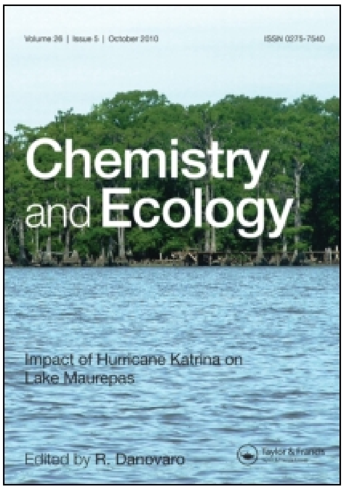

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Relationships between dissolved and particulate carbohydrate temporal patterns and mucilaginous aggregates formation: evidences from time series analysis and biochemical composition of aggregates

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RELATIONSHIPS BETWEEN DISSOLVED AND PARTICULATE CARBOHYDRATE TEMPORAL PATTERNS AND MUCILAGINOUS AGGREGATES FORMATION: EVIDENCES FROM TIME SERIES ANALYSIS AND BIOCHEMICAL COMPOSITION OF AGGREGATES

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A 3-year monitoring of dissolved and particulate carbohydrate concentrations in four transects located in the Adriatic and Tyrrhenian Seas was performed in order to get information on the role played by carbohydrates in the mucilage formation in these areas. The application of time series analysis pointed out that the concentration of dissolved carbohydrates does not vary significantly in coincidence of the mucilage appearance showing an almost constant state with respect to time. In contrast, wider temporal variations of carbohydrate amounts, either increase or decrease, were observed when mucilages were lacking or reduced. This almost constant state of carbohydrate amounts observed in presence of mucilages that we define 'steady state' could be associated to an alteration of the complex chemical equilibrium between synthesis and degradation (either hydrolysis or oxidation) reactions of the organic polymers which are typical of the humification processes in the marine environment. The results of this study suggest that the monitoring of carbohydrates can represent an useful tool for the comprehension of the most relevant phenomena of mucilage appearance in the Northern Adriatic Sea.

Keywords: Mucilage aggregates; Marine snow; Carbohydrates; Adriatic Sea; Tyrrhenian Sea; Time series analysis

1 INTRODUCTION

Several studies have been carried out in the Adriatic Sea in order to identify causes inducting the formation of suspended macro aggregates of organic matter along the water column called mucilages. These anomalous aggregates are present in several forms such as small flocs, strings, clouds and scums, with dimensions covering several orders of magnitudes from centimetres to hundreds of meters (Fogg, 1995; Mingazzini and Thake, 1995). In Figure 1 we report a photo of one of these aggregates floating along the water column during the mucilage events on summer 1997 in the Northern Adriatic Sea. This phenomenon, also occurring in the Tyrrhenian Sea, has been investigated according to different approaches.

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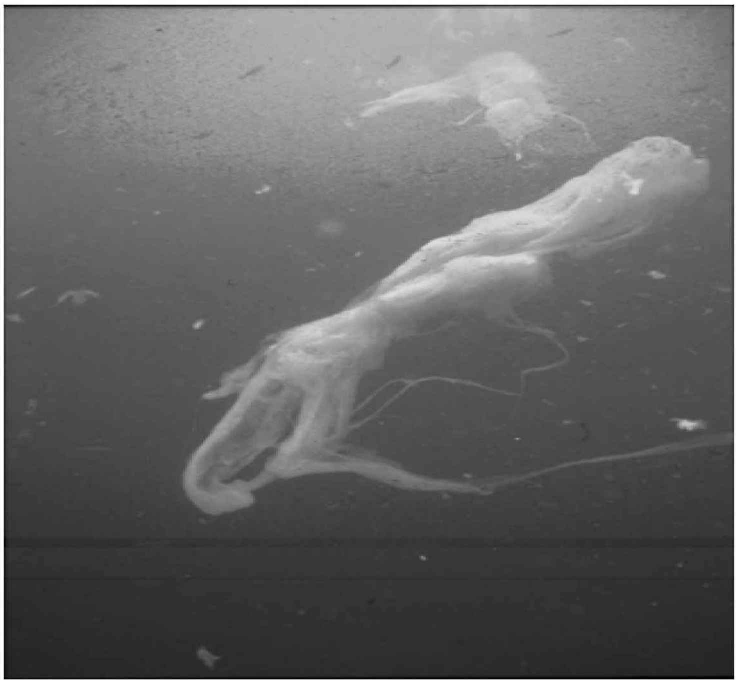

FIGURE 1 Example of mucilage aggregates observed in Adriatic Sea during summer 1997. The length of the samples was about 15 cm.

Some authors investigated the relationships between the hydrological conditions and mucilages appearance (Degobbis *et al.*, 1995; Lancelot, 1995); others investigated the relationships between phytoplankton (Myklestad, 1995), micro and macro algae (Innamorati, 1995), microbial activities (Decho and Herndl, 1995) bacteria (Serratore *et al.*, 1995) and the mucilage formation. Other studies have been carried out to assess the contribution of the carbohydrate and humic fractions to the whole mucilage composition (Leppard, 1995; Myklestad, 1995; Mingazzini *et al.*, 1995; Mecozzi *et al.*, 2001; 2002). The possible causes of the phenomenon are still controversial though some hypothesis have been already proposed on the basis of previous studies. According to the humin structure of mucilages, the initial formation of the aggregates could follow the natural humification process of marine organic matter (Mecozzi *et al*., 2001; 2002) without being a process depending on a pollutant event 'a priori' (Zitko, 2001). In fact, recent studies show that anoxic conditions (Ciglenečki *et al.*, 2000) and reduced bacterial activity, (Azam *et al.*, 1999; Manganelli and Funari, 2003) can enhance the formation of mucilaginous aggregates. In particular, anoxic conditions and reduced bacterial activity could be some of the main factors leading to the transition of micro-aggregates (*i.e.*, particulate and marine snow) towards mucilagenous macro-aggregates (Leppard, 1999).

In order to have additional information on the chemical, physical and biological processes involved in the mucilage formation, a 3 year research program (June 1999–May 2002) was sponsored by the Italian Minister of Environment. In this framework, dissolved and particulate carbohydrate concentrations were measured in five different areas in the Adriatic and Tyrrhenian Seas.

The aim of the present study was to provide new insight on the role of carbohydrates in the mucilage formation. To do this, we examined temporal variability of particulate and dissolved carbohydrates from June 1999 to June 2002 using a powerful statistical tool such as the time series analysis.

2 MATERIALS AND METHODS

2.1 Sampling and Sample Pre-treatment

Sampling areas in the Adriatic and Tyrrhenian Seas are illustrated in Figure 2. The samples of transect ‘A’ were analysed by the scientists of the Ruder Boškovič Institute (Roviny, Croatia), partner of the research program. All the samples for dissolved and particulate carbohydrates were collected by a Niskin bottle at 5 depths, 0, 5, 10, 20, 50 m. For dissolved carbohydrates

FIGURE 2 Map of Italian Seas showing the transects included in the study. B1 transect is the ‘short transept of Cesenatico’ including 6 sampling stations; B2 is the ‘long transept of Cesenatico’ including 9 sampling stations. C is the transect ‘Senigallia’. D is the transect ‘Isola delle Femmine’. E is the place in central Tyrrhenian sea where marine snow samples were sampled.

25 mL of seawater were subsampled into plastic tubes; for particulate carbohydrates, 1 L was filtered on glass fiber filter, pore size 47 μm, previously treated in an oven at 350 °C. All the samples were stored frozen at −25 °C until analysis. As the beginning of the sampling was on June 1999, each year of monitoring ranges between each month of June and May of the following year.

Marine snow and mucilage samples were collected along the coasts of Isola del Giglio and Isola d'Elba (Central Tyrrhenian Sea, Fig. 2) by means of a Niskin bottle deployed by the ship. All the samples were dialysed by a Spectrum 1000 dialysis membrane to eliminate inorganic salts and lyophilized before infrared spectroscopic analysis.

2.2 Carbohydrate Analysis

The determination of carbohydrates as glucose equivalent was performed according to a modified protocol of the phenol–sulphuric acid method (Mecozzi *et al.*, 1999). The colorimetric analysis was made by means of a double beam DMS 200 Varian UV-VIS spectrophotometer.

2.3 Infrared Analysis of Mucilage and Marine Snow Samples

Qualitative analysis of samples was performed by the transmission mode on pellets prepared adding 1 mg of lyophilized mucilage or marine snow to 200 mg of KBr previously dried in an oven at 150 °C. The spectra were collected at 4 cm^{-1} resolution from 400 to 4000 cm^{-1} after 200 scans by using the cosine function as apodization process. The second derivative of each spectrum was determined according to the Savitzky Golay algorithm to enhance the spectral resolution of the observed bands (Brereton, 1989). All the infrared measurements were performed using a Jasco 410 Fourier transform spectrophotometer.

2.4 Time Series Analysis for Carbohydrate Data in Seawater and Particulate Samples

Time series analysis was performed by means of the autocorrelation functions (Brereton, 1989) calculated on the median values of carbohydrate concentrations determined at five sampling depth. The autocorrelation functions were determined by the spreadsheet Excel for Windows as follows: for an arbitrary time series X_0 with n data collected monthly, new $n - 3$ time series are obtained (Tab. I). Then the correlation coefficients among the ori-

TABLE I Example of Determination of the Autocorrelation Coefficients for a Time Series X_0.

X_0	X_1	X_2	X_3	X_4	r
2.1	2.2	2.5	1.9	1.8	0.265
2.2	2.5	1.9	1.8	2.5	−0.907
2.5	1.9	1.8	2.5	3	−0.262
1.9	1.8	2.5	3		0.929
1.8	2.5	3			
2.5	3				
3					

Note: The time series X_0 considered for this example is arbitrary and dimensionless. r values are the correlation coefficients determined by the specific function obtained by using any specific statistical software or spreadsheet.

ginal X_0 time series and all the other X_i series are determined. The plot of the new $n - 3$ correlation coefficients represent the autocorrelogram plot showing the real time trend of the X_0 series filtered by the reduction of noise (Brereton, 1989).

3 RESULTS AND DISCUSSION

3.1 Time series analysis of dissolved and particulate carbohydrates

In Table II we report the range of the average values of carbohydrate amounts measured in the water column at the five depths for all transects and sampling areas.

These data indicate that carbohydrate concentrations fluctuated for more than one order of magnitude. Such wide variability does not allow a correct analysis of variance because in this condition the data distribution cannot obey to a normal distribution (Ignatiedes *et al.*, 1992) and moreover, the average and the variance values used for the analysis of variance cannot represent the real data distribution (Miller and Miller, 1989).

Because some studies report the amount of carbohydrates in Adriatic Sea (Sarà *et al.*, 1998; Albertelli *et al.*, 1999; Pettine *et al.*, 1999), a general approach, consisting of comparing the amount of carbohydrates of previous studies with the amount of our study is possible, but in any case this approach cannot clarify some details about the dynamics of the carbohydrate cycle in seawater.

A more useful approach consists of the examination of the temporal variation (*i.e.* time series analysis) of carbohydrate amounts by comparing their real cyclic trends in the years under investigation. This approach arises from the differences among the years of the study because they were characterized by three different conditions as far as the presence of mucilage concerns. In fact, in the first year of monitoring, mucilages were absent, in the second year the phenomenon was very relevant in Adriatic and Thyrrenian Seas and in the third one, mucilages were observed with a lesser relevance than the previous year only in Adriatic Sea.

A simple tool to examine the real temporal trend of a physicochemical variable enhancing the presence/absence of cyclic events is to determine the autocorrelation coefficients of a time series (Brereton, 1989; Ildiko and Todeshini, 1994). This statistic technique has some peculiar advantages because it reduces the effect of noise in the data evidencing the real time trend of the variable under investigation and moreover, it is computationally easy.

So, the study of the dynamic events associated to carbohydrate concentrations among the years of the same trasect and the different transects, becomes a simple comparison among plots, each one showing the real temporal and cyclic trend of carbohydrates in the years

TABLE II Range of Carbohydrate Amounts Determined in the Years of the Study Taking into Account for Each Month, the Average Values of the Five Levels of Depth.

Transect	*Dissolved*			*Particulate*		
	1999–2000	2000–2001	2001–2002	1999–2000	2000–2001	2001–2002
Long Cesenatico	5.61–15.75	1.87–12.92	3.18–14.75	0.12–1.88	0.71–1.87	0.23–1.94
Short Cesenatico	0.33–16.81	1.24–11.71	2.33–20.24	0.01–3.70	0.68–5.58	0.24–1.19
Senigallia	1.91–13.01	1.63–7.61	3.84–15.24	0.18–2.35	0.32–1.14	0.06–1.87
Isola delle Femmine	0.33–29.33	1.81–7.39	2.01–29.56	0.02–0.79	0.14–0.98	0.07–0.22

Note: The values are expressed as μmol L^{-1} of seawater (dissolved carbohydrates) and μmol L^{-1} of filtered seawater (particulate carbohydrates).

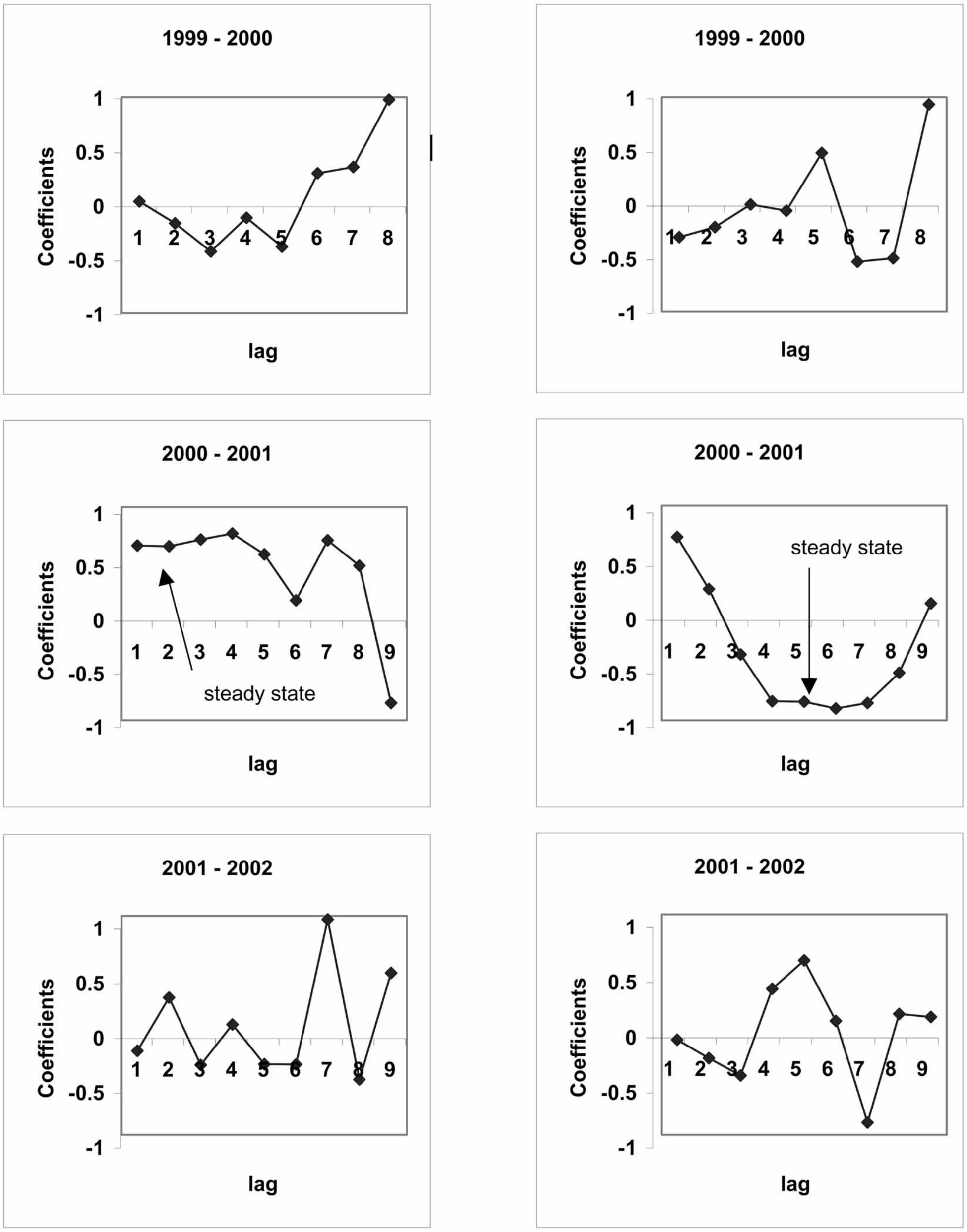

FIGURE 3 Time series plot of the median values of dissolved (left side) and particulate (right side) carbohydrates for the long transept of Cesenatico. The plots of each row represent the single year of the monitoring.

of the study. In Figures 3–6 we report the results of the time series analysis performed on the three transects located in Adriatic Sea and in the transect located in the South Tyrrhenian Sea.

In the long transect of Cesenatico (Fig. 3) the temporal trends of carbohydrates vary considerably among years. As far as the dissolved carbohydrate content concerns, the plots of the first year (absence of mucilage) and the third year (low evidence of mucilage) show a trend with alternate cyclic events. In contrast, in the second year, in correspondence of a massive presence of mucilages, carbohydrate contents show an initial state where significant

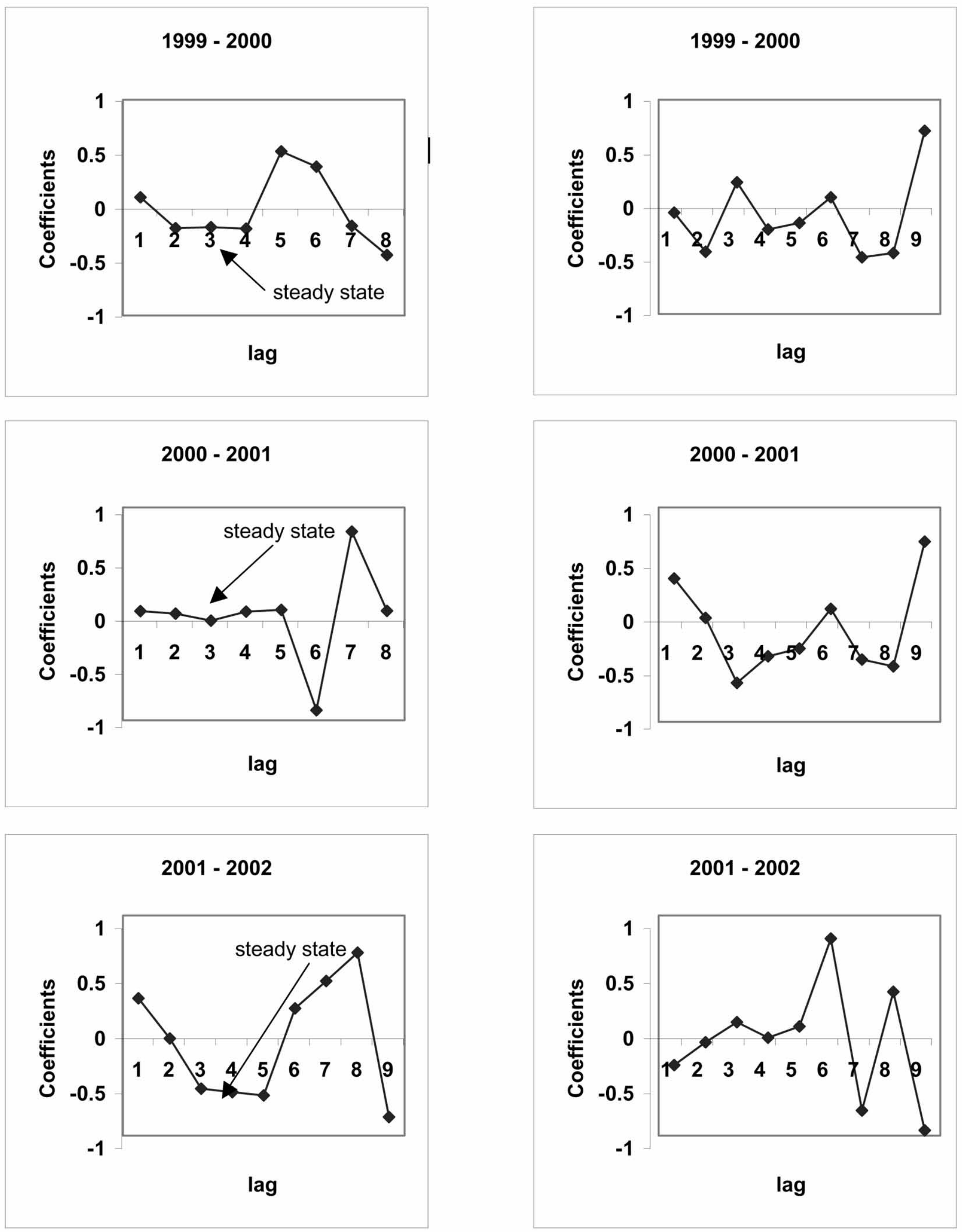

FIGURE 4 Time series plot of the median values of dissolved (left side) and particulate (right side) carbohydrates for the short transept of Cesenatico. The plots of each row represent the single year of the monitoring.

variations of carbohydrates (either increase or decrease) are absent. This period where carbohydrate concentrations seem to be constant or time-invariant can be considered as a temporal steady state which is located approximately in the first lags (months) of the screening and corresponding to the end of spring and the beginning of the summer season. Analogous evidences result from the autocorrelogram of particulate carbohydrates determined for the same year.

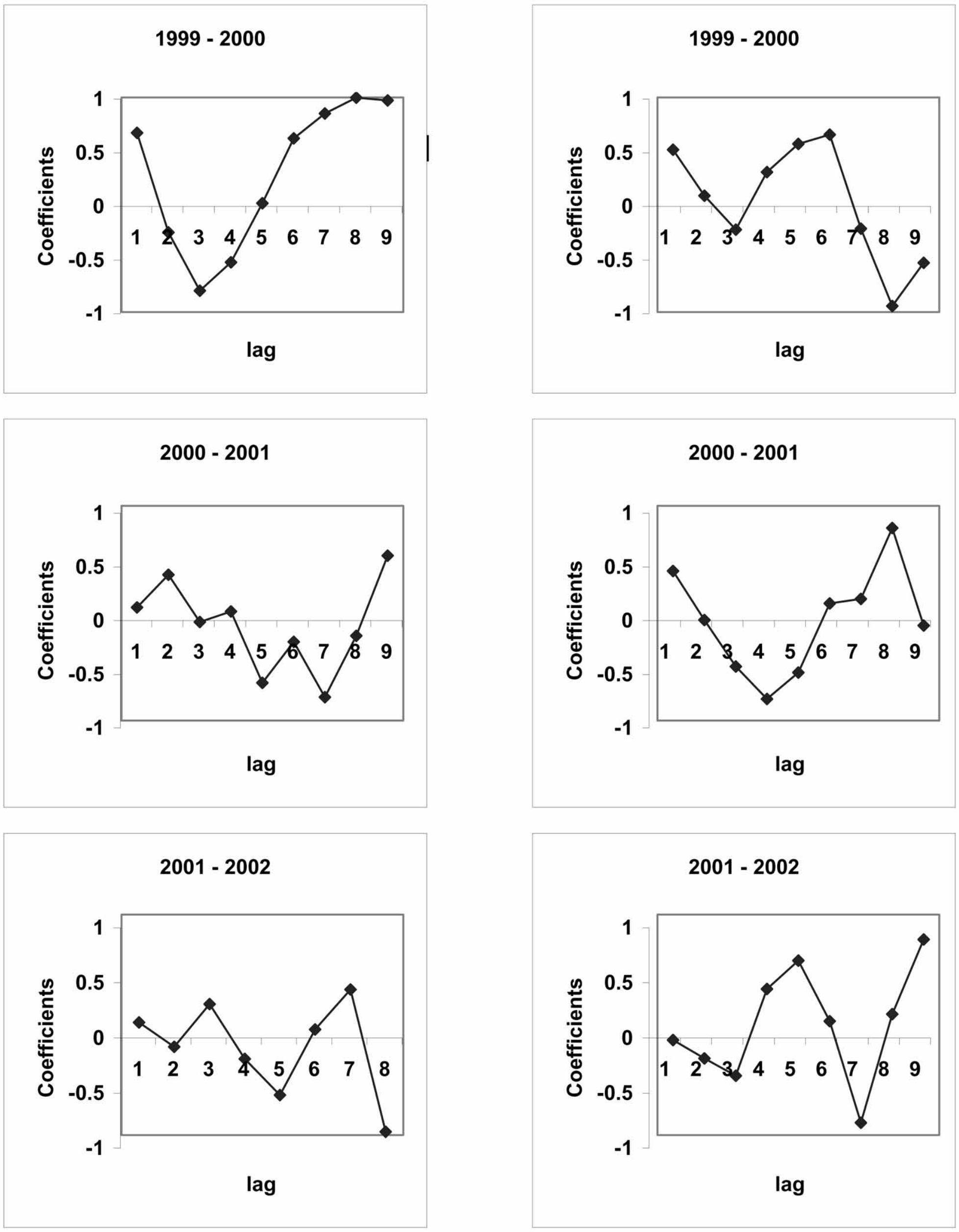

FIGURE 5 Time series plot of the median values of dissolved (left side) and particulate (right side) carbohydrates for the transept of Senigallia. The plots of each row represent the single year of the monitoring.

The autocorrelogram plot of the short transect of Cesenatico (Fig. 4) shows the presence of a time state almost steady without relevant variation. The time steady state is placed in the beginning of autocorrelogram of dissolved carbohydrates for the second year of monitoring (*i.e.* the time of the major presence of mucilages). In the other 2 years, the plot of the autocorrelograms always shows the presence of a time steady state though a little time-shift was observed with respect to the same period of the second year. Vice versa, no one steady state is observed in the autocorrelograms of particulate carbohydrates.

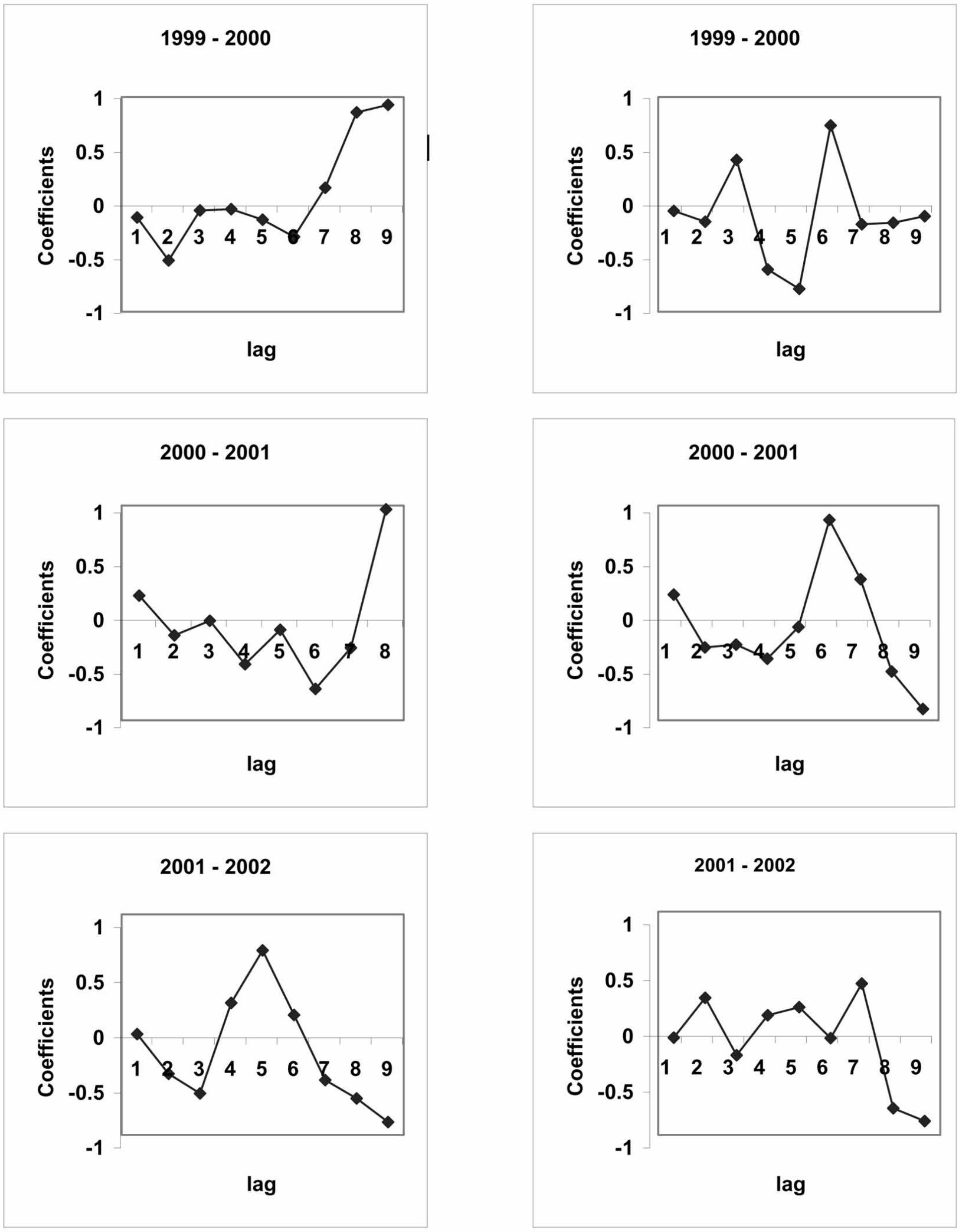

FIGURE 6 Time series plot of the median values of dissolved (left side) and particulate (right side) carbohydrates for the transept of Isola delle Femmine. The plots of each row represent the single year of the monitoring.

In the transects of Senigallia (Fig. 5) and Isola delle Femmine (Fig. 6) where the presence of mucilage aggregates resulted negligible if compared to the other two transects (Russo, 2001), the plot of the autocorrelograms either of dissolved or particulate carbohydrates show more regular cyclic trends without the presence of a time steady state. In more details as far as the transect of Isola delle Femmine concerns, mucilages were observed only on October 2000, during the second year of the monitoring.

The findings of a time steady state corresponding to the periods where mucilages were more relevant either for size or time, allows several considerations about the general characteristics of the phenomenon and the role played by carbohydrates in the aggregation and the coalescence of the organic matter. The tendency of dissolved carbohydrates to remain almost constant during the presence of mucilage aggregates is a peculiarity of the transects located in the Northern Adriatic Sea where the first historical presence of mucilages is dated back to 1729 and where the phenomenon is generally particularly relevant (Innamorati, 1995).

How can we interpret the temporal steady state of carbohydrate amounts observed in presence of mucilage events? By the point of view of environmental aquatic chemistry, a temporal state almost steady can be interpreted as an alteration of the natural humification processes. In fact, carbohydrates resulted involved neither in synthesis nor in degradation reactions of the organic matter because otherwise, a variation of carbohydrates, either increase or decrease, should be observed. After a time steady state, the autocorrelation values tend either to diminish or to increase (Figs. 3 and 4) showing the restoration of the natural conditions of humification which consists of simultaneous synthesis and degradation of organic matter (Ishiwatari, 1992).

A re-examination of the data in Table II gives additional information about the characteristics of carbohydrates in the years of mucilage phenomenon. In fact, Table II shows that the range of dissolved carbohydrates in the year of mucilage events is not higher than the other 2 years when the phenomenon was absent or negligible. The results of Table II are static results because they do not show the dynamic processes involved in the carbohydrates presence but in any case, they suggest that the carbohydrate concentrations present in the years of mucilage events are not necessarily higher than those observed in the years when the presence of mucilage aggregates is negligible or absent. So, the joint results of the time series analysis with the evidences of Table II suggest that the mucilage phenomenon defined in some studies as ‘hyperproduction of exudates’ could be also defined as ‘hyper aggregation of exudates’ because the observed temporal steady state is likely dependent on an alteration of the equilibrium between dissolved and aggregated organic matter where hydrological factors (Decho and Herndl, 1995), bacteria (Azam *et al.*, 1999) and anoxic conditions (Ciglenečki *et al.*, 2000) can determine a delay of the degradation of organic matter.

Moreover, the above result suggests and confirms several other hypotheses about the causes of the phenomenon. If mucilages are due to an alteration of the humification process, which are the main causes of such an alteration? The aggregation of organic matter in the marine environment from small colloids ($<0.2\,\mu$m) to higher size aggregates ($>1.0\,\mu$m) is supported by diffusion limited process (Wells and Goldberg, 1993) when low turbulence regimes support the aggregation of organic matter into marine snow (Herndl *et al.*, 1999) and mucilage (Degobbis *et al.*, 1995). So, the role of carbohydrates in the mucilage formation has to be re-considered within all the proved evidences of the natural causes supporting the aggregation of natural organic matter (Alldredge and Croker, 1995).

On the base of our results and on the specific studies reported in the scientific literature we believe that the observation of a temporal steady state is a potential marker of the mucilage events in the Adriatic Sea because it could represent the first step of an anomalous aggregation of carbohydrates leading to mucilage formation.

3.2 The Presence of Carbohydrates in Marine Snow and Mucilage Samples

A further confirmation of the role played by carbohydrates in the mucilage formation arises from the study on samples of marine snow from an area of the Central Tyrrhenian Sea between Isola del Giglio and Isola d’Elba (Fig. 2). In this area, hydrological conditions of high temperature and salinity, that seem to be the most favourable to support the formation

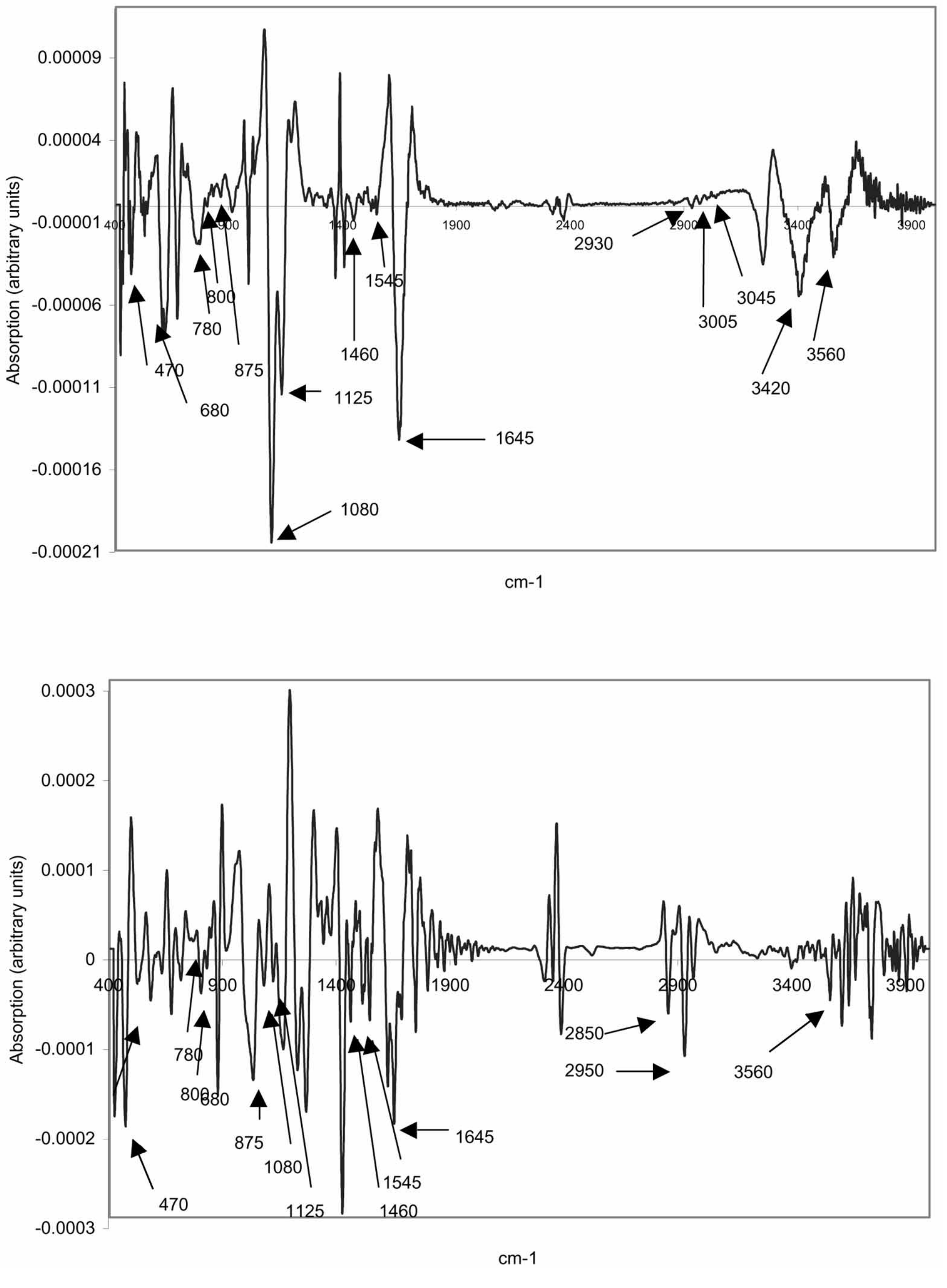

FIGURE 7 Comparsion between the second derivative FTIR spectra of a marine snow sample (upper spectrum) and a mucilage sample from Isola del Giglio (lower spectrum). The structural similarities between the two different states of aggregation of the organic matter are evident as shown by the common bands of infrared absorption. See the list of Table III for the assignement of the bands.

of extracellular polymeric substances (Liu and Buskey, 2000) and mucilages (Russo, 2001) were observed on May 2002. Some authors (Alldredge *et al.*, 1993; Fogg, 1995) suggest that mucilage formation can begin from marine snow which progressively aggregates forming structures with increased dimension and molecular weight. This hypothesis agrees very

TABLE III Principal FTIR Absorption Bands Common to Marine Snow and Mucilage Sample with the Relative Identified Compounds.

Functional group	*Wavelength* (cm^{-1})	*Compound*
Stretching OH*	3400	Carbohydrates
Stretching C—O*	1030	Carbohydrates
Stretching C—O—C†	1160–1140	Carbohydrates
Stretching C=O* AmideI*	1650–1640	Proteins
Stretching C—N Amide II*	1545–1540	Protein
Stretching C=O*	1460	Carbonate
Bending C=O†	875	Carbonate
Stretching Si—O*	1080	Quartz
Intertetraedric O—Si—O†	780 and 800	Quartz
Bending Si—O†	470 and 680	Quartz
Bending C—H†	2930–2950	Aliphatic and protein

*Strong band.
†Weak band.

well with the other hypothesis that consider mucilages as a consequence of an altered marine humification process because the humification process also consists of the formation of organic matter aggregates starting from low molecular weight compounds such as fulvic acids, to higher molecular weight compounds such as humic acids and humin (Ishiwatari, 1992). A simple and fast way of verifying the hypothesis of the evolution of the aggregate from marine snow to mucilage is to compare the structure of both types of aggregates by means of the FTIR spectroscopy, a powerful tool for elucidating the structure of organic matter (Kögel-Knaber, 2000). In Figure 7, we report an example of this comparison by means of the second derivative FTIR spectra of a marine snow sample and a Tyrrhenian mucilage sample from Isola del Giglio. Though the dimension of mucilage aggregates (>1 cm) is two order of magnitudes higher than the dimension of the marine snow sample (<0.01 cm), the structural analogies are relevant. Carbohydrates and proteins are significantly present in each type of sample as results by the typical bands of absorption (Tab. III). However, an additional and interesting similarity between the two samples arises from the presence of the bands of silica (quartz) and carbonate. The presence of inorganic compounds such as quartz and carbonate after the prolonged treatments of dialysis and lyophilization, shows that they are bonded to the organic matter by means of strong chelating interactions which are typical of humin, the insoluble fraction of the natural organic matter (Schulten and Schnitzer, 1995); in this type of interactions between organic and inorganic compounds, carbohydrates always play a fundamental role (Leppard, 1995; Yang *et al.*, 2002). So, the structural similarity between mucilage and marine snow samples shows that their pathway of formation is reasonable. This and the observed steady state referred to carbohydrate amount in concomitance to the presence of mucilages, is the first step of the aggregation of organic matter which evolves forward to mucilage formation.

4 CONCLUSION

The results of the time series analysis applied to the data of dissolved and particulate carbohydrate amounts in the four transects of the Adriatic and Tyrrhenian Sea allow to confirm that these compounds play a fundamental role in the coalescence of the marine organic matter forming the mucilage macroaggregates. In the year where mucilages were more evident, the transects located in Northern Adriatic Sea always showed a time interval, corresponding to the spring and summer periods, where the amount of dissolved carbohydrates was almost constant without significant variation.

According to characteristics of the humification processes in the aquatic environment, this almost temporal steady state could depend reasonably on the altered chemical equilibrium between the depolymerization and polymerization reactions which produce the humic substance of the marine environment. In fact, we suggest that the temporal steady state observed for carbohydrates represents the unbalanced aggregation of organic matter which leads to the mucilage formation where, in the altered humification process, the degrading reactions of the organic matter become slower than the polymerization (*i.e.* aggregation) reactions. This unbalanced equilibrium between aggregation and degradation reactions of the organic matter could explain why in presence of the mucilage events, the amount of carbohydrates is not higher than the amount of carbohydrates observed in the years where the presence of mucilage is negligible or absent. The central role of carbohydrates in all the aggregation processes leading to the mucilage formation is confirmed by the study on the structure similarity existing between mucilage and marine snow samples.

These results reveal for the first time, that the mucilage appearance is not necessarily connected to an extra formation of carbohydrates with respect to the period without any mucilage appearance; vice versa, the interpretation of these results suggests that the presence of mucilages can be related to a complex mixture of events producing anomalous conditions within the humification processes leading to a stationary state where the natural degradation of organic matter is delayed.

At last, the results of this study suggest that the monitoring of carbohydrates in seawater can be an helpful marker for the comprehension of mucilage appearance in the Italian Seas.

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