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ORIGINAL RESEARCH PAPER

PHYSICAL-CHEMICAL CHARACTERIZATION OF SOME GREEN AND RED MACROPHYTE ALGAE FROM THE ROMANIAN BLACK SEA LITTORAL♦

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Abstract: Algae are a group of organisms, *Thallophytes*, containing specific bioactive compounds (i.e. brominated phenols, heterocyclic oxygen compounds, sterols, terpenes, polysaccharides). Their excessive growth has negative consequences on marine organisms; on the other hand, they have a crucial role in food, pharmaceuticals, cosmetics, agriculture. In this paper were studied the main physical-chemical characteristics correlated with the biological specificity of three species of multicellular algae, *Enteromorpha intestinalis* and *Ulva rigida* (green), *Ceramium rubrum* (red), frequently encountered along the Romanian Black Sea coast. Generally mixtures of *thallophytes* algae from the Black Sea were collected, processed and characterized. Density, *p*H, conductivity, anions, loss on drying, ash, total nitrogen, protein, lipids, carbohydrates, carotenoids were determined. The results emphasized the possibility of using these marine resources as biofertilizer in agriculture.

Keywords: *green macroalgae, red macroalgae, physical-chemical composition, Romanian Black Sea coast*

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INTRODUCTION

It is well-known that ocean is a rich source of biological and chemical diversity. It represents more than 70% of the entire planet, and hosts more than 300,000 species of plants and animals.

Diversity and productivity of marine ecosystems are very important in preserving health of aquatic and terrestrial environment and provide important sources of food to humans and animals, additives in food and cosmetic industry and medicine, as well.

Some of the marine bioactive substances can be used in industry as technological components, laboratory tools or ingredients in cosmetics. They are already marketed with profits and benefits. It may be also profitable to obtain medicines from marine organisms. For example, a small number of marine plants and animals and bacteria as well provided approximately 12,000 pharmaceutical products. Among commercially available marine byproducts, arbinosides extracted from *Tethya crypta* sponge are Ara-A (acyclovir) as antiviral drug used in herpes infections from marine sponges, Ara-C (cytosar-U, cytarabine) as anticancer drug used in leukemia and non-Hodkin's lymphoma [1].

Research and industry of marine products have developed in the last 25 – 30 years. The starting point was the use of products from fish processing industry. Chitin and chitosane obtained from shrimp wastes, proteolytic enzymes from fish intestines, alkaline phosphates from lipids of marine fish and shrimps are only a few examples [2 – 4].

Investigations upon marine bacteria and algae were done afterwards. Because of the harsh living conditions they live in, these organisms have unique properties.

Organisms from the tropical waters were the basis of screening and development of chemical products of marine origin [7]. Production of marine chemical products was based on the extraction from raw materials. If a bioactive substance has properties which are of economic interest, the commercial source for pharmaceutical industry must rely on organic synthesis or fermentation, which allows a company to control the entire process of production.

Based on genetic nature of marine environment, methodology for effective screening of marine organisms represents a huge potential, taking into account the market available worldwide and the great possibilities to prospect marine resources.

The goals of the integrated policy of marine environment protection are the following [10]:

- to protect and preserve the marine environment, to allow its reconstruction or to reconstruct, if possible, its structure, functions and processes of marine biodiversity and ecosystems;
- to prevent and gradually eliminate pollution in water and on the coast not to put marine ecosystems and human health at any significant risks.

In this context, our researches on using marine bio-resources were treated very seriously. We kept on monitoring the marine ecosystem, characterizing bio-resources and providing marine biomass at the same time.

This study aims to point out one part of the ecosystem on the Romanian seashore, which is not known or explored enough.

Seaweeds are part of systematic *Thallophyta.* They are lower plants with a mere organization, with a slightly differentiated body. Depending on the color of the algae, which depends on prevailing pigment cells, they are classified as: blue algae - *Cyanophyta*, green algae - *Chlorophyta*, and brown-red algae - *Phaeophyta-Rhodophyta* [1 – 3, 11 – 14].
Seaweed is of major economic importance; due to chemical and biochemical composition were used as a source for active pharmaco-dynamic substances, food and fodder or feed additive, agricultural fertilizer, ingredients in food and cosmetics industry. Research on chemical and biochemical content of some species of algae in the Black Sea have been undertaken some time in our country [4, 5].
The general chemical composition of algae consist of polysaccharides, cyclic polyols, vitamins, pigments, sterids, phenolic compounds and amino acids, metals and metaloides (chlorine, bromine, iodine). The chemical composition of algae shows differences even within the same plant, recorded significant variations, depending on morphological or anatomical element isolated after harvest. Algae contain many of the elements presented in sea water, which can accumulate in considerable quantities. Biochemical changes that occur during the vegetative period, depending on the season, are closely reflected in the variation of chemical composition of algae; finds a maximum content: lipids, protein, ash, carbohydrates [2, 4, 7 – 9].

MATERIALS AND METHODS

Biological samples have been collected from three locations, directly from the marine environment: Mamaia - Pescărie Gulf - Constanța area, Capul Turcului Gulf - Eforie Sud area and Vama Veche - 2 Mai, in the period January - April 2010, following the standard methodology for marine sampling; total number of processed samples: 12 macrophytobenthos and 16 marine fauna samples. Sampling frequency was depending of weather conditions and the biological cycle of the species, especially macroalgae. The methods been used were differentiae, according to the pursued goal. Marine samples were sorted, obtaining the following fractions:
- algal material (red algae harvested in winter and green algae harvested in warmer spring months);
- a rich material consisting of marine organisms, shells, exoskeleton remains and tissues.
Studies regarding biological macroscopic and microscopic characterization were carried out. Marine samples collected from the shallow area between January - April 2010 were analyzed depending on their nature, vegetal and animal biomass (Table 1) [15].
Full samples (sediments, macroalgae) were subjected to processing and analysis procedures which aimed separation, shunting and identification of key taxons from deposits on the beaches.

Table 1. *General characterization of marine biomass collected in March - April 2010 from the shallow are of the Mamaia – Vama Veche coastal segment*

Month	Characteristics (for a sample)	Vegetal biomass	Animal biomass
March	No. of recently ashore deposited species	3	6
	Ratio between abundance of	Macroalgae : Invertebrate fauna – 2:5	
	Ratio between biomass of	Macroalgae : Invertebrate fauna – 3:7	
	Ratio biomass	Mollusks : Crustacea – Decapoda – 9:1	
April	No. of recently ashore deposited species	14	16
	Ratio between abundance of	Macroalgae : Invertebrate fauna – 7:9	
	Ratio between biomass of	Macroalgae : Invertebrate fauna – 7:17	
	Ratio biomass	Mollusks : Crustacea – Decapoda – 9:5	

Sampling

Samples were collected from collection points situated on the lower beach area, stored in plastic bags and containers and transported to the laboratory (Figure 1) [16].

February March April

Figure 1. *Sampling from littoral sector Constanța –Vama Veche, February -April 2010 (Photo: Negreanu-Pirjol, 2010)*

Separation

Sedimentary material was separated from the biological one by integral sample washing through granulometric sieves (mesh size: 5, 1, and 0.5 mm; Figure 2).

Figure 2. *Sample processing (Photo: Paraschiv, 2010)*

The procedure followed to collect quantitative samples and their processing in the laboratory is in accordance with the Standard Methodology for Biology Samples Analyses [17], so that results can be statistically processed.

Triage and identification of biological material

This operation is essential to identify the dominant species and for further chemical and biochemical analysis. In a first stage is carried out the separation of vegetal/macroalgae; after separation follows the triage and proper identification of organisms, identification made on the basis of taxonomic characters emphasized by stereomicroscope and Nikon microscope (Figures 3 and 4); considered quantitative parameters were occurrence frequency and dominance of a low number of species.

Figure 3. *Triage and identification of biological samples (Photo: Negreanu-Pirjol, 2010)*

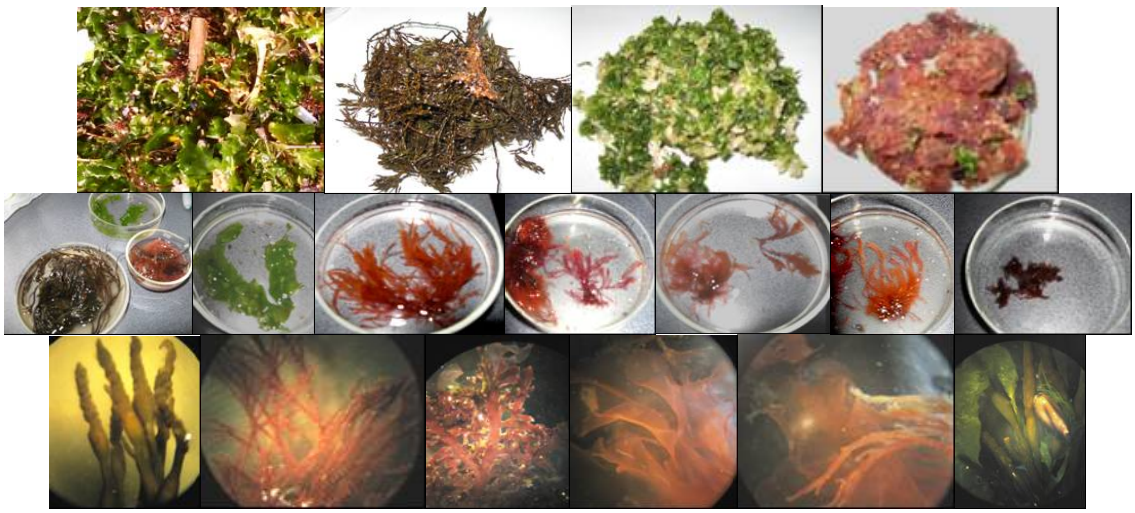

Figure 4. *Triage and identification of macroalgae samples (Photo: Paraschiv, 2010)*

Obtaining algal powder material

Preliminary washing was performed with sea water in plastic tanks with a shaking device. The operation aims to remove impurities, gravel and sand from the mass of raw material collected. Seawater was preferred because the environment does not change its native characteristics and avoid cell lyses, a phenomenon that would lead to loss of organic matter.

After washing, the material was dried at room temperature. Algae mass is a fragile constitution, and chemical composition and biochemical attributes are affected by thermal treatment at temperatures above 50 °C.

Dried material was ground. The grinding device has two working compartments, one for rough fragmentation, and one for fine grinding. Material resulting from the grinding process was separated through vibrating granulometric sieves. The device is equipped

with a set of sieves with openings from 0.045 to 6.3 mm. The fraction greater than 1 mm was returned to grinding. From the obtained algae powders a quantity of 50 g of each species has been taken in order to analyze water content, ash, protein, carbohydrate and fat [18].

Determination of loss on drying and ash

Loss on drying was determined by drying the sample at 105 ± 2 ºC (thermoregulation oven Caloris EC100, temperature range: 50 – 240 ºC) for 12 hours. The minerals content was determined by sample calcinations at 550 ± 10 ºC for 12 hours. Organic substance (OS) was calculated as the difference between 100% and the sum of loss of drying and ash percentages [19, 20].

Determination of total nitrogen and protein

Total nitrogen - protein content in particulate algal substances was determined by Kjeldahl method, using the Digestor UdK DK6 equipment and distillation unit 127, Velp program. The method is based on the determination of total nitrogen in the sample after it has been mineralized in the presence of sulfuric acid under the catalytic action of mercury and selenium. After alkalinization, ammonia is steam driven and captured in a boric acid solution which is dosed by titration with hydrochloric acid. Results were expressed as percentage relative to the amount of algae powder used in the test [19, 20].

Determination of lipids

Lipids from samples were extracted with dichloromethane in Soxhlet apparatus for 5 hours. After solvent evaporation fats were determined gravimetrically. Results were expressed as percentage relative to the amount of algae powder used in the test [21].

Determination of carbohydrates

Carbohydrate extraction was performed with acetic acid solution 15%. Carbohydrate content was determined by the method of Dubois (1956), with an absorption maximum at 490 nm, using an UV-VIS spectrophotometer Specord 205 (measuring range 190 – 1000 nm). Results were calculated based on a standard calibration curve with glucose [10, 11].

Quantitative determination of carotenoids

For quantitative determination of total carotenoidic content, the spectrophotometric method has been applied, using as a reference substance a solution containing 2.5 mg β-carotene in 100 mL benzene [22]. Sample absorbance is determined against the solvent at λ = 470 nm. Readings were done with a Jasco V 630 spectrophotometer (Table 2) and the β-carotene concentration was determined by graphical method, using standard curve of β-carotene, where: $Y = A \times X$; A = 0.0860326; correlation coefficient R^2 = 0.992366.

Table 2. *Standard curve determination for β-carotene*

Sample no.	Volume of 2.5 mg β-carotene/100 mL benzene solution [mL]	Volume of benzene [mL]	β-carotene concentration [μg.mL^{-1}]	Absorbance [relative units]
1.	0.1	difference up to 10 mL	0.25	0.013
2.	0.2		0.50	0.034
3.	0.3		0.75	0.055
4.	0.4		1.00	0.078
5.	0.5		1.25	0.112
6.	0.6		1.50	0.142
7.	0.7		1.75	0.147

Sample preparation and physical – chemical parameters determination

Coding samples

M1 - seaweed, collected in February 2010, Mamaia - Pescărie Gulf - Constanța area;
M2 - green algae, collected in March 2010, Capul Turcului Gulf - Eforie Sud area;
M3 - red and brown algae collected in March 2010, Capul Turcului Gulf - Eforie Sud area;
M4 - green algae, collected in April 2010, Vama Veche - 2 Mai Gulf areas;
M5 - red and brown algae, collected in April 2010, Vama Veche - 2 Mai Gulf areas.

Collected samples were dried and then subjected to maceration, 6 g of dry sample per 100 g distilled water. Mixture samples were centrifuged 10 min at 3000 rpm, and supernatant was separated by filtration. Opalescent samples were subjected to decantation for 16 hours. On the resulted solutions the following physical-chemical parameters were determined: *p*H, temperature, electrical conductivity, relative density, phosphates, total phosphorus, chlorine, free chlorine, total chlorine, nitrates, nitrites, sulfides, sulfates, ammonium, total dissolved salts. Each parameter was determined three times for each sample, was recorded separately and averaged the results (Table 3).

RESULTS AND DISCUSSION

On the seaside of Black Sea the most frequent macrophyte algae belong to the chlorophytes from the group *Enteromorpha* spp., *Ulva* spp. and *rhodophytae* from the group *Ceramium* spp. The increased values of the primary production include also the species of the groups *Enteromorpha* and *Ceramium*. This assertion is due to the distribution of this kind of algae in all the seaside areas with rocky substratum, almost in all seasons of the year (Figure 6).
The period searched by us was extremely limited, as the first propagules started to be seen only at the end of March and developing of the macro algae flora started to be noticed in April (due to the meteorological conditions with a different evolution this year comparing to previous years).

Table 3. *Methodology for physical-chemical analyses*

No.	Parameter	Method of analysis	Apparatus
1	*p*H, temperature, electrical conductivity	potentiometric	WTW82362 portable *p*H-meter (Germany)
2	Relative density	pycnometer	50 cm^3 glass pycnometer; thermometer; electronic scales with two decimals AND EK 300i (Korea)
3	Total phosphorus/ phosphates	colorimetric	Kit Aquaquant 1.14445.0001 (Merck)
4	Chlorides	colorimetric	Kit Aquaquant 1.14401.0001 (Merck)
5	Free chlorine and Total chlorine	spectrophotometric, measuring range 0.05 – 5.00 $mg.L^{-1}$, λ = 560 nm	Kit Spectroquant 1.00597.0001 (Merck)
6	Nitrates	spectrophotometric, measuring range 0.5 – 15.00 $mg.L^{-1}$, λ = 530 nm	Kit Spectroquant 1.14542.0001 (Merck)
7	Nitrites	colorimetric	Kit Aquamerck 1.11170.0001 (Merck)
8	Sulphides	colorimetric	Kit Aquaquant 1.14416.0001 (Merck)
9	Sulfates	spectrophotometric, measuring range 5 – 250.00 $mg.L^{-1}$, λ = 530 nm	Kit Spectroquant 1.14548.0001 (Merck)
10	Ammonium	colorimetric	Kit Aquaquant1.14428.0001 (Merck)
11	Total dissolved salts	conductimetric	Portable TDS-meter (Merck)

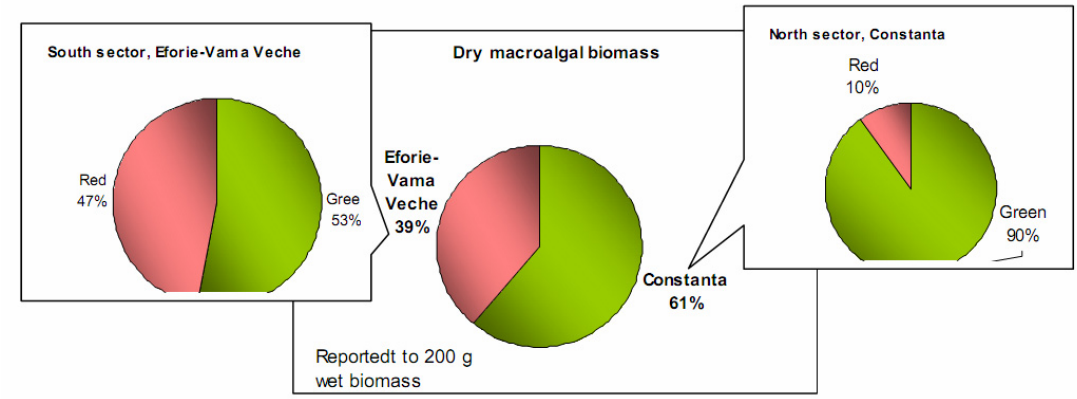

Figure 6. *The ratio of the dried macro algae from northern and southern sector of Romanian seaside (dried algae/200 g mixture of humid macrophyte algae)*

If in the northern sector of the Romanian seaside were noticed more organisms of the group of green algae, in the southern sector, red algae development is almost comparable to that of green algae; this fact is explained firstly by the great expansion of

the calcareous platforms in this sector, platforms that represent the necessary attachment substratum.
The results of the physical-chemical, chemical and biochemical analyses are presented depend on algae species (Table 4) and depend on specific collected station area (Table 5). The average levels of lipids, proteins, carbohydrates and minerals content in powder from three macrophyte algae species, *Enteromorpha* spp., *Ulva* spp. and *Ceramium* spp. are presented in Table 4.

Table 4. *The chemical and biochemical composition of algal powder from green, red and brown algae collected from the Romanian seaside*

Parameter	***Enteomorpha* spp.**	***Ulva* spp.**	***Ceramium* spp.**
Loss on drying, [%]	9.14	12.86	11.01
Ash, [%]	30.28	18.38	13.83
Organic substance, [%]	60.57	68.86	75.16
Total nitrogen, [%]	1.94	2.32	3.19
Proteins, [%]	12.10	14.58	19.94
Lipids, [%]	1.69	0.69	3.43
Carbohydrates, [%]	46.57	54.95	51.90
Beta-carotene in liposoluble substances, [mg/100 g]	0.1372	0.1287	0.2199

Powder mixtures obtained from the three algal species: *Enteromorpha* spp., *Ulva* spp., and *Ceramium* spp. presented the following chemical composition: carbohydrates average (51.14%); proteins average (15.54%); lipids average (1.93%).
The average content of mineral substances in green algae (*Enteromorpha* spp. and *Ulva* spp.) 24% is statistically different compared to that of red algae (*Ceramium* spp) 13.83%. Proteins content in algal powder from green algae (13.34%) is statistically lower than that in red algae powder (19.94%). The average lipids content presents statistically insignificant differences between green algae (1.19%) and red algae (3.43%). Carbohydrate content of algal powder obtained from green algae species (50.76%) showed statistically insignificant differences compared to that of powders obtained from red algae (51.90%). The carotenoids content of algae, expressed as β-carotene, is generally small, being slightly increased in red algae compared to the green ones.
Concluding, in the red macroalgae species were registered increased values for proteins, lipids and carbohydrates compared with the ones of the green macroalgae species. On the other hand, for the mineral substances content, increased values for green algae species compared with the red algae species were registered.
The results of the physical-chemical analyses of macroalgae samples distributed on collected stations (M1 - M5) are presented in Table 5.

Table 5. *The physical-chemical parameters of the analyzed macroalgae samples*

Parameter	Sample station				
	M1	**M2**	**M3**	**M4**	**M5**
*p*H	7.09	7.26	7.36	7.25	7.21
Temperature, [°C]	4	7	7	10	10
Electrical conductivity, [mV]	-1	-8	-16	-19	-3
Relative density [against water at 20 °C]	1	0.9997	1.0001	1.0008	1.0006
Total phosphorus, [mg/g dry sample]	0.00025	0.018	0.117	0.00031	0.00025
PO_4^{3-} [mg/g dry sample]	0.00076	0.055	0.361	0.211	0.350
Cl^- [mg/g dry sample]	< 0.00014	0.00255	0.0012	0.00255	< 0.00014
Free Cl_2, [mg/g dry sample]	0	0	10.638	0	1
Total chlorides, [mg/g dry sample]	0	0	0	0	0.0033
NO_3^-, [mg/g dry sample]	1.666	56.265	106.382	54.32	80.45
NO_2^-, [mg/g dry sample]	0.04	0.1	0.21	0.01	0.18
S^{2-}, [mg/g dry sample]	0.022	0.032	0.056	0.054	0.049
SO_4^{2-}, [mg/g dry sample]	0.03	0.031	0.076	0.018	0.030
Free CO_2, [mg/g dry sample]	1.43	0	0	2.53	1.54
NH_4^+, [mg/g dry sample]	0.005	0	0	0.0025	0.01-0.015
Total dissolved salts, [mg/g dry sample]	21.5	209.63	236.17	37.2	36.9

Regarding the physical-chemical and biochemical composition of the green and red macroalgae species, there are no standard or values limits, for these types of biological samples. Several remarks may be outlined:

- *p*H values recorded are within acceptable limits, without significant variations; Samples M3 (red and brown macroalgae) and M5 (red seaweed) were slightly elevated;
- Electrical conductivity has negative values for all the analyzed samples;
- Total phosphorus was obtained in greater quantity in sample M5 (red and brown algae), 0.117 mg/g dry sample, due to water eutrophication, specific low salinity for Black Sea waters and marine streams missing;
- Samples containing red algae (M5) shows higher values for other parameters: phosphates, free chlorine, nitrates, nitrites, sulfates, total dissolved salts, due to water eutrophication, specific low salinity and marine streams missing;
- Sulfate ion shows values in larger quantities especially in sample M5, due to the presence of invertebrates organisms in algal biomass;
- The amount of ammonium ions shows low content with slightly variations especially in samples M5, due to organic matter degradation in gulf area and marine streams missing.

CONCLUSIONS

- Considering that in the first quarter of 2010 the meteorological conditions evolved differently than previous years, the marine macroalgae brought by streams or waves to the shore (during the strong storms) were significantly more reduced quantitatively;
- Due to the same meteorological conditions, the developing of macro flora of algae was not early or significant either; generally, in March the species of macro algae of cold temperature starts to develop; the first fragments of algae were noticed at the shore at the beginning of April.
- Compared to literature data and taking into consideration nonentity of the standard limits values for the analyzed parameters in macroalgae, no differences regarding qualitative composition were registered. The values of physical-chemical and biochemical parameters obtained for the three categories of macrophyte algae, generally were comparable and was not recorded a significant variation between the sampling stations; all parameters values are in the acceptable limits for this type of samples. Considering that the algae are natural sea water biofilter the measured biochemical parameters shows non accumulation of inorganic compounds in macroalgae and no polluted areas were reported.
- Regarding the biological, physical-chemical and biochemical analyzed composition of the green and red macroalgae from the Romanian Black Sea coast, this biomass could be improved and recommended to be used in agriculture, as potential fertilizer.

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