	0.4 ± 0.1	2.7 ± 0.3
Phytoplankton proteins (µg L^{-1})	33.9 ± 3.6	8.9 ± 1.4	114.0 ± 7.5

Average, minimum and maximum values ± standard error are reported.
[a]Phytoplankton RNA/DNA and taxon-specific 18S rRNA/rDNA ratios are obtained by different methods, and therefore, they represent different estimations. Data can't be compared based on absolute ratio values.

and GAPDH, respectively) and a linear relationship over six orders of magnitude ($r^2 = 0.99$). The standard curve slopes were used for calculation of diatom and dinoflagellate 18S rRNA/rDNA ratios as reported below.

Data distribution analysis

Total phytoplankton, diatom and dinoflagellate biomass, chl *a*, and molecular variables, such as phytoplankton RNA/DNA, diatom and dinoflagellate 18S rRNA/rDNA ratios and proteins were analyzed (Table I). Obtained data generally showed positively skewed asymmetrical distributions (Fig. 1). Diatom 18S rRNA/DNA ratio and proteins showed a less pronounced asymmetry, but the only exception was represented by dinoflagellate 18S rRNA/DNA ratio distributions, showing an almost normal distribution. Most variables showed direct proportionality to each other, although some cases involved monotonic rather than linear relationships. Only a small number of outliers was detected. In fact, most variables only had 2 or 3 out of 40 values exceeding the mean by >2 SD units, whereas diatom and dinoflagellate 18S rRNA/rDNA ratios showed no or only one outlier, respectively.

In our samples, diatoms included various taxa, among which *Chaetoceros* spp., *Pseudo-nitzschia* spp. and *S. marinoi* were the most abundant (Table II). Dinoflagellate taxa were represented mostly by *A. minutum*, *Gymnodinium* spp. and *Heterocapsa niei*. In the studied period, the contribution of various taxa abundance was different within the phytoplankton assemblages. In autumn–winter (November–February), diatoms were the most abundant group being responsible for the highest biomass values due to *S. marinoi* and *Chatoceros* spp. bloom events. Diatom biomass was also relevant (>60%) in April with highest abundance of *S. marinoi*, *Chatoceros* spp. and *Pseudo-nitszchia* spp. In this

Table II: List of main phytoplankton diatom and dinoflagellate taxa found in the assemblages at the sampling stations of Foglia and Metauro in the study period

Diatoms	Dinoflagellates
Asteromphalus flabellatus	*Alexandrium* spp.
Cerataulina pelagica	*Ceratium furca*
Chaetoceros affinis	*Ceratium fusus*
Chaetoceros decipiens	*Ceratium trichoceros*
Chaetoceros spp.	*Dinophysis rotundata*
Cyclotella sp.	*Dinophysis sacculus*
Cylindrotheca fusiformis	*Diplopsalis* sp.
Dactyliosolen fragilissimus	*Gonyaulax* sp.
Ditylum brightwellii	*Gymnodinium* sp.
Guinardia striata	*Gyrodinium fusiforme*
Hemialus hauckii	*Gyrosigma* sp.
Leptocylindrus danicus	*Heterocapsa niei*
Leptocylindrus minimus	*Heterocapsa* sp.
Leptocylindrus sp.	*Minuscola bipes*
Lioloma pacificum	*Noctiluca scintillans*
Melosira sp.	*Oxytoxum scolopax*
Navicula sp.	*Oxytoxum variabile*
Pleurosigma sp.	*Prorocentrum cordatum*
Pseudo-nitzschia spp.	*Prorocentrum lima*
Pseudo-nitzschia pungens	*Prorocentrum micans*
Rhizosolenia sp.	*Prorocentrum triestinum*
Skeletonema marinoi	*Protoperidinium diabolus*
Thalassionema nitzschioides	*Protoperidinium* sp.
Thalassionema sp.	*Protoperidinium steini*
Thalassiosira rotula	*Scrippsiella* sp.
Thalassiosira sp.	*Scrippsiella trochidea*
	Und. dinoflagellates

period, dinoflagellates were also present showing biomass value up to one order of magnitude lower than those of diatoms. In late spring–early autumn (May–October), dinoflagellates were more frequent giving highest contribution to the total biomass with exception in August and September (Fig. 2).
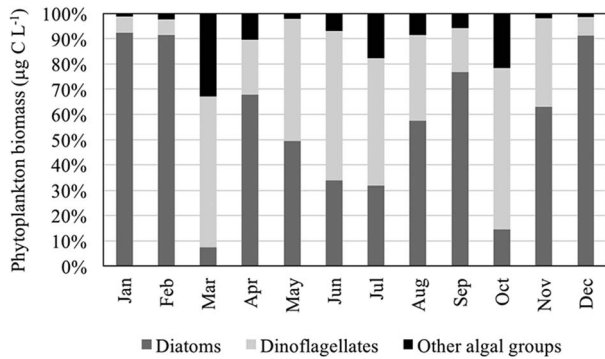
Fig. 2. Mean percent composition of main phytoplankton group assemblages in terms of biomass (μg C L^{-1}) on a monthly basis.

Relationship between biomass and RNA/DNA and 18S rRNA/rDNA ratios and proteins within phytoplankton assemblages

The hypothesis of independence between biomass and RNA/DNA ratios and proteins obtained from phytoplankton assemblages, including the main class specific groups of diatoms and dinoflagellates, was tested for each pair of variables by means of Spearman's rank correlation. A significant positive correlation was found between chl *a* and all variables (Fig. 3), namely total phytoplankton ($r_s = 0.93$, $P < 0.001$), diatom and dinoflagellate biomass ($r_s = 0.83$, $P < 0.001$ and $r_s = 0.51$, $P < 0.001$, respectively), total phytoplankton RNA/DNA ratio ($r_s = 0.80$, $P < 0.001$), 18S rRNA/rDNA ratios of diatoms and dinoflagellates ($r_s = 0.74$, $P < 0.001$ and $r_s = 0.64$, $P < 0.001$, respectively) and total phytoplankton proteins ($r_s = 0.75$, $P < 0.001$). Moreover, total phytoplankton RNA/DNA ratio showed a significant positive correlation with both diatom and dinoflagellate 18S rRNA/rDNA ratios ($r_s = 0.75$, $P < 0.001$ and $r_s = 0.54$, $P < 0.001$, respectively) and with phytoplankton proteins ($r_s = 0.75$, $P < 0.001$). Phytoplankton proteins showed a significant positive correlation with both diatom and dinoflagellate 18S rRNA/rDNA ratios ($r_s = 0.62$, $P < 0.001$ and $r_s = 0.52$, $P < 0.001$, respectively).

Total phytoplankton RNA/DNA ratio varied in accordance with the main traits of phytoplankton biomass, in terms of chl *a* and carbon content. Further, total phytoplankton RNA/DNA ratio agreed significantly with proteins in the phytoplankton assemblages, and details about those relationships were shown in Figure 4. All the Spearman's rank correlations were significant and ranged from 0.75 to 0.83.

Taxon-specific measurements of diatom and dinoflagellate 18S rRNA/rDNA ratios were carried out using taxon-specific qPCR assays and the details about the relationships among diatom and dinoflagellate 18S
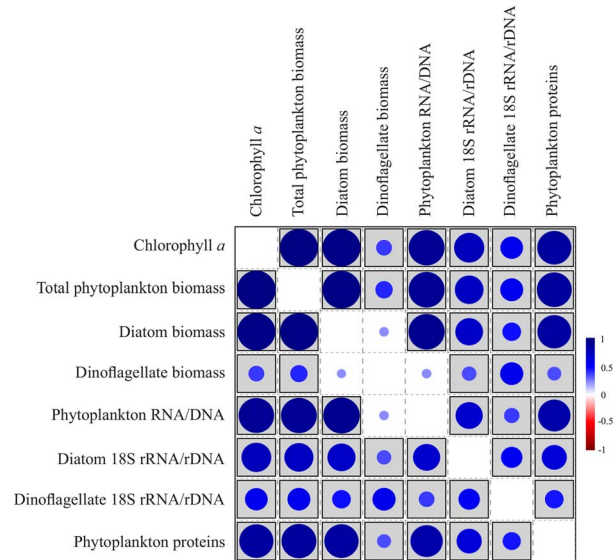

Fig. 3. Spearman's correlation values among chl *a* (μg L^{-1}), total phytoplankton, diatom and dinoflagellate biomass (μg C L^{-1}), phytoplankton RNA/DNA ratio, diatom and dinoflagellate 18S rRNA/rDNA, phytoplankton proteins (μg L^{-1}) obtained in this study. Gray boxes indicate significant correlations.

rRNA/rDNA ratios and the other variables were shown in Figure 5. While the resulting data patterns showed some scattering around a perfect linear agreement, diatom and dinoflagellate 18S rRNA/rDNA ratios clearly varied according to phytoplankton biomass in terms of chl *a* ($r_s = 0.74$ and 0.64, respectively, $P < 0.001$). Taxon-specific 18S rRNA/rDNA ratios were significantly correlated also to biomass in term of carbon content with r_s values of 0.66 ($P < 0.001$) and 0.53 ($P < 0.001$) for diatoms and dinoflagellates, respectively. The relationships between diatom and dinoflagellate 18S rRNA/rDNA ratios and total phytoplankton RNA/DNA ratio and protein content showed similar levels of agreement.

DISCUSSION

Estimation of RNA/DNA ratios have been used to provide a fast and easy method to assess *in situ* functioning status of a wide range of aquatic organisms (Chícharo and Chícharo, 2008). Although, these molecular variables have been applied as indicator of biomass and growth in natural communities (Yebra *et al.*, 2011; Bowman *et al.*, 2017; Mewes *et al.*, 2017), few data are available for the autotrophic component of marine phytoplankton assemblages (Nicklisch and Steinberg 2009).

In our study, significant correlations between total phytoplankton RNA/DNA ratio and taxon-specific 18S
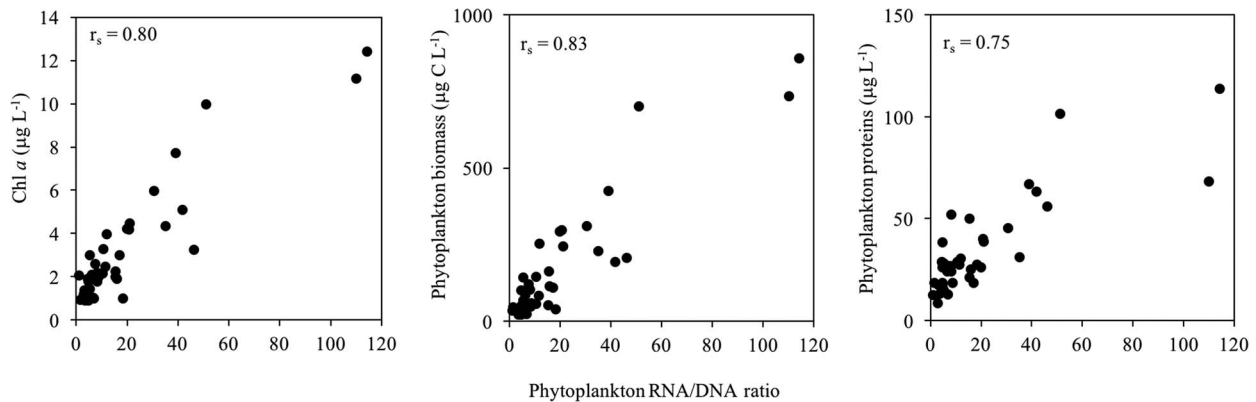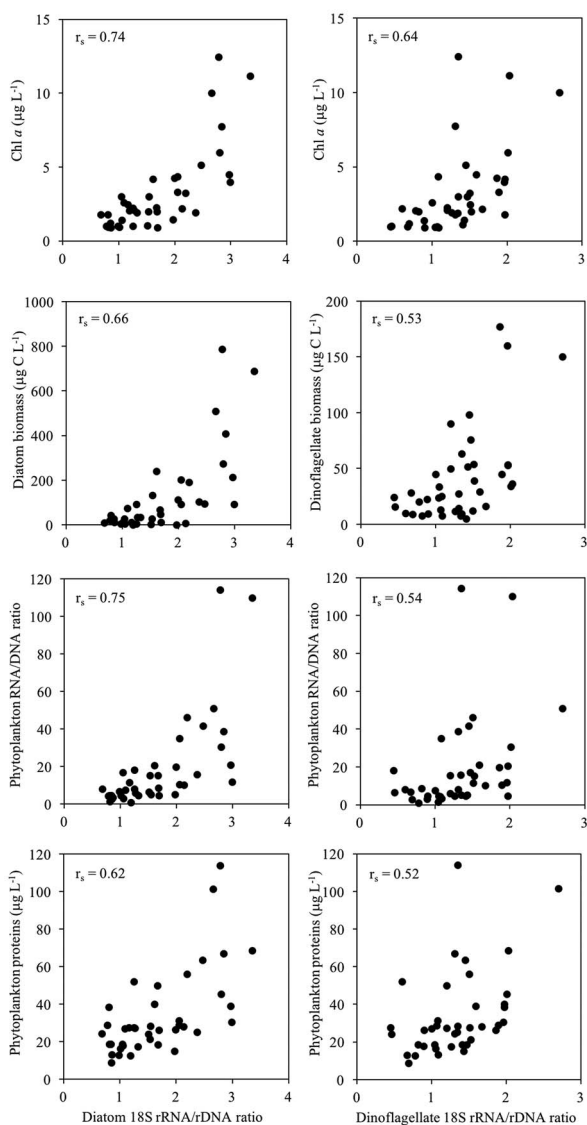
Fig. 4. Relationships between total phytoplankton RNA/DNA ratio and chl *a*, phytoplankton biomass and proteins. All relationships were statistically significant ($P < 0.001$).

rRNA/rDNA ratio of diatom and dinoflagellate and biomass in term of chl *a* and total and taxon-specific carbon content in phytoplankton assemblages were found. The correlation coefficient values were generally high, representing significant relationships between molecular ratios and biomass of the phytoplankton community in the examined period. These findings are in agreement with previous RNA/DNA ratio studies of natural planktonic communities and cultured strains (Pommier *et al.*, 2010; Ning *et al.*, 2013; Lin *et al.*, 2018). Significant correlations were detected with chl *a* and carbon content, which were both used to quantify the autotrophic phytoplankton biomass component. In fact, especially dinoflagellates can have a mixotrophic behavior (Park *et al.*, 2013) or they are obligate heterotrophes (Anderson and Menden-Deuer, 2017). However, considering the autotrophic phytoplankton assemblage, the significant positive correlation between diatom and dinoflagellate 18S rRNA/rDNA ratios and carbon content suggests that the changes in biomass of the heterotrophic components within the phytoplanktonic assemblages were related to the changes in phototrophic biomasses or negligible (Stoecker *et al.*, 2017). In any case, the correlation coefficient values between diatom and dinoflagellate 18S rRNA/rDNA ratio and phytoplankton chl *a*, biomass or RNA/DNA ratio may depend on the phytoplankton assemblage composition, as the correlation coefficients between diatoms and dinoflagellates were different. In fact, lower, although still significant, values of the correlation coefficients among molecular and biological variables were obtained for dinoflagellates. Diatom 18S rRNA/rDNA ratio showed higher correlations with all variables measured. In the studied period, diatoms seemed to be the dominant group, more abundant than dinoflagellates, and therefore having more influence on the relationship between 18S rRNA/rDNA ratios and

biomass than dinoflagellates. In the NW Adriatic coastal ecosystem, in the winter–spring period, various diatom species are dominant in turbulent rich nutrient waters, whereas dinoflagellates are present with the highest biomass prevalently in late spring–summer period when waters are stratified and nutrient depleted (DeGobbis and Gilmartin, 1990; Socal *et al.*, 2008; Penna *et al.*, 2013; Bernardi Aubry *et al.*, 2017; Totti *et al.*, 2019). Moreover, in autumn–winter period, high biomass values due to diatom bloom event, which was usually reported in this area, were observed (Bernardi Aubry *et al.*, 2004; Totti *et al.*, 2005).

We found that diatom and dinoflagellate 18S rRNA/rDNA ratios were correlated with phytoplankton RNA variability ($r_s = 0.68$; $P < 0.001$ and $r_s = 0.62$; $P < 0.001$, respectively), whereas no correlation was found with phytoplankton DNA variations ($P > 0.05$), suggesting that these molecular ratios were likely related to the growing and metabolic activity of the phytoplankton assemblage (Pommier *et al.*, 2010). Similar relationships were obtained between total phytoplankton RNA/DNA ratio and phytoplankton RNA variability ($r_s = 0.76$, $P < 0.001$); no correlation was found with phytoplankton DNA in the assemblages ($P > 0.05$), data not shown. Indeed, DNA content per cell is relatively constant, whereas total RNA is involved in protein synthesis and its cellular concentration varies reflecting transcription (mRNA transcript concentration) and translation (rRNA concentration) intensity. Growth rate and biomass production of phytoplankton assemblages are linked to the ribosomal availability required for protein synthesis, which is related to growth rate through conserved proteins and synthesis rate of ribosomes (Sterner and Elser, 2002; Allen and Gillooly, 2009; Flynn *et al.*, 2010; Loladze and Elser, 2011). Thus, higher 18S rRNA/rDNA ratios may indicate higher biomass production and metabolism

was also found in zooplankton copepod community after diatom blooms: RNA/DNA ratio was applied as indices of growth and nutritional conditions of copepods, as it was correlated with egg production rate (Ning *et al.*, 2013). Since phytoplankton biomass variation can be linked to primary production, and since phytoplankton RNA/DNA ratios was correlated to biomass, these ratios could be applied as potential primary production indicators in further investigations (Richardson *et al.*, 2005; Mayot *et al.*, 2017; Lewis *et al.*, 2020).

Our results showed significant relationships between the total phytoplankton RNA/DNA, taxon-specific 18S rDNA/DNA ratios and total phytoplankton or diatom and dinoflagellate biomass fluctuations during our routine monitoring program. These observed relationships were a preliminary, but strictly necessary, step toward the future application of the taxon-specific 18S rRNA/rDNA ratio to potentially provide an estimation of growth rate or primary production in the phytoplankton assemblages. However, further investigations are needed to assess the use of these RNA/DNA ratios as indicators of dynamic processes in the phytoplankton communities. In fact, both experimental and field studies based on the use of taxon-specific rRNA/rDNA ratios and higher sampling frequency should be performed in order to demonstrate that these molecular ratios could be used as indicators of phytoplankton functional activity.

The use of diatom and dinoflagellate 18S rRNA/rDNA ratio together with RNA/DNA ratio and protein content of the phytoplankton community, as both growth rate and production indicators, may be useful in monitoring and ecological studies, because they are direct, fast and easier approach than radioactive, fluorescent chemical methods or models. Furthermore, in an ecological based approach monitoring, the 18S rRNA/rDNA ratios may be applied to assess seasonal variations of taxon-specific activity in comparison to changes in phytoplankton assemblages.

Fig. 5. Relationships between diatom and dinoflagellate 18S rRNA/rDNA ratios and chl *a*, diatom and dinoflagellate biomass, phytoplankton RNA/DNA ratio and proteins. All relationships were statistically significant ($P < 0.001$).

through protein biosynthesis (Foley *et al.*, 2016; Bowman *et al.*, 2017). Our results agree with those of previous studies as both total phytoplankton RNA/DNA ratio and taxon-specific 18S rRNA/rDNA ratio showed positive significant correlation with the protein content of phytoplankton assemblages. Ribosomal RNA constitutes >80% of RNA cellular content, and it closely related to the cellular growth and biomass (Warner, 1999). Thus, rRNA and/or taxon-specific 18S rRNA/rDNA ratio might be a proxy of overall metabolic activities within an active cell (Blazewicz *et al.*, 2013). Similar correlation

CONCLUSIONS

The taxon-specific 18S rRNA/rDNA and overall RNA/DNA ratios and proteins were correlated with phytoplankton biomass variations in the NW Adriatic coastal ecosystem. The phytoplankton assemblage composition was mainly dominated by diatoms in the range of ~60–90% of biomass, thus explaining higher correlation values between diatoms 18S rRNA/rDNA ratios and biological variables respect to dinoflagellates.

In general, the RNA/DNA ratios express the growth rate and metabolic activity of a community, because these are linked to the RNA activity in the cells. We have demonstrated that the molecular ratios were significantly

correlated to biomass. Now it remains to investigate the relationship between RNA/DNA ratios and growth rate of phytoplankton assemblage in order to develop new biological indicators of coastal ecosystem primary productivity. The 18S rRNA/rDNA ratio approach might improve the analysis of phytoplankton productivity over well-established methods providing more direct and easier tool for the study of phytoplankton assemblage functioning in marine ecosystem.

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