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Communication

Composition of Colloidal Organic Matter in Phytoplankton Exudates

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Abstract: The colloidal organic matter (COM) was isolated from the exudates of three cultured phytoplonkters, namely the chlophyte nanoflagellate *Tetraselmis* sp., the diatom *Chaetoceros socialis* and the dinoflagellate *Prorocentrum minimum*, from the Gulf of Trieste (northern Adriatic Sea). The isolation of COM was performed by ultrafiltration with molecular weight cut-off membranes of 5 kDa and final desalinisation by dialysis. The composition of the COM was characterised using C elemental analysis and ^{1}H NMR spectroscopy and compared with COM isolated from a marine sample from the same area (Gulf of Trieste). By using ^{1}H NMR spectroscopy, it was possible to semi-quantitatively determine the concentrations of the main biochemical constituents present in the COM samples. The results showed that the phytoplankton COM was predominantly composed of polysaccharides, with minor contributions from proteins and especially lipids. Therefore, the phytoplankton COM mainly contributes to the marine COM pool in the polysaccharide fraction and less in the protein and lipid fractions.

Keywords: colloidal organic matter; phytoplankton exudates; NMR spectroscopy; chemical composition; northern Adriatic Sea

Citation: Klun, K.; Šket, P.; Beran, A.; Falnoga, I.; Faganeli, J. Composition of Colloidal Organic Matter in Phytoplankton Exudates. *Water* **2023**, *15*, 111. https://doi.org/10.3390/w15010111

Academic Editor: Jun Yang

Received: 30 November 2022
Revised: 15 December 2022
Accepted: 26 December 2022
Published: 29 December 2022

1. Introduction

Dissolved organic matter (DOM) is an important component of the marine carbon cycle [1] operationally defined as a fraction that passes through a filter of a 0.22 (0.45) µm nominal pore size and which also encompasses colloidal organic matter (COM). COM (from 1 kDa (5 kDa) to 0.22 (0.45) µm) is composed of important biochemical constituents such as lipids, carbohydrates, proteins [2] and humates [1]. Colloidal organic matter (COM) represents approximately 25% of dissolved organic matter (DOM) [3,4]. Recent research has shown the various biochemical functions of COM, especially the role of its biological degradable component in the marine microbial loop [5,6]. Marine COM can act as a precursor in the formation of aggregates and macroaggregates [3,7,8] (i.e., macrogels, presumably of phytoplankton origin, which have periodically occurred in the northern Adriatic Sea in the past, usually in the period of late spring–early summer) [9]. In this context, the composition of phytoplankton exudates could be an important step in decoding the origin of marine COM, most of which has not yet been chemically characterised. It is well known that due to the complexity of marine COM's composition, it is difficult to elucidate its chemical composition and structure [1,4].

Nuclear magnetic resonance (NMR) spectroscopy has been shown to be a crucial, powerful method for the compositional and structural characterisation of aquatic COM and DOM at the molecular level [4,10–13]. NMR is viewed as highly complementary and not overlapping with mass spectrometry (MS), which has become the dominant technique in this research field due to its greater sensitivity [14]. However, NMR is simple, non-destructive, quantitative and reproducible for the analysis of DOM in various aquatic

media [10], including those in the exudates of phytoplankton and bacterial cultures [14]. As the high salt content interferes with NMR as well as MS, and due to the low DOM concentration, ultrafiltration isolation or solid phase extraction are used for NMR analysis [2].

The aim of this study is to provide a better understanding of the origin and chemical composition of COM (and DOM) in coastal waters, decoding the composition of COM released by some cultured phytoplankters isolated from the coastal area of the Gulf of Trieste (northern Adriatic Sea) in terms of organic C and ^{1}H NMR spectroscopic analyses. Comparisons are made with the marine COM and the macroaggregate compositional data, which are thought to be a product of the COM agglomeration in this area [13,15,16].

2. Materials and Methods

2.1. Culture Methods

The phytoplankton species of the chlorophyte nanoflagellate *Tetraselmis* sp., the diatom *Chaetoceros socialis* and the dinoflagellate *Prorocentrum minimum* isolated from the Gulf of Trieste were grown in batch cultures with enriched seawater (medium ESAW [17]) under 75 µEinstein m^{-2} s^{-1} of fluorescent light in a cycle of 12 h of light and 12 h of dark at a constant temperature of 16 °C. The cultures were not axenic. They were first preconditioned in 100 mL of ESAW medium and inoculated in 1 l of ESAW medium after 2 weeks.

In the stationary phase of growth, the cultures, with cell densities ranging between 3.5×10^5 and 1.5×10^6 cells mL^{-1}, were filtered through preignited 0.7 µm pore size Whatman GF/F glass-fibre filters. The filtrates were then filtered through 0.22 µm Nucleopore filters. In order to isolate the COM, the filtrates were ultrafiltered through membranes with a nominal molecular weight cut-off (MWCO) of 5 kDa using a Vivascience Vivaflow 200 unit (Sartorius) with a Masterflex S/L membrane pump (Cole-Palmer) at a flow rate of 300 mL min^{-1} at 2.5 bar and 20 °C. The average concentration factor (CF) was 6. The retentates were freeze-dried and desalted by dialysis [18] in Milli-Q water for 18 h at 4 °C using MWCO 1 kDa RC membranes (Spectrapor 7, Spectrum Lab). The surrounding milliQ water was exchanged twice after 2 and 4 h to reduce the salinity from PSU 37 to PSU < 0.2.

2.2. Analyses

DOC Analysis

Analyses of the DOC in the filtrates, retentates and permeates were performed with a high-temperature catalytic method using a Shimadzu TOC-L analyser after acidifying the samples with 6 M HCl to eliminate CO_2 [19]. The reproducibility was between 1.5 and 3.0%.

2.3. NMR Spectroscopy

The ^{1}H-NMR spectra of the dialysed COM samples were obtained using an Agilent Technologies VNMRS 800 MHz NMR spectrometer in D_2O at a temperature of 298 K using a cold probe. Standard 1D ^{1}H-NMR spectra were acquired with the use of DPFGSE solvent suppression. The number of scans was 256, the pulse width was 7.7 ms, the spectral width was 16,000 Hz, the acquisition time was 1 s, and the relaxation delay was 1.5 s.

The ^{1}H-NMR spectroscopy enabled quantitative analysis because the signal intensity was proportional to the amount of compounds present in the sample. In this study, semi-quantitative analysis was performed by integrating ("trajectory lines" in Figures 1–4) specific areas in the ^{1}H-NMR spectra that could be assigned to the main biochemical groups. The sum of all 4 integrals was 100%. The integrals were expressed as the relative abundance (in%) of the main biochemical groups (lipids, proteins and CRAM, polysaccharides and formate) in each sample.

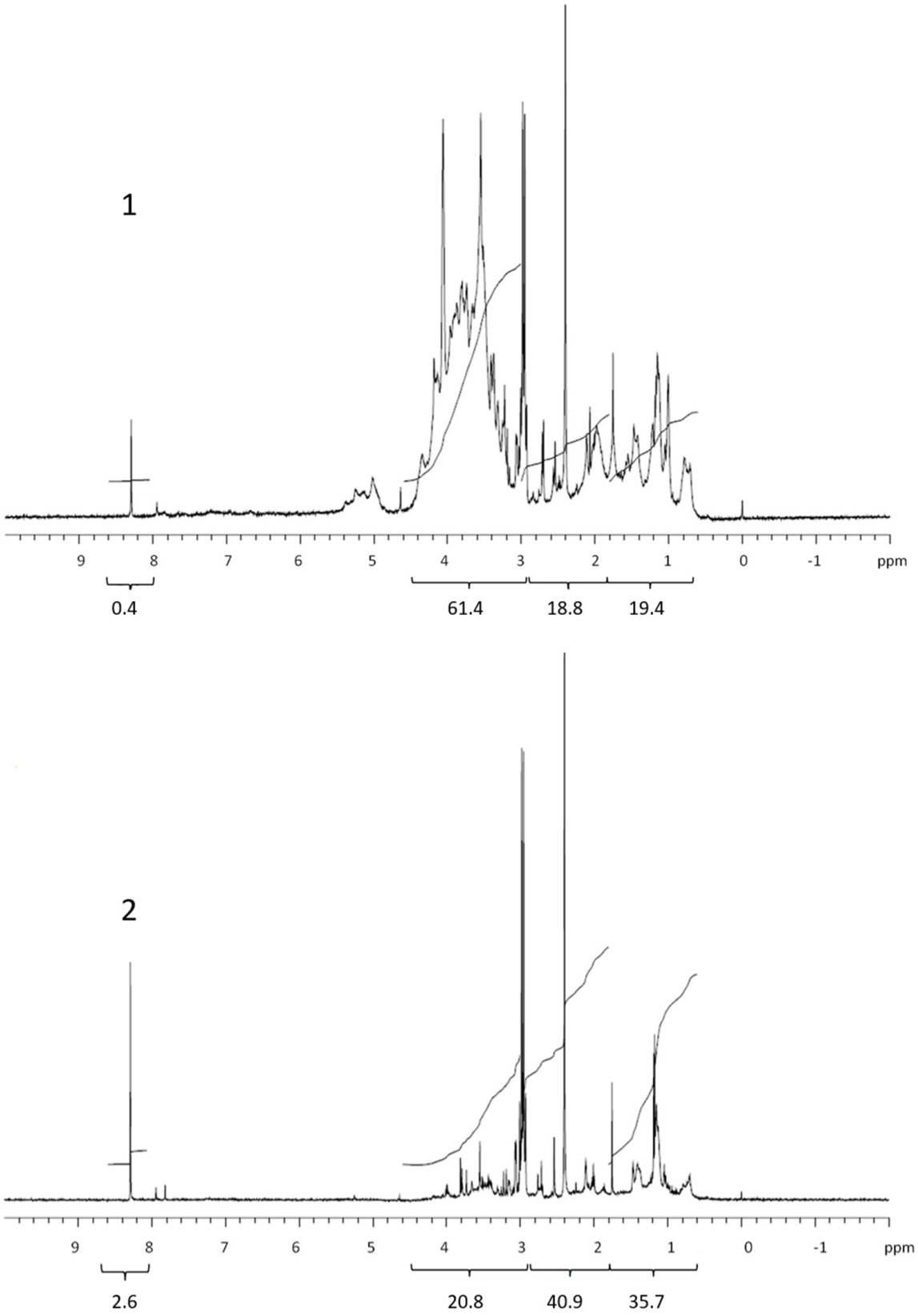

Figure 1. ^{1}H NMR spectra of retentate (**1**) and permeate (**2**) isolated from exudate of the nanoflagellate *Teratselmis marina.* Values below brackets are integrated values.

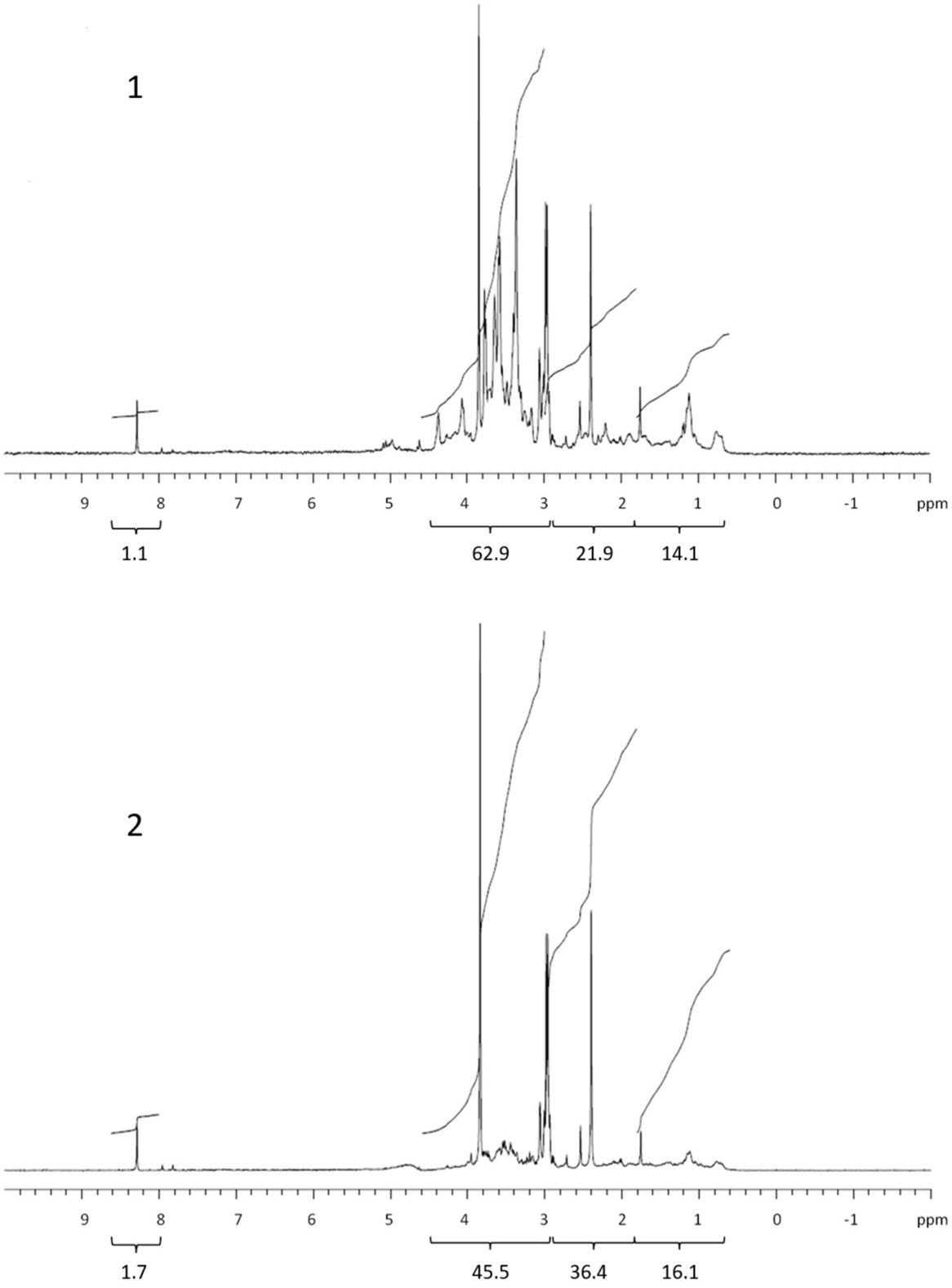

Figure 2. ^{1}H NMR spectra of retentate (**1**) and permeate (**2**) isolated from exudate of the diatom *Chaetoceros socialis.* Values below brackets are integrated values.

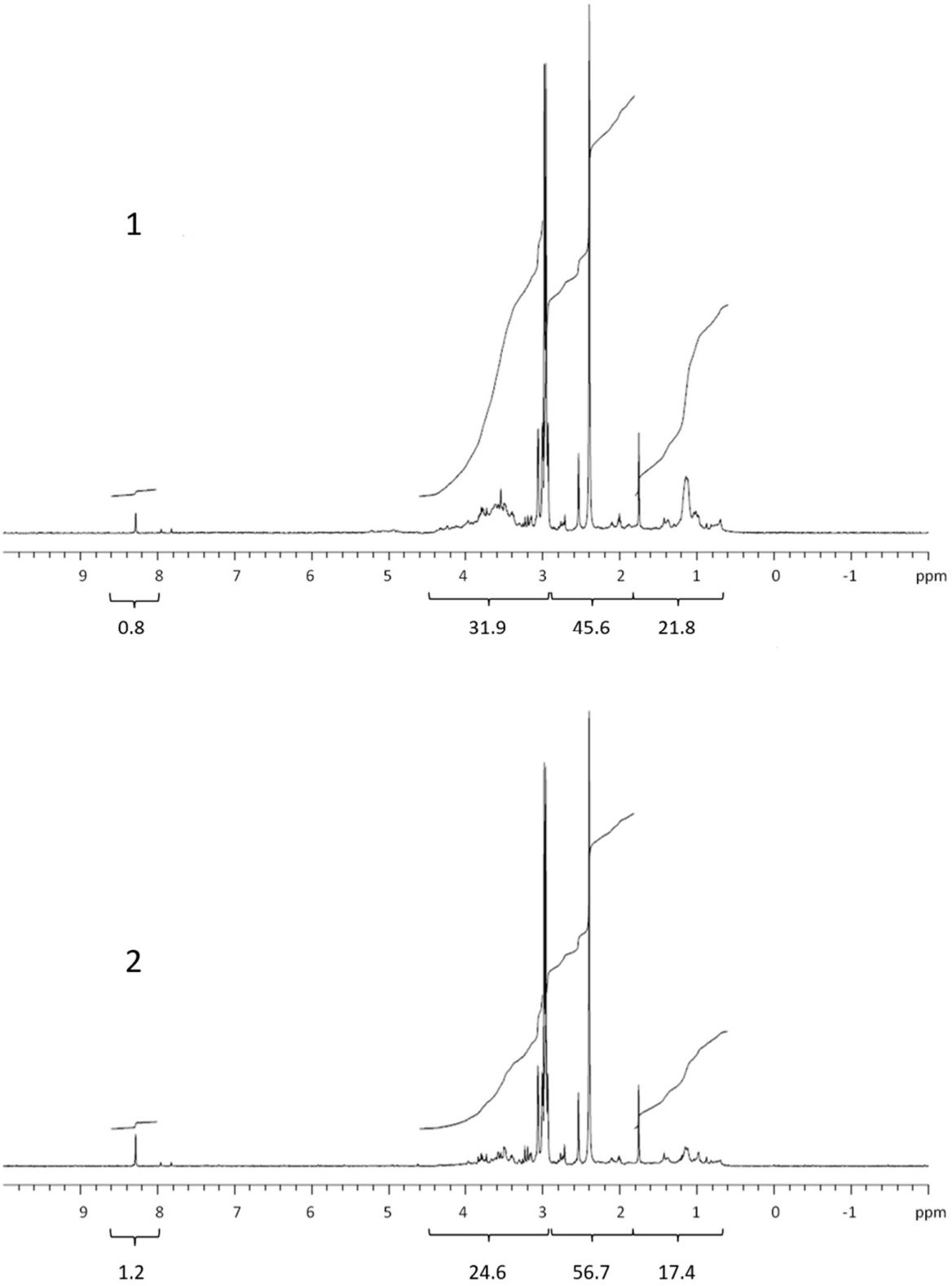

Figure 3. ^{1}H NMR spectra of retentate (**1**) and permeate (**2**) isolated from exudate of the dinoflagellate *Prorocentrum minimum*. Values below brackets are integrated values.

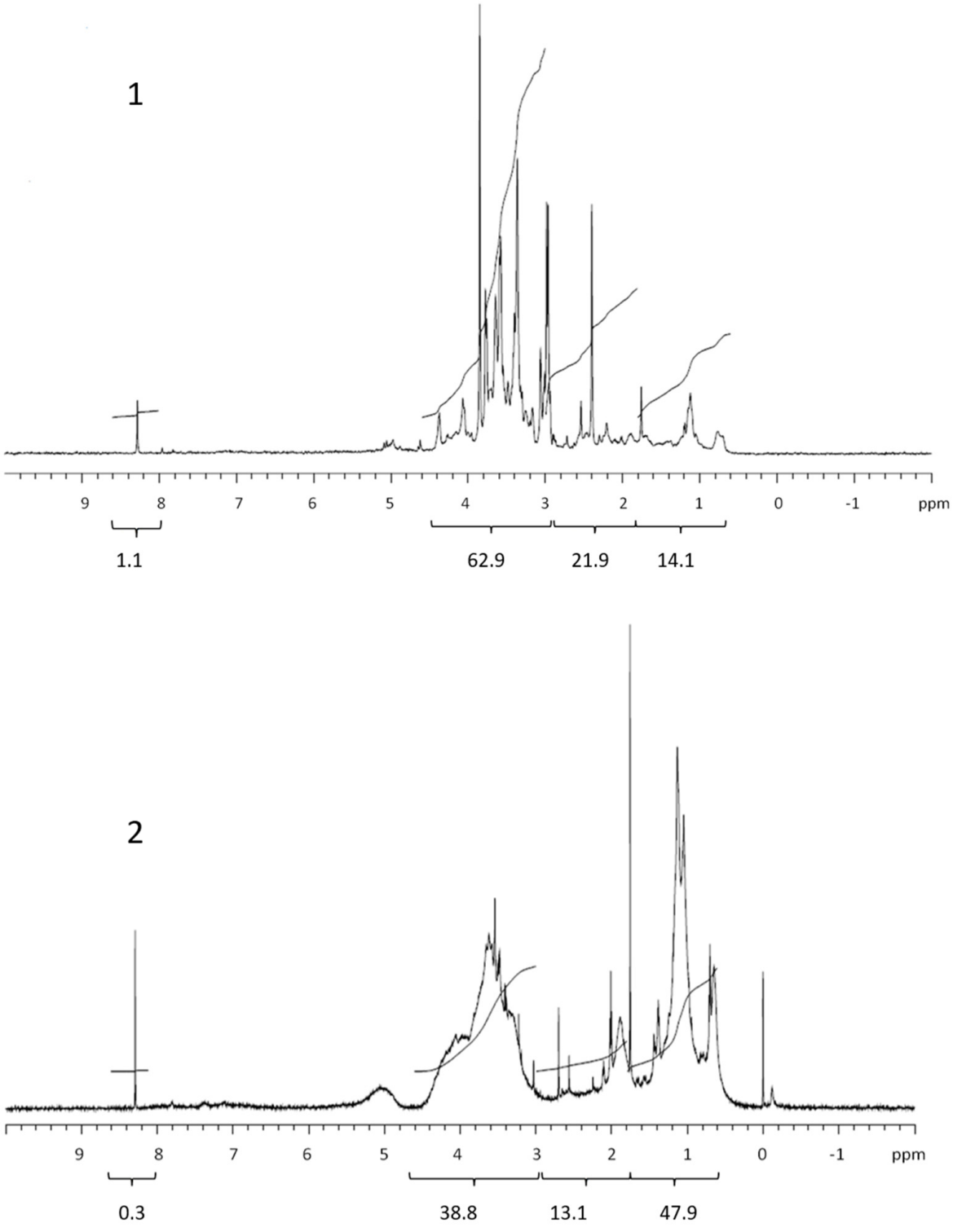

Figure 4. Comparison between ^{1}H NMR spectra of marine COM from July 2012 (**1**) and COM isolated from exudate of *Chaetoceros socialis* (**2**). Values below brackets are integrated values.

3. Results and Discussion

3.1. COM Concentration

The total extracellular $C_{org.}$ (DOC) content results in the exudates of the three phytoplankton cultures (Table 1) showed that the highest concentration was found in the diatom *Chaetoceros socialis*, followed by the nanoflagellate *Tetraselmis* sp. and the dinoflagellate *Prorocentrum minimum*. This was the opposite of the previously published $C_{org.}$ elemental

composition of cultured *T. marina* (60% dry weight) and *C. socialis* (25% dry weight) [20]. The isolated colloidal organic carbon (COC) (calculated as % COC) also showed the opposite order: 39.8% in the nanoflagellate, 35.1% in the dinoflagellate and 23%, in the diatoms, releasing the lowest percentage of extracellular colloidal organic matter. The latter value was similar to the generally reported value that COM represents on average (about 25% of dissolved organic matter (DOM)) [4]. Previous studies showed that in summer 2012, the COM began to accumulate in the Gulf of Trieste, when the percentage of COC in the bulk DOC rose twofold from 16.3 to 32.4% [13]. The COM accumulation from late spring to early summer indicated an increase in refractory COM in the water column and the possibility of the formation of aggregates leading to the episodic (periodic) occurrence of macroaggregates in the northern Adriatic Sea, as has occurred in the past [15,16]. This is in accordance with the progressive increase in DOC concentrations in the Gulf of Trieste towards summer [13,21,22] after the early spring (primary) productive period, while the bacterial production reached its peak in summer [23]. Similar temporal dynamics were reported for the total dissolved carbohydrates and particularly for dissolved polysaccharides in the water column of the Gulf of Trieste, which were hypothesised to be the macroaggregate precursors [24].

Table 1. Distribution of the integrated main groups of proton resonances (lipids, proteins and CRAM, polysaccharides and formate) in ^{1}H MNR spectra (δ/ppm), concentrations of $C_{org.}$ (μmol L^{-1}) in retentates and permeates and % COC from exudates of cultured phytoplankters. Protons that resonated in certain chemical shift range of integrated main groups are in bold (second row).

	Lipids	**Proteins and CRAM**	**Polysaccharides**	**Formate**	**$C_{org.}$**	**% COC ***
	HCH_2-CH_2-	**H**C-**H**COR	**H**C-OH **H**C-O-C	**H**COO		
δ **/ppm	0–1.8	1.8–3.0	3.0–4.6	8.0–9.0	μmol L^{-1}	%
			Teratselmis marina			
0.2 μm filtrate					915.1	
Retentate	19.4	18.8	61.4	0.4	364	39.8
Permeate	35.7	40.9	20.8	2.6	507	
			Chaetoceros socialis			
0.2 μm filtrate					2285	
Retentate	14.4	21.9	62.9	1.1	526	23.0
Permeate	16.4	36.4	45.5	1.7	1765	
			Prorocentrum minimum			
0.2 μm filtrate					439.3	
Retentate	21.8	45.6	31.9	0,8	154	35.1
Permeate	17.4	56.7	24.6	1.2	418	

Note(s): * Colloidal organic carbon percentage: %COC = $C_{org.}$ (retentate) × 100%/$C_{org.}$ (0.2 μm filtrate). ** Chemical shift range in ^{1}H NMR spectra for each group of compounds.

3.2. COM Composition

The ^{1}H NMR spectra of the dialysed marine COM (Figures 1–4) can be divided into the main groups of proton resonance, which can be assigned to major biochemical components present in the marine environment: protons bonded on or near carbon in alcohol or ether functional groups (polysaccharides; **H**C-OH, **H**C-O-C-: δ = 3–4.6 ppm), protons near the amide, ketone and carboxyl functional groups in alicyclic organic molecules (CRAM) (proteins, CRAM; **HC-H**COR: δ =1.8–3 ppm) and aliphatic protons (lipids; **H**CH_2-CH_2-: δ = 0.6–1.8 ppm) [10,13,15,25,26]. The spectra of all samples exhibited a peak in formate (δ = 8.3 ppm) [27], with higher abundances in the permeates compared with the retentates, which probably originated from the formate esters in the DOM. The spectra, corresponding to the studied phytoplankton exudates, showed peaks for all the main COM components (Figures 1–3). Integration revealed that the main difference was represented

by polysaccharides and proteins, while lipids and especially formate represented the minor fractions (Table 1). When comparing the samples of cultured phytoplankton species, the polysaccharide fraction was the highest in the retentate of the nanoflagellate *Tetraselmis* sp. (61.4%), while lipids and proteins accounted for 19.4 and 18.8%, respectively (Table 1). The composition of permeate was different, as proteins represented the highest portion (40.9%). The diatom *Chaetoceros socialis* also contained the highest polysaccharide level for retentates (62.9%) and permeates (45.5%), followed by proteins and lipids. The retentates and permeates of the dinoflagellate *Prorocentrum minimum* were quite different, exhibiting the highest levels of proteins and the lowest levels of lipids.

The quantity and composition of the phytoplankton extracellular DOM depend on the organism, its physiological status, the physical and chemical environmental properties and the presence of other organisms [28]. The $C_{org.}$ levels, ^{1}H NMR spectra and their integrals differed among the studied phytoplankton species. The colloids from the nanoflagellates and diatoms contained mainly polysaccharides, in accordance with the high carbohydrate concentrations in the cell [20], supporting previous conclusions that carbohydrates are major components of phytoplankton exudates [29]. Conversely, the colloids from the dinoflagellates contained mainly proteins. The COM of all phytoplankton cultures exhibited lower lipid contents in comparison with marine COM [13]. Kovac et al. (2002) [15] reported on the presence of lipids (aliphatic) followed by polysaccharide fractions as the major constituents of macroaggregates of phytoplankton origin, with lower concentrations of proteins. The compositional differences between COM in phytoplankton cultures and marine COM and macroaggregates indicate the refractory nature of lipids in the marine environment [30]. However, the differences observed could also be due to different solvents used in ^{1}H NMR analysis of COM and macroaggregates. Comparing the ^{1}H NMR spectrum of the nanoflagellate *Tetraselmis* sp. COM with marine COM from the Gulf of Trieste [13] revealed a similarity, since nanoflagellates together with diatoms represent the majority of the phytoplankton found in the Gulf of Trieste [31].

4. Conclusions

The COM isolated from the exudates of three cultured phytoplankters, namely the nanoflagellate *Tetraselmis* sp., the diatom *Chaetoceros socialis* and the dinoflagellate *Prorocentrum minimum*, from the Gulf of Trieste is predominantly composed of polysaccharides, with minor contributions from proteins and particularly lipids. The phytoplankton COM contributes to the composition of marine COM, particularly through an increase in the polysaccharide fraction during COM agglomeration and possible macroaggreagate formation, as observed in the past in the northern Adriatic Sea, while the accumulation of lipids is due to their refractory nature and resistance to microbial and chemical degradation.

Author Contributions: J.F. designed the study and drafted the manuscript; K.K. drafted and submitted the manuscript and obtained and analysed the data; P.Š. performed ^{1}H NMR analysis and revised the manuscript; I.F. helped with the study design and revised the manuscript; A.B. provided the phytoplankton cultures and revised the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Slovenian Research Agency (research core funding no. P1-0143, P4-0432 and P1-0242).

Data Availability Statement: During this study no additional data were created.

Conflicts of Interest: The authors declare no conflict of interest.

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