EJMN

January - June 2019
EUROPEAN JOURNAL OF
Volume 2, Issue 1
MEDICINE AND NATURAL SCIENCES

ISSN 2601-6397 (Print)

ISSN 2601-6400 (Online)

REVISTIA

PUBLISHING AND RESEARCH

EUROPEAN JOURNAL OF

MEDICINE AND NATURAL SCIENCES

January - June 2019

Volume 2, Issue 1

Typeset by Revistia

ISSN 2601-6397 (Print)

ISSN 2601-6400 (Online)

Address: 11, Portland Road, London, SE25 4UF, United Kingdom

Tel: +44 2080680407

Web: https://ejmn.revistia.com

Email: office@revistia.com

Indexed in Elsevier's Mendeley, WorldCat, RePEc & Ideas, Google Scholar, Microsoft Academics, Crossref

TABLE OF CONTENTS

CONSTANTIN LIPSA
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ISSN 2601-6397 (Print)
ISSN 2601-6400 (Online)
European Journal of Medicine and Natural Sciences
January - June 2019
Volume 2, Issue 1

Marine Algae from Black Sea - Important Resources in the Pharmaceutical and Medical Research

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Abstract

During the past years, it became obvious that the ecosystem presents a marine algae surpluses, which should be turned valuable in one way or another. The importance of the macrobenthic flora – algae and phanero-gammes – for the general productivity of the marine environment, especially in shallow waters, is becoming more and more obvious from the biological as well as from the economical point of view. The macrophytes also represent a particular life form. The benthic macroflora includes 33 species 4: 16 Chlorophyta, 10 Rhodophyta, 5 Phaeophyta, and 2 Phanerogama. The quantity of green algae (Chlorophyta) was higher in the Constanta – Eforie area, as red algae are predominant in the southern part of the littoral. Brown algae (Phaeophyta) were encountered in the Constanta city area (Punctaria) and in Vama-Veche (Cystoseira barbata). Yet, this fact indicates a slight amelioration of the marine ecosystem, after many years of eutrophication. The superior capitalisation of the marine biomass represents a highly important resource for the pharmaceutical industry, supplying raw material for the extraction of bioactive substances and various other substances, the purity of which is strongly connected to the state of the marine ecosystem.

Keywords: marine algae, Chlorophyta, Rhodophita, Phaeophyta, Phaeophyta

Introduction

The importance of the macrobenthic flora – algae and phanerogames – for the general productivity of the marine environment, especially in shallow waters, is becoming more and more obvious from the biological, as well as from the economical point of view[1÷3]. The macrophytes also represent a particular life form. The interspecific relationships between the benthic macro- and microphytes, as well as the relationship between these and the associated fauna ensure the existence of a wide interspecific type of relationships. Yet, this fact indicates a slight amelioration of the marine ecosystem, after many years of eutrophication. The superior capitalisation of the marine biomass represents a highly important resource for the pharmaceutical industry, supplying raw material for the extraction of bioactive substances (vitamins, sterols, and collagen) and various other substances, such as agar-agar, the purity of which is strongly connected to the state of the marine ecosystem.

Research Methods

In 2012 it was conducted the qualitative and quantitative analysis of phytobentic samples on a range of 75 samples collected during the summer season. The samples were taken from profiles and stations considered representative in terms of algal flora and follow the coastline: Năvodari, Constanta Casino, Eforie Nord, Eforie Sud, Tuzla, Costineşti, Mangalia, 2 Mai and Vama Veche[1]. Following the qualitative analysis, in summer 2014 were identified 20 taxa assigned to filums as follows: 9 species belonging to the phylum Chlorophyta, 1 species – phylum Phaeophyta (Cystoseira barbata), 8 species belonging to the Rhodophyta phylum (7 species and a variation, respectively Ceramium rubrum var. barbatum) [3]. In 2014 in view of the monitoring of the Black Sea water quality there were investigated the physico-chemical indicators. They were obtained from the analysis of surface water samples and of the water column (0-20 m) collected during two oceanographic expeditions (in May and November), from two stations situated on 5 and 20 m isobath[2] . There were analysed the main physical – chemical and status indicators that characterize and control the eutrophication level, namely: salinity, pH, dissolved oxygen, inorganic nutrients. The salinity was measured in-situ. The dissolved oxygen was determined by the Winkler method. The pH was measured by the potentiometric method. The nutrients in seawater were quantified by analytical spectrophotometric methods, internally validated in the laboratory with reference to the textbook "Methods of Seawater Analysis" [4] The benthic macroflora included 33 species [5]: 16 Chlorophyta, 10 Rhodophyta, 5 Phaeophyta, 2 Phanerogama. The quantity of green algae (Chlorophyta) was higher in the Constanta – Eforie area, as red algae are predominant in the Southern part of the littoral. Brown algae (Phaeophyta) were encountered in the Constanta city area (Punctaria) and in Vama-Veche (Cystoseira). During the past years, it became obvious that the ecosystem presents a marine algae excess, which should be turned valuable in one way or another. The following species stand out: Cladophora vagabunda (L.), Enteromorpha intestinalis (L.), and Ulva Lactuca Species (Ulvae rigida). From the read and brwoun algae we have Ceramium rubrum and Cystoseira barbata. From the green algae of the Black Sea, the systemic clasification of the Class Chlorophyceae was presented [5,6,7]. The methods are from botanical phytochemical and physico-chemical area.

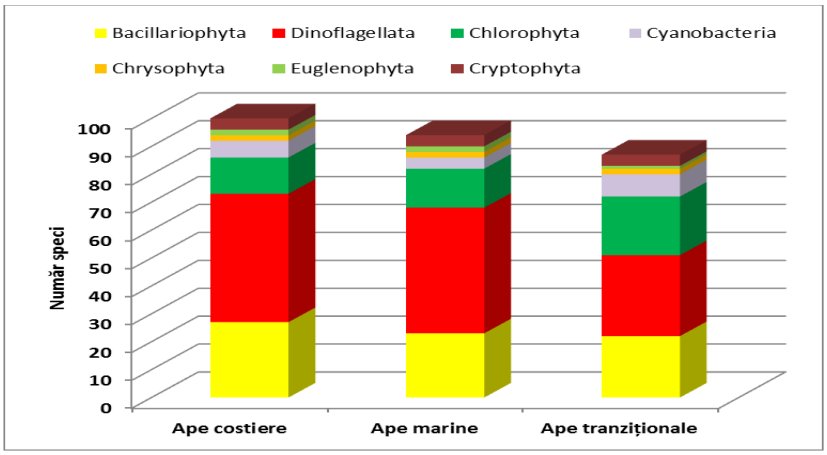
Results and Discussions

It resulted that algal biomass grew abundantly in summer 2012 as a result of favourable environmental conditions (respectively, high temperature water, amount of nutrients, photosynthesis transparency favorable to photosynthesis). Among green algae, Cladophora vagabunda (1.800 g/m^2 fresh biomass) and Cladophora sericea (1.700 g / m^2 bp) and from the red algae - Ceramium rubrum var. barbatum (approx. 1,000 g / m^2b.p.) developed high biomass. These species were identified during the whole summer season, both in samples and in storage on shore, particularly in the northern part of the coast, because in the southern part prevailed specimens of Ulva (Ulva lactuca - 1200 g/m^2 b.p., Ulva intestinalis - 770 g/m^2 b.p.) as associated species for Cystoseira barbata fields existing in these areas. [1,3]. Fresh medium biomasses for quantitative dominant groups between 2005-2012 (summer seasons).[1].

In 2014 it can be noted the dominance of opportunistic green algae in the northern sector of the Romanian seaside and the presence of brown algae Cystoseira barbata in Mangalia, 2 Mai and Vama Veche, where it is known that marine waters have a higher quality, which allowed the rehabilitation and the existence of this key marine species for the marine ecosystem (Fig.1). In Mangalia during the summer there were observed Cystoseira barbata well-developed shrubs, epiphytic vagabunda of Cladophora, Ceramium diaphanum var. elegans and Callithamnion corymbosum. The fresh biomass recorded by it was raised (4300g / m^2b.p.), similar to that reported in the previous year (4700g / m^2b.p.), which shows that this species maintain a stable direction of development in this area. In 2 Mai, on a rough rugged substrate, it is found a field of Cystoseira (between 1-3m deep) well developed, with tall Cystoseira barbata specimens, epiphytic generally by small specimens of Ceramium virgatum, at the base of the tals being encountered Ulva lactuca, the dominant species associated to the field. On the elastic surface of the tals were present mussels, which draws attention on the great importance of this perennial brown algae for the ecosystem, as a species that provides life for other organisms. Vama Veche is known from the previous studies as being the area where Cystoseira barbata forms a vast field (between 1-3m) with mature specimens and rich associated fauna.

Fig. 1: The taxonomic composition of the Romanian sector

Black Sea phytoplankton in 2014 [2]



Likewise, in Vama Veche was observed the presence of red algae Corallina officinalis. In the south of the coast, the biomass of the opportunistic species were reduced more compared to other analyzed areas, areas where the perennial species Cystoseira barbata stood out [3]

The salinity recorded homogeneous values between 15.94 to 18.53 PSU, specific values for the brackish waters of the Black Sea. The higher values were recorded offshore, in the water column (Vama Veche station 20m), during spring, due to shaping the thermocline and stratification of water bodies. In autumn, the stratification is not visible, the salinity being homogeneous in the water column. The pH values were in the range of 8.20 to 8.55, normal values, falling within the limits accepted by the Order No.161 / 2006 (The normative concerning the classification of surface waters in order to determine the ecological status of water bodies) namely 6,5 – 9,0. In general, a good water oxygenation was observed in the studied area. In spring, in the surface layer was revealed the photosynthetic production of oxygen, the saturation values being within the range from 103.1 to 124.2% . In autumn, the values were homogeneous throughout the water column, slightly lower at the water-sediment contact area (80%). There were no hypoxia phenomena recorded, all values being within the accepted limit of Order no. 161/2006. **The nutrients** usually registered normal values, specific to the variability domain specific to the area. During spring, were recorded high values of the phosphates (1.87 µM) and ammonium concentration (17.43 µM) at surface. The concentrations of phosphate $(PO_4)^{3-}$, showed concentrations ranging from 0.06 µM, and 1.87 µM. Except the surface value, measured in dots during spring, the other values are low, compared to the 1960's results, reference period for good water quality status of the Romanian coast. The nitrates concentrations (NO3) – varied between 0.04 and 0.93 µM, very low values that do not exceed the maximum concentration permitted by Order no. 1061/2006, or 1.5 mg / dm3 (107.14 µM). Generally, it was observed a homogeneous distribution of nitrates throughout the water column with slightly higher values during fall when, with the decrease of the biological activity, the nutrients stock began to recover. Nitrites (NO 2), intermediate redox process forms involving inorganic nitrogen species reported low concentrations in the range 0.13 - 0.95 µM. All values fall within the maximum allowed by Order no. 161/2006 respectively 0.03 mg / dm3 (2.14 µM). The ammonium $(NH_4)^+$, the polyatomic ion in which the nitrogen has the maximum oxidation number, +3, is the most easily assimilable form of inorganic nitrogen. Its concentrations showed values ranging from 0.51 to 17.43 µM. The maximum value was recorded in May at surface near the shore and it exceeds the accepted limit for both ecologic status and the impact area of the anthropogenic activity in Order no. 161/2006 - 0.1 mg / dm^3 (7.14 µM). The silicates, $(SiO_4)^{4-}$, had low concentrations situated within the range 4.8 - 10.2 µM. The highest values were determined in the water-sediment contact area during spring, as a result of the stratification of the water masses. Regarding the **contamination indicators** in the marine reservation area Vama Veche – 2 Mai, in 2014, the polychlorinated biphenyls and the majority of organochlorine pesticides had, in water, values below the detection limit.

The **macroscopical examination for marine algae** was realized, which represents the first stage in the investigation of the known or untested vegetal products. This was done through the examination of the entire plant (rizoid, celluloid and filoid), with the human eye, as well as with a magnifyong glass, in order to observe its aspect, dimensions, colour, taste

and smell [4, 5]. The **microscopic exam** of the collected algae was realized through the use of specific for the pharmacobotanical researches, [5, 6]. For the global chemical analysis, the extraction of the active principles is very important [6,7,8]. The qualitative chemical analysis involves the successive and selective analysis of the vegetal products, using solvents with opposing polarities, and the separation by the means of chemical methods, followed by specific reactions which help to identify different groups of active principles or certain chemical constituents [7].

Out of the vegetal sample sprinkled with a non-polar solvent (ethylic ether, petroleum ether, benzene, hexane, clorophorm etc), then with a medium polarity solvent (ethanol, methanol) and only in the ned with water, the following fractions were obtained, [6,7,8]:

- etheric extractive solution (A)
- alcoholic extractive solution (B)
- water based extractive solution (C)

Each extract is the analysed for the identification of the active principles of pharmaceutical interest. For the identification of the chemical compounds of the three extracts, they are analysed separately, using the methods fit for the physical and chemical properties of each group of active principles. In the etheric extract we can identify lipophyle chemical compounds, and in the other two extracts hydrophyle chemical compounds.

As a follow up of the identification reactions previously discussed, the following results were obtained, summarized in Table 1, 2, 3, 4.

Table 1The selective reactions used in chemical analysis and identified active principles from seaweeds

Analyzed solution	Reactions used	Identified active principles
Etheric extracts	Lieberman-Burchard	Steroles and triterpenes
	Fluorescent UV. (λ = 365 nm	Cumarines
Alcoholic extracts	Iron Chlorure reaction	Catehic Tanin
	Fehling	Reducing compounds
	Liebermann Bourchard	Triterpenic heterozides
	Borntrager	Antracenozide
	UV (λ = 365 nm)	Cumarines
Water based extracts	Fehling	Reducing compounds
	H_2SO_4 conc. + tymol	Ozes and poliozes
	Foaming	Soapozides
	$FeCl_3$	Catehic tanin

In etheric solution (A):

- a fraction of the etheric extract was evaporated up to dryness; the residuum obtained was dissolved in alcohol - the resulting solution does not have the specific smell of volatile oils for any of the algae species;
- the residuum obtained from the evaporation of the etheric extract was processed with HCl 2% ; the solution undertook the Mayer and Bertrand reactions; the reactions were negative; the analyzed algae do not contain basic alcaloids;
- the residuum obtained from the evaporation of the etheric extract was processed with methilic alcohol; the alcoholic solution undertook the Shibata reactions; the reaction was negative for all algae species, which do not contain flavonoic aglicones
- the residuum obtained from the evaporation of the etheric extract was processed with amonium hydroxyde; the Borntrager reactive did not colout the solution orange; the analyzed algae do not contain endemoles;
- the Lieberman - Burchard reaction on the etheric extract was positive, indicating the presence of steroles and triterpenes in all analyzed algae;
- the Carr – Price reaction was positive, thus they contain carotenoids, for the species Cladophora vagabunda, Ulva rigida, and negative for the other species;
- the residuum obtained from the evaporation of the etheric extract was processed with amonium hydroxyde; the solution did not present an intense fluorescence on UV radiation, thus no species contains cumarines.

Table 2 The active principles traced as a follow up of chemical analyses on the etheric extracts

The seaweed species	Analyzed solution	Steroles and triterpenes	Cumarines
Cladophora vagabunda	Etheric extracts	++	++
Enteromorpha intestinalis		++	++
Ulva lactuca		++	++
Cystoseira barbata		++	-
Ceramium rubrum		++	-

In non-hydrolized alcoholic solution (B):

- the reaction withe the Styassny was negative;
- the reaction with iron chlorure is dark green, thus positive form catehic tanin for the Cladophora vagabunda, Enteromorpha intestinalis, Ulva rigida, Cystoseira barbata species, and negative for Ceramium rubrum;
- In hydrolyzed alcoholic solution:
- through the Fehling reaction, a brick-red precipitate was obtained for all species. Reducing compounds are present in all specie;
- the reaction with ninhydrine of the water based solution obtained from the residuum of alcoholic solution was positive for Cladophora vagabunda, Enteromorpha intestinalis şi Ulva rigida, which cotain aminoacids, and negative for Cystoseira barbata, Ceramium rubrum:
- the residuum obtained through the evaporation of the non-hydrolized alcoholic solution is processed with a water based solution of HCl 2% , then turned alcaline with amonium hydroxyde and extracted with ether; after the evaporation of the etheric solution and the recuperation of the HCl 2% residuum, the Mayer and Bertrand reactions are done; the reactions were negative for all the species. The analyzed specie do not contain salt alcaloids.
- the residuum obtained after the evaporation of the alcoholic hydrolized solution is processed with 50% methilic alcohol; the Shibata reaction on alcoholic solution was negative for all the species; none of the algae analyzed contains flavonozides;
- the Liebermann – Bourchard reaction on the residuum of the alcoholic hydrolized solution caused a green-violet coloration, thus it signalled the presence of triterpenic heterozides in Cladophora vagabunda, Enteromorpha intestinalis şi Ulva rigida ;
- the Borntrager reaction was negative for all species. Antracenozides lack in all species.
- The acid solution is dark brown and does not indicate the presence of antocianozides in any of the species;
- The solution becomes fluorescent under UV radiations, thus cumarines are present in Cladophora vagabunda, Enteromorpha intestinalis and Ulva rigida ;

Table 3 The active principles traced as a follow up of chemical analyses on the alcoholic extracts

The seaweed species	Analyzed solution	Catehic Tanin	Reducing compounds	Triterpenic heterozides	Antracenozide	Cumarines
Cladophora vagabunda	Alcoholic extracts	++	++	++	++	++
Enteromorpha intestinalis		++	++	++	++	++
Ulva lactuca		++	++	++	++	++
Cystoseira barbata		++	++	-	-	-
Ceramium rubrum		++	++	-	-	-

In the water based extractive solution (C):

- The reaction for the identification of starch (with Lugol reactive) was positive for Cladophora vagabunda, Enteromorpha intestinalis şi Ulva rigida;
- We have obtained a flaky precipitate – poliuronides present in Cladophora vagabunda, Enteromorpha intestinalis and Ulva rigida;

- The Fehling reaction was positive for all the species analyzed – reducing compouds are found in all analyzed species;
- The water based extractive solution evaporates into residuum, adding a few drops of concentrate sulphuric acid and tymol alcoholic solution – there results a red coloration, which demonstrates the presence of ozes and poliozes in all analyzed species;
- The foaming reaction of soapozides was negative; soapozides lack in all analyzed species
- The water based extractive solution reacts with diluted $FeCl_2$ and a dark green coloration emerges, which confirms the presence of catehic tanin in all species, except Ceramium rubrum;
- the Mayer şi Bertrand reactions were negative – basic alcaloids lack in all analyzed species.

Table 4 The active principles traced as a follow up of chemical analyses on the water based extracts

The seaweed species	Analyzed solution	Reducing compounds	Ozes and poliozes	Soapozides	Catehic tanin
Cladophora vagabunda	Water based extracts	+	+	++	+
Enteromorpha intestinalis		+	+	++	+
Ulva lactuca		+	+	++	+
Cystoseira barbata		+	+	-	+
Ceramium rubrum		+	+	-	+

Conclusion

The general conslusions of the studies are the following:

As a follow up of the pharmaceutical studies, we have identified and dosed compounds such as flavonoic aglicoles, cumarines, streroles and triterpenes, which are valuable active principles for the pharmaceutical industry.

In addition, we have identified ozes and poliozes, catehic tanin and reduction compounds.

The results obtained enhance the possibility of opening new directions in the process of valorification of the resources offered by the Black Sea, in the research of medicines produced from natural resources.

Acknowledgement

This paper is supported by the Sectoral Operational Programme Human Resources Development (SOP HRD), financed from the European Social Fund and by the Romanian Government under the contract number POSDRU/187/1.5/S/156040/

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European Patients' Rights to Be Protected Against Counterfeit Medicines

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Abstract

Because the falsification of medicines is a global problem, requires increased and effective international coordination and cooperation to ensure the effectiveness of the strategies to combat counterfeiting, especially in relation to the sale of such products on the Internet. In the context of people's health and life rank foremost among the values and interests protected by the TFEU, this article examines the evolution of the legislative process regulating the internal market for medicinal products in order to ensure a high level of protection of public health against falsified medicines and to present the legislative initiatives that have been taken at EU level taking account of new risk profiles, measures meant to ensure, at the same time, the functioning of the internal market of medicinal products. However, this article aims to address consumers' right to have access to safe, effective, quality and innovative medicinal products as a right of the European patient. Ensuring the free movement of medicinal products on the EU market must not violate or restrict this fundamental right of the patient. The threat that falsified medicines pose to public health is also recognized by the World Health Organization (WHO), which has established the International Medical Products Anti-Counterfeiting Taskforce ("IMPACT"). IMPACT has developed the Principles and Elements for National Legislation against Counterfeit Medical Products, which were endorsed by the IMPACT General Meeting in Lisbon on 12 December 2007.

Keywords: patient rights, European legislation, the European Union, counterfeit medicinal products, public health, legislative initiatives, internal market, legal supply chain

1. Introduction

To reduce the disparities in the field of medicinal products for human use, between certain national provisions, which directly affected the functioning of the internal market, was necessary to draw near the relevant laws, establishing rules for

monitoring medicinal products and specifying the obligations of the competent authorities of the Member States to ensure compliance with the legal requirements.

Considering its importance for health services, the pharmaceutical sector is subject to strict regulations. Although the main objective of rules governing the production, distribution and use of medicinal products must be to safeguard public health, however, the means by which this goal is achieved, should not prevent the development of the pharmaceutical industry or of the trade in medicinal products in the European Union. In this context, the existing regulatory framework in this sector should not include unnecessary regulatory constraints that restrict and limit competition.

Overseeing the rules regulating and governing the freedom of competition on the pharmaceutical market and the direct and clear intervention if violations of the regulatory framework are found guarantees the existence of a competitive environment in the pharmaceutical sector in the European Union, the complex mechanisms of the pharmaceutical sector being subject to constant and careful analysis both at the level of the European Commission and of the Competition Council.

2. Theory

The pharmaceutical sector is vital to the health of European citizens, who must have access to innovative, safe and affordable medicinal products. In terms of regulation, the EU level concerns in terms of competition in the pharmaceutical market pay particular attention to the rules on authorization and marketing, on pricing and reimbursement of medicinal products and to those relating to patents.

When examining the compatibility with Community law of the conditions for the retail supply of medicinal products, the Court of Justice recognized the specific nature of medicinal products, whose therapeutic effects distinguish them substantially from other goods. The Court also stated that the health and life of humans rank foremost among the values and interests protected by the TFEU and that Member States are responsible for deciding on the level of public health protection they wish to provide and the measures to be implemented to achieve this level [1].

Directive 2001/83/EC [2], has been an important step in achieving the objective of free movement of medicines. On average, consumers do not have access to generic medicines earlier than seven months after the date on which innovative medicines have lost exclusivity. This is due, in part, to pharmaceutical companies that use various techniques to extend the commercial life cycle of their products. When the original products compete with generic medicines, prices go down and become accessible to a larger number of patients. In some cases, prices may decrease considerably.

Given the experience, especially by the Committee for Proprietary Medicinal Products, since the adoption of the Community code relating to medicinal products for human use additional measures have been necessary in order to cancel any remaining barriers to the free movement of patented drugs.

In 2005 came into force significant changes in the pharmaceutical regulatory framework, which had the objective of facilitating the market entry of generic medicines [3], for example, the introduction of so-called Bolar provisions [4].

Any action by public authorities in the pharmaceutical sector should aim at creating a competitive environment to ensure the access to medicinal products for European citizens to innovative, safe and affordable medicinal products, without unnecessary delay. In this respect, both competition law enforcement and regulatory measures can improve market performance for the benefit of consumers and should be considered in this regard.

To facilitate the movement of medicinal products and to prevent the duplication of controls from one Member State to another, the minimum requirements for the manufacture and imports from third countries were established, as well as the conditions for granting their authorization.

Because the new protection periods were applied for the innovative product for which authorization was applied for and approved after these rules became effective in 2005, some new rules - namely the new harmonized rules on data and market exclusivity, basically entered into force only in 2013.

Regarding medication, when necessary information to protect public health is already available to the competent authorities of the Member State of destination as a result of first placing on the market of a product in that Member State, a parallel imported product is subject to licenses granted on the basis of a proportionally „simplified" procedure (as opposed to a procedure for granting marketing authorization), if the imported product has been granted a marketing authorization in the Member State of origin and whether the imported product is essentially similar to a product which has received marketing authorization in the Member State of destination.

3. Results and Discussions

The parallel trade in products is a legal form of trade on the internal market. It is „parallel" as it involves products that are essentially similar to products marketed through the sales networks of original producers or suppliers, but which takes place outside and often parallel to those networks.

Parallel trade is a result of differences in prices between pharmaceutical products [5], for example, when Member States establish or otherwise control the price of products sold on their markets. In principle, parallel trade creates healthy competition and price decreases for consumers and is a direct consequence of the development of the internal market which guarantees the free movement of goods.

Although the safety and the first marketing of medicines are regulated by EU law, the principles of legality of parallel trade in these products have been established as a result of decisions of the Court under the provisions of the Treaty on the free movement of goods [6].

In an attempt to balance the rights of parallel traders and the need to maintain public interest objectives such as public health, the Commission has introduced guidelines on parallel imports in the *Commission Communication on parallel imports of proprietary medicinal products for which marketing authorizations have already been granted* (2003) [7].

In addition, we must distinguish between parallel trade and reimport. For example, in the case of pharmaceutical products, re-importation designates transactions through which are imported medicinal products from a Member State in which they are authorized, after having been previously obtained by a pharmacy in another Member State from a wholesaler in the Member State of import. In this regard, the Court held that a product manufactured in a Member State which is exported and then reimported into the concerned Member State is an imported product in the same way as a product manufactured in another Member State [8]. The Court noted, however, that these findings do not apply if it is found that such products were exported solely for the purpose of re-importation in order to avoid legislation such as that at issue [9].

The threat that falsified medicines pose to public health is a global problem and requires increased and effective international coordination and cooperation to ensure the effectiveness of strategies to combat counterfeiting, especially in relation to the sale of such products on the Internet. Taking account of new risk profiles, legislative initiatives which have been taken at EU level include measures to ensure, at the same time, the internal market of medicines.

An impact assessment conducted in 2008 by the European Commission [10] brought before the authorities alarming elements regarding falsified medicinal products entering the legal supply chain. The analysis has highlighted the increasing number of falsified medicines seized in customs (2.7 million in 2006 to 2.5 million in 2007, representing an increase of 384% compared to 2005 [11]), the counterfeiting with fatal effects of medicines for serious diseases (heart, cancer) and the introduction to the legal supply chain of fake drugs, including online purchase.

The European Commission estimated that annually are sold to Europe, through legal distribution circuit, 1.5 million boxes of counterfeit medicinal products. The fact that their volume increases on average by 10-20% per year is even more worrying. With a growth rate of 10% , the number of boxes of falsified medicines in the legal distribution circuit could reach 42 million by 2020. According to other, more pessimistic estimates, the growth rate is 30% , which would bring this number to 192 million.

Past experience shows that no falsified medicines reach patients only through illegal means, but also via the legal supply chain. This poses a particular threat to public health and can lead to the distrust of patients, including in the legal supply chain. To respond to this increasing threat, Directive 2001/83/EC had to be changed.

Considering all these alarming aspects, the European Parliament and the Council adopted Directive 2011/62 /EU on the prevention of the entry into the legal supply chain of falsified medicinal products [12].

As the stated aim of the Directive is to protect public health, it provides the legal basis for which the *counterfeiting of medicinal products is a criminal act* which deprives patients of safe and quality medical treatment.

The measures of the Directive to include the mandatory application on the packaging of medicinal products of safety features [13], the increased controls and inspections of factories producing active pharmaceutical substances [14], increasing the strictness of distributor records, the obligation of producers and distributors to report medicinal products presumed fake, and the centralized regulation of online pharmacies.

However, the distribution network of medicinal products is increasingly complex and involves many players which are not necessarily wholesale distributors as referred to in that Directive. This includes not only wholesale distributors, whether or not they physically handle drugs but also intermediaries who are involved in the sale or purchase of medicinal products without selling or purchasing those products themselves, and without owning and physically handling the medication.

The illegal sale of medicinal products to the public via the Internet constitutes a serious threat to public health because in this way counterfeit drugs may reach the public. It was therefore necessary to address this threat in Directive 2011/62/EU.

In this regard, account was taken of the fact that specific conditions for the supply of medicinal products to the public have not been harmonized at EU level and, therefore, Member States may impose conditions for supplying medicinal products to the public within the Treaty on European Union (TFEU).

The Court of Justice of the European Union, analyzing the compatibility with Community law of the conditions for the supply of retail drugs, held that the Member States are responsible for deciding on the level of public health protection they wish to provide and means to be implemented to achieve this level [15].

The Court also stated [16] that Member States should have discretion as regards the supply of medicinal products to the public on their territory. Given the particular risks to public health and the power given to Member States to determine the level of protection of public health, the Court has recognized that Member States may, in principle, restrict the retail sale of medicinal al products to pharmacies only.

Without prejudice to national legislation prohibiting the remote offer for sale to the public of medicinal products subject to medical prescription via the Internet [17], the Member States must ensure that products are offered for sale remotely to the public by means of information society services as defined in Directive 98/34/EC [18].

So that the functioning of the internal market not be unduly restricted, but also for public health protection, for the retail supply of medicinal products sold online, the Directive 2011/62/EU proposes the creation of a common logo that can be recognized throughout the Union and allowing the identification oft he Member State of establishment of the person offering the medicinal products for remote sale to the public. The logo shall be clearly displayed on the Internet site offering the medicinal products for remote sale to the public.

To prevent drugs that are suspected to present a danger to health from reaching the patient, Member States use a system that includes the receipt and handling of notifications of suspected falsified medicinal products, as well as suspected quality defects of medicinal products. If it is suspected that falsified medicinal products have reached patients, urgent public announcements are made within 24 hours to recover these products from the patients. Such notices shall contain sufficient information on the suspected quality defect or falsification and the risks involved.

Enlightening in this respect is the case of Pegasys, which we will present in the following. In November 2013, the Police and the National Agency for Medicines and Medical Devices (MAMD) started an investigation after three pharmacies in the country were found counterfeit syringes with serum hepatitis B and C.

The Syringes with counterfeit hepatitis serum, on which the investigation was initiated, were found in two pharmacies in Pitesti and in one of Ialomita County. The investigation was initiated as a result of complaints received from patients.

The drug manufacturer, Roche Romania SRL, informed the National Agency for Medicines and Medical Devices (NAMMD) in September 2013 on the identification in Germany by the quality department of F. Hoffmann-La Roche, Basel Ltd. of a box of counterfeit Pegasys 180 mg / 0.5 ml. After this information, constant communication with NAMMD continued related to occurrence of suspected counterfeit boxes in Romania. As well, Roche Romania SRL has shown that by November there were no reported cases of counterfeit suspicions of possible penetration in Romania.

After dozens of boxes of counterfeit Pegasys were released in November on prescription in pharmacies in several counties and irregularities were noticed by several people with hepatitis, the forgery came to the attention of the national authorities.

In the context of the investigation started, the Ministry of Health recommended that patients using the product Pegasys 180 µg/0.5 ml solution for injection in pre-filled syringe, when buying it in the last two weeks, to immediately contact the treating physician to determine the appropriate therapeutic management.

In this case, the counterfeiting of a drug for a serious chronic disease, we can speak of a criminal act because for patients with hepatitis B and C, interferon vials mean life expectancy. Counterfeit drugs could endanger patient response to

treatment and even his/her life. The interest in counterfeiting this medicinal product is obviously economic because one vial has an average price of 750 RON, the entire sum being paid by the state through national health programs. Therefore, the investigation of the national health authority was doubled by that of the Organized Crime.

Conclusion

Fighting the penetration of falsified medicinal products in the legal supply chain without hampering the functioning of the internal market of medicinal products is a goal that can not be sufficiently achieved by the Member States and can therefore be better achieved by the Community.

When referring to the implications of a falsification of on public health, we must consider both the specific characteristics of the products in question, and the severity of the conditions intended to be treated with such medicinal products. Another aspect to be taken into consideration when introducing counterfeit medicines into the legal distribution chain is the price of counterfeit medicines. Counterfeiting of medicines for serious chronic diseases, for example, lead to reimbursement by the state of the full price of the medicinal product in question and, in addition, to tax evasion produced by the people introducing the forgeries to the legal distribution network, which endangers the lives of patients, by the lack of the necessary treatment.

Counterfeit drugs are illegal in terms of EU pharmaceutical legislation as they do not comply with EU rules on medicinal products. They pose a major threat to European patients and European industry and the public and stakeholders are deeply concerned about the steady increase of these products detected in the European Union in recent years.

Another concern is the fact that the risk profile has changed. The number of falsifications of innovative and life-saving medicines is increasing.

Even if you the exact number of existing or future cases is unknown, there is a noticeable trend clearly threatening the high level of public health protection in the European Union. *We believe that this trend can have disastrous consequences for consumer patient confidence in the pharmaceutical industry and the policy makers.*

The assessment of policy options, starting from a baseline of "non-action" on falsified medicinal products entering the legal distribution chain and estimates based on existing data, which are limited, were revealed the direct and indirect costs to society of non action, which could reach, depending on the scenario, between 9.5 billion and 116 billion by 2020.

The European Commission compared the costs of non-action costs for achieving the chosen policy options, namely the elimination, by all means, of the risk of falsified medicines entering the legal supply chain, and estimated the costs which will be incurred by 2020 by all actors involved in the distribution of medicines on the internal market.

Unfortunately, the costs the patients consumers of these counterfeit medicinal products have to bear can not be estimated, the danger that they have on human health and life can not be quantified.

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Heavy Metals Existing in the Seaweed from the Romanian Coast of the Black Sea

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Abstract

Seaweed is a natural treasure that can be intensely evaluated for therapeutic purposes. It is well known that the seaweed is an indicator of accumulation of heavy metals. In order to be used for medical purposes, kelp has to meet certain conditions concerning the content of heavy metals accumulated. In this work are presented the results of the analyzes concerning the content of heavy metals in two algae: Cystoseira barbata and Ceramium rubrum.

Keywords: marine algae, Black Sea, Cystoseira barbata, Ceramium rubrum

Introduction

Seaweed is a natural treasure that can be intensely evaluated for therapeutic purposes. During the past years, it became obvious that the ecosystem presents **a marine algae excedent**, which should be utilized in one way or another. Marine algae have been intensely studied [1] In the context of restructured economic activities and of increased exigencies with respect to the implementation of environment politics, a slight but continuous recovery process of the marine ecosystem has been taking place during last years. The superior exploitation of the marine biomass represents a highly important resource for the pharmaceutical industry, supplying raw material for the extraction of bioactive substances (vitamins, sterols, and aminoacids) and various other substances, the purity of which is strongly connected to the state of the marine ecosystem [2, 3]. Several studies regarding marine habitats where large amounts of seaweed can be found have been conducted [4, 5, 6]. Seaweed's rich content in bioactive compounds was outlined through a series of studies conducted by various researchers and published in diverse papers [7,8,9]. The antioxidant abilities of marine algae, as revealed by modern laboratory testing [10] and the attempts of obtaining seaweed gels [11], show the increased interest regarding the use of algae in medicine. It is well known that algae are an indicator of heavy metal accumulation. In order to be used for medical purposes, algae must comply with certain characteristics regarding content of heavy metals. This paper presents the results concerning the heavy metal content of 2 marine algae: *Cystoseira barbata si Ceramium rubrum.*

Research Methods

Atomic absorption spectroscopy was used on seaweed samples, following these stages:

- Mineralization of the sample using the dry mineralization method;
- Establishment of calibration curves for the metals which will be determined;
- Exposure of mineralized solutions to flame ionization and measurement of specific absorbance, followed by calculation of heavy metal content; Cd (λ = 228.8 nm), Cu (λ = 324.7 nm), Zn (λ =213.9 nm), Cr (λ = 357.9 nm), Mn (λ = 279.5 nm), Ni (λ = 232 nm) şi Pb (λ =217 nm).

Drying and calcination in the programmed oven

An established quantity of the sample is weighed (usually 10 to 20g) in a crucible with an accuracy of 10mg, according to the type of sample. The sample is treated with 3mL of $Mg(NO_3)_2$, after which it is kept for two hours at a temperature of 100^0C. The temperature is increased with a maximum speed of 50°C/hours to 350^0C, where it is kept for 3 hours for complete carbonization. The temperature is increased to 450^0C and kept for 3 hours until a white ash is formed.

Ash dissolution

The crucible is removed from the oven and left to cool. The ash is moistened with 5 mL HCl (1: 1), with an added mL of glacial acetic acid and a drop of perhydrol and is evaporated using a water bath or a hot sieve. After cooling, 5mL of HCL and added acetic acid are added to the crucible, so that the ash comes into contact with the acid, after which it is heated for 5 minutes on a water bath. The residue is diluted in a volume of 5mL of water and it is transferred in a 100mL balloon. The mark is reached using warm water. After cooling, the mark is reached using cold water.

Atomic absorption spectroscopy

The analysis of mineral content was performed through flame atomic absorption spectroscopy (FAAS), an analysis method which is based on the property of an atom in fundamental electronic state to absorb radiant energy which is tallied to the wavelength of one its resonance radiations. Microelements were determined from the algae. This method can be used for the following metals which can be found in algae samples: Zn, Cu, Pb and Cd.

Work equipment

For this method, the following work equipment was used:

- Atomic absorption spectroscope GBC AVANTA (air/acetylene flame) and hollow-cathode lamps for all the analyzed elements;
- MLW112 calcination oven, thermo adjustable with a maximum temperature of 1100^0C;
- Mettler Tolede analytic scale with a measurement domain of 10mg-200g;
- Thermo adjustable electric water bath with a temperature domain of 100^0C;
- Thermo adjustable stove.

Reactants and materials used:

- Reactants for sample mineralization: hydrochloric acid, d=1,18 g/mL, azotic acid, d=1,40 g/mL;
- Materials: porcelain crucibles, graded balloons, polyethylene vials, porcelain capsules, Berzelius glasses, quantitative filter paper with low porosity;
- Calibration stock solutions: solutions with a 1000 ppm concentration in the analysed element, from which different concentration solutions are obtained in order to draw calibration curves for each measured microelement.

Calibration solutions

- *Lead calibration solution* 1000 mg/L. 1000g of Pb are diluted in 7mL azotic acid (4:3) in a 1L graded balloon.
- *Cadmium calibration solution* 1000 mg/L. 1000 g of Cd are diluted in 14mL of water + 7mL of azotic acid (4:3) in a 1L graded balloon.
- *Zinc calibration solution* 1000 mg/L. 1000g Zn are diluted in 14 mL water + 7 mL azotic acid (4:3) in a 1L graded balloon.

- *Copper calibration solution* 1000 mg/L. 1000g Cu are diluted in 7 mL azotic acid (4:3) in a 1L graded balloon.

Results and Discussions

Cystoseira barbata is part of the brown algae (Phaeophyta phylum), which represents a group of pluricellular macroscopic algae, the vast majority of them being found in seas and oceans. The low mineralization capacity of the tallus and the lack of fossils make appreciating their age and phylogenetic links quite hard. The vast majority of algae researchers believe that, due to the type of flagella and assimilating pigments, they are related with golden algae, which might actually represent their origin group. Brown algae have evolved, specialized and have adapted a lot, exclusively in the aquatic medium. They have not lead to the formation of other plant groups, as they are considered a closed phylogenetic group.

Morphology and structure

It is a large alga, characterized by polymorphism with a tallus that can reach 1.5-2m in height. It latches to the substrate through a powerful disc-like spike, from which one or more cylindrical rods stem. On these rods, a high number of branches are formed, which gives the tallus a tree-like appearance.

Both primary and secondary branches can be cylindrical or flattened and can present with terminal receptacles. Receptacles are either cylindrical or cone-shaped and they contain conceptacles which produce reproductive elements. Alongside the branches, numerous air-containing vesicles arranged in a chain can be found.

Growth cycle

Even though the growth cycle of *Cystoseira barbata* is a monogenetic type, it does have certain particularities linked with the hydrobiologic conditions innate to the Black Sea. These are represented by two more active vegetation and fructification periods. The first and more intense one during spring (March-May) and the second one during autumn (September-November). Another particularity is represented by the continuous formation of new branches and receptacles containing ovogones and antherida.

Spread

The species is spread in the Black Sea, where it forms a perennial association on a hard substrate, which is very valuable from an ecologic point of view. Currently, this association is very reduced, partly due to past freezing periods and partly due to pollution, increased water turbidity and substrate warping.

Ceramium rubrum is part of the red algae (Rhodophyta phylum), which encompasses uni- or pluricellular algae, which live in the aquatic medium, especially maritime environments, very few of them being found in sweet waters. Due to the membrane's mineralization ability, some species have fossilized and traces of red algae can be traced back to the Jurassic. The vast majority of algae researchers believe that they are related to blue algae, a theory sustained by the presence of phycoerythrin and phycocyanin pigments in both groups. They have evolved towards a superior vegetative apparatus and a specific reproductive mechanism, but they are still considered a closed phylogenetic group, as they have not lead to the formation of other plant groups.

Fig. 1- *Cystoseira barbata*

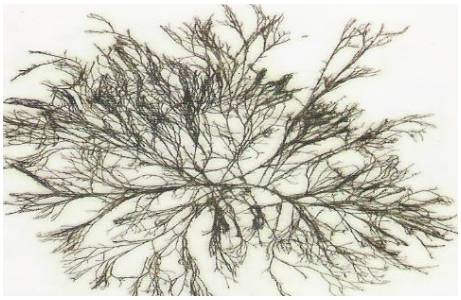

Fig. 2 - *Ceramium rubrum*

Morphology and structure

Ceramium rubrum presents as a dark red filamentous bush, fixed to the substrate through rhizoids. The filaments have a dihotomic ramification and growth begin with a single cell, which can be found at the apex and may sometimes be hidden. Each ramification ends with two short arms which form a tiny pliers. Filaments are made up of a single head to head row of cells, which makes it appear as an axial structure with the ends of the cells meeting at the knots. At each knot, a variable number of cells called periaxial cells is formed. These cells continue to divide leading to the formation of cortical cells. The species is characterized by a special type of polymorphism, which has lead to the identification of several varieties.

Growth cycle

Ceramium rubrum is characterized by a trigenetic growth cycle. The first generation is represented by the haploid gametophyte on which sexual organs can be found on the superior aspect. On the male gametophyte, spermatocytes are small and oval shaped. Through the emergenece of new haploids the cycle beings again and this succession of three generations can occur several times a year.

Spread

This alga is widely spread in the Atlantic Ocean, the Pacific, the Mediterranean Sea, and the Black Sea. It is an annual species, which sometimes widely colonizes the rocky substrates from the medio- and infraseaside and areas exposed to waves. On the Black Sea shore, it is found alongside the entire coastal area, on rocks, at depths of 0.5 to 4-5 m, all throughout the year, with a more accelerated growth during spring and summer.

Figures 1-4 present the calibration curves for the heavy metal content measured in the two types of algae.

- Sample 1 – *Cystoseira Barbata (C.B);* 11,9289 g were analyzed and mineralized according to the above mentioned methods.
- Sample 2 – *Ceramium rubrum* (C.R.); 14,2778 g were analyzed and mineralized according to the above mentioned methods.

Heavy metal content was determined using the following formula:

$$[conc] = \frac{C_{curve} x V_{sample}}{m_{sample}}; mg / kg$$

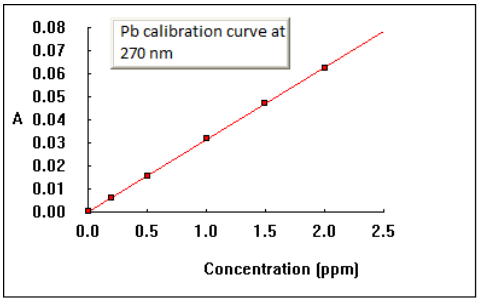

Fig. 1 Pb calibration curve (λ = 270 nm)

Relationship: Linear Straight line calibration formula:

$A=0{,}03129 \times C+0{,}00048$

Correlation coefficient (r): 0,99988 $(r)^2$:0,99975

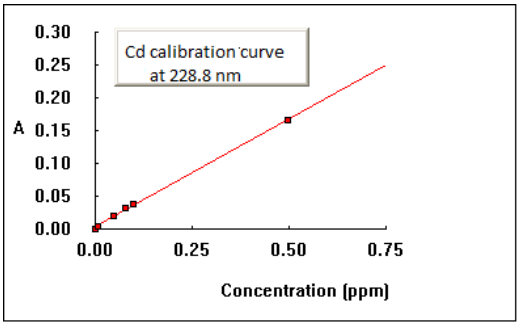

Fig. 2 Cd calibration curve (λ = 228,8 nm)

Relationship: Linear Straight line calibration formula:

$A=0{,}32804 \times C+0{,}00412$

Correlation coefficient (r): 0,99932 $(r)^2$:0,99864

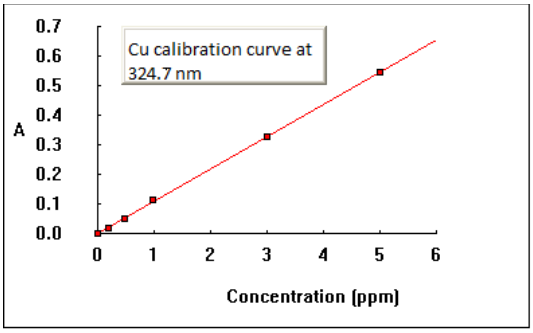

Fig. 3 Cu calibration curve(λ = 324,7 nm) Relationship:

Linear Straight line calibration formula: $A=0{,}10941 \times C+0{,}00083$

Correlation coefficient (r): 0,99984 $(r)^2$: 0,99968

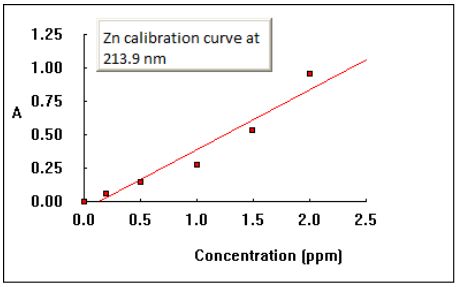

Fig. 4 Zn calibration curve(λ = 231,9 nm)

Relationship: Linear Straight line calibration formula: A=0,45089 × C-0,05999

Correlation coefficient (r): 0,97070 $(r)^2$: 0,94226

The results obtained for heavy metal content are presented in table 1.

Table 1. Heavy metal content of algae

Type of alga	Pb (mg/kg)	Cd (mg/kg)	Cu (mg/kg)	Zn (mg/kg)
Cystoseira barbata	2,51	0,201	4,19	1,93
Ceramium rubrum	5,04	0,231	6,58	6,72

It can be observed that the red alga – *Ceramium rubrum* – has higher content of heavy metals compared to the brown alga – *Cystoseira barbata*. This is especially due to the biogeographic localization of these algae in the sea. *Cystoseira barbata* can be found in the shoreline area, at shallow depths, where solar radiation can penetrate, meaning that a part of the metals will become part of the biogeochimic circuit and used for photosynthesis. On the other hand, *Ceramium rubrum* lives at higher depths, where solar radiation cannot reach and photosynthesis does not occur. In addition, due to specific Black Sea currents, sediments of heavy metals are deposited and formed. Compared to the studies performed by biology and chemistry researchers from the Ovidius University of Constanta, an increase in heavy metal concentration can be observed for each of the alga species [21].

Table 2. Results regarding heavy metal content of red and brown algae studied in the year 2013 (ppm) in the Ovidius University of Constanta laboratories

Species/location	Cd	Pb
Ceramium rubrum		
Constanta - Modern	5,76	3,77
Constanța – Cazino	8,03	-
Constanta – 3 Papuci	11,21	-
Cystoseira barbata		
Constanta – Pescărie	0,44	-
Constanta – 3 Papuci	2,84	-
Constanta - Modern	4,11	-

Table 3. Results regarding heavy metal content of red and brown algae studied in the year 2014 (ppm) in the Ovidius University of Constanta laboratories

Species/location	Cd	Mn	Zn
Ceramium rubrum			
Constanta – Pescărie	0,003	0,145	0,075
Constanta – Cazino	0,002	2,403	0,420

There is a series of factors that influence the chemical composition of algae and that is: alga species, anatomical and morphological characteristics of the alga, harvesting area, harvesting season, and storage conditions. In what regards alga species, research has demonstrate that both the amount of alginic acid contained by the alga, as well as metal content vary according to species and even type, as is the case of red and brown algae.

Table 4 Cr, Cu, and Fe concentration as determined by molecular absorption spectroscopy on algae from the Black Sea shore in the years 2013, 2014, and 2015

Harvested algae	Harvest year	Cr concentration mg/Kg	Cu concentration mg/Kg	Fe concentration mg/Kg
Cystoseira barbata	2013	14.8	6.45	429.0
Ceramium rubrum		3.92	22.43	363.8
Cystoseira barbata	2014	14.47	8.30	254.70
Ceramium rubrum		2.86	7.28	497.2
Cystoseira barbata	2015	-	4,19	-
Ceramium rubrum		28.47	6,58	263.4

Compared to previous studies, we can observe that, throughout the years of analysis, the chrome concentration in brown algae has decreased, while for red algae it has increased to a worrisome level. Copper concentration in both species has decreased, while iron concentration is very important for the growth cycle and the development of the phytoplankton.

Copper concentration in *Cystoseira barbata* has decreased during one year, from 8,30 µg/g in 2014 to 4,19 µg/g 2015. Copper concentration in *Ceramium rubrum* has decreased from 7,28 µg/g in 2014 to 6,58 µg/g in 2015. The results obtained in 2015 for the two algae species is between STAS approved limits (3,87 – 41,37 µg/g).

Conclusion

It has been observed that concentration limits for heavy metals in the algae analysed in this study are comparable with results obtained in previous studies and are in accordance with the limits imposed by current legislation. The use of algae in the medical and pharmaceutical field can be optimized through the monitoring of heavy metal content in both the algae and their environment.

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Polyelectrolyte Complexes Based on Chitosan and Natural Polymers

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Abstract

For many years chitosan has been the subject of interest for its use in different medical fields due to its appealing properties such as biocompatibility, biodegradability, low toxicity and relatively low production cost from abundant natural sources. Chitosan is positively charged at low pH values, so it is spontaneously associated with negatively charged polyions in solution to form polyelectrolyte complexes. These chitosan based polyelectrolyte complexes exhibit favourable physicochemical properties with preservation of chitosan's biocompatible characteristics. These chitosan based complexes are a good candidate for excipient materials for the design of different types of dosage forms. The aim of this review is to describe polyelectrolyte complexes of chitosan with selected natural polyanions and also to indicate some of the factors that influence the formation and stability of these formed complexes.

Keywords: chitosan; polyelectrolyte complex; natural polymers

Introduction

Chitosan is a combination of a series of polymers that are deacetylated derivatives of chitin,a natural polysaccharide,and presents different degrees of deacetylation and molecular weights. It consists of deacetylated units of β-1,4-linked glucosamine and acetylated units of *N*-acetyl-D-glusoamine (Figure 1). Chitosan presents typical degrees of deacetylation between 70 and 95% and molecular weights between 10 and 1,000 kDa [1,2] . It was reported that highly refined grades of chitosan have been used in pharmaceutical formulations as a release-controlling agent [3].

It was shown that the cationic amino groups on the C2 position of the repeating glucopyranose units of chitosan can interact electrostatically with the anionic groups (usually carboxylic acid groups) of other polyions to form polyelectrolyte complexes. Many different polyanions from natural origin (e.g. pectin, alginate, carrageenan, xanthan gum, carboxymethyl cellulose, chondroitin sulphate, dextran sulphate, hyaluronic acid) or synthetic origin (e.g., poly (acrylic acid)), polyphosphoric acid, poly (L-lactide) have been used to form polyelectrolyte complexes with chitosan in order to provide the required physicochemical properties for the design of specific pharmaceutical formulations [4].

Thoughout the years chitosan complexes have been used in a wide range of pharmaceutical applications such as complexes formed between chitosan and anionic polymers for use as biosensors, scaffolds in tissue engineering, for waste-water treatment and for drug delivery in different forms [5,6].

Most of the investigated and studied polycomplexes that involve chitosan are those formed with other polysaccharides, which are divided into natural polysaccharides (including phytopolysaccharides, zoopolysaccharides and microorganism polysaccharides) and synthetic polysaccharides [7]

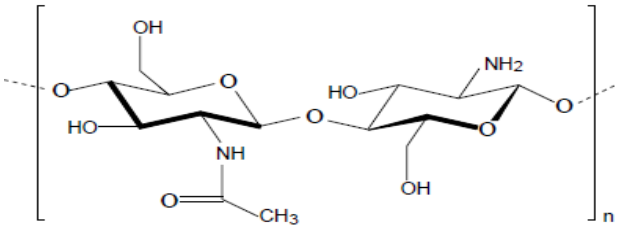

Figure 1. Chemical structure of chitosan consisting

of *N*-acetyl-D-glucosamine and glucosamine units.

Research Methods

Natural polymers are widely used in the regenerative field of medicine, for wounds and burns dressing because of their unique properties such as biocompatibility, biodegradability and similarity to macromolecules recognized by the human body.

The selection of the natural polymers in this review was based on the complexing capacity with chitosan. The structural characteristics are presented, as well as the therapeutical effects and data on the biodegradability of these complexes in comparation with data from ohter reseaches and specialty literature.

Results and Discussions

Polyelectrolyte Complexes between Chitosan and Natural Polymers

1. *Chitosan-alginate polyelectrolyte complex*

Alginate is a natural, linear, unbranched, biodegradable polysaccharide and it consists of 1,4-linked β-D-mannuronic acid and α-L-guluronic acid monomers in varying proportions (Figure 2). Alginates are extracted from brown seaweeds and marine algae such as *Laminaria hyperborea*, *Ascophyllum nodosum* and *Macrocystis pyrifera* [8,9].

In order to form a polyelectrolyte complex, the negatively charged carboxylic acid groups of manuronic and guluronic acid units in alginate interact electrostatically with the positively charged amino groups of chitosan.

Due to the biodegradability and biocompatibility of the polyelectrolyte complex formed between these two polymers, alginate is one of the most studied anionic polyelectrolytes in complexation with chitosan. This polyelectrolyte complex is mechanically stronger at lower pH values where chitosan dissolves [10]. A study on the biodegradation of chitosan-alginate polyelectrolyte complexes showed that while chitosan alone, with a low degree of deacetylation, was effectively degraded by lysozymes, the effect of these enzymes on the polyelectrolyte complex was negligible. The ability of the polyelectrolyte complex of lysozyme absorbtion was high, but enzymatic degradation was hindered by the strong interaction between the chitosan and alginate polymeric chains.

Since it was demosntrated that the rate of biodegradation may be regulated by changing the polymer ratio, it indicates that this particular polyelectrolyte complex has a high potential in tissue engineering for scaffolds and support materials [11].

Chitosan-alginate polyelectrolyte complex fibers showed promising results for controlling the release of charged molecules and exhibited high encapsulation efficiencies of these molecules [12].

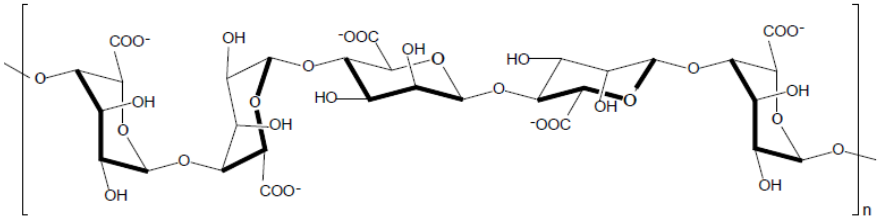

Figure 2. Chemical structure of alginate

2. *Chitosan-carrageenan polyelectrolyte complex*

Carrageenan is the generic name for a family of high molecular weight sulphated polysaccharides obtained from certain species of red seaweeds. There are three basic types of carrageenan, namely kappa (κ), iota (ι) and lambda (λ) carrageenan (Figure 3) [13,14].

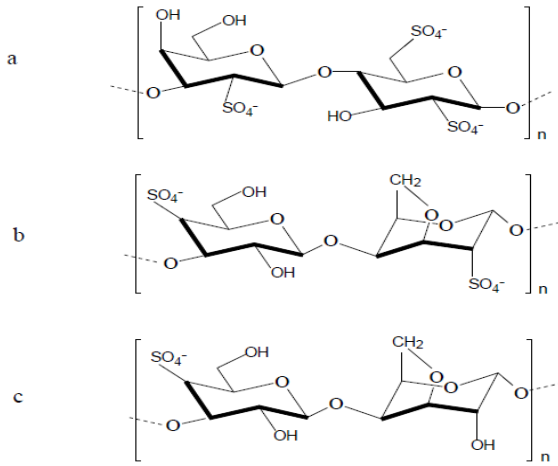

Figure 3. Chemical structures of (a) λ-carrageenan, (b) ι-carrageenan and (c) κ-carrageenan

Recent reports have shown that the nature or type of carrageenan considerably influence the characteristics of the polyelectrolyte complex that is formed with chitosan. The mechanical strength of polyelectrolyte complex gels formed between chitosan and different carrageenans were in the order λ- > ι- > κ- carrageenan. The gels obtained for ι- and κ-carrageenan were temperature sensitive because of the helix–coil conformational transitions in their molecules [15].

3. *Chitosan-pectin polyelectrolyte complex*

negatively charged carboxylic acid groups of pectin and the positively charged amino groups of chitosan can occurre. Furthermore, by adjusting the pH of this mixture a polyelectrolyte complex could be obtained.

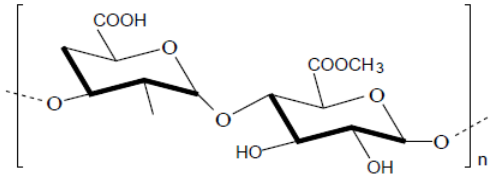

Figure 4. Chemical structure of pectin.

As expected, the extent of this interaction depended on the pH of the surrounding medium which determined the extent of ionization of the polymers [6].

4. *Chitosan-xanthan gum polyelectrolyte complex*

Xanthan gum is an exopolysaccharide secreted from *Xanthomonas campestris*.

It consists of β-(1,4)-D-glucopyranose glucan, a cellulosic backbone, and presents on every second glucose residue a trisaccharide side chain, namely (3,1)-α-D-mannopyranose-(2,1)-β-D-glucuronic acid-(4,1)-β-D-mannopyranose, (Figure 5) [18].

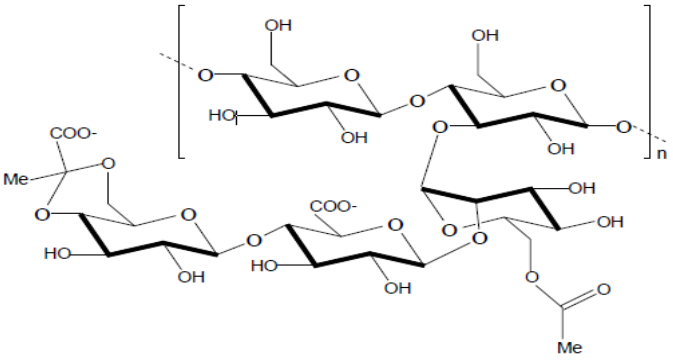

Figure 5. Chemical structure of xanthan gum

Results obtained from a modulated differential scanning calorimetry analysis and the swelling degree of microcapsules prepared from chitosan-xanthan gum polyelectrolyte complexes indicated that the cross-linking density was interdependent on xanthan concentration, chitosan concentration and chitosan solution pH [19].

5. *Chitosan-hyaluronic acid polyelectrolyte complex*

Hyaluronic acid or hyaluronan or hyaluronate is the only nonsulfated glycosaminoglycan found in the extracellular matrix throughout connective, epithelial and neural tissues.

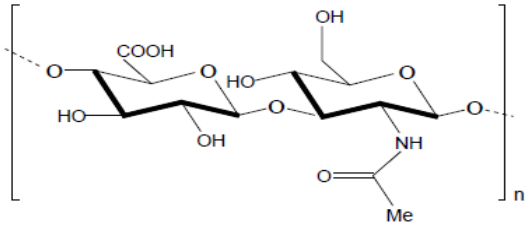

Figure 6. Chemical structure of hyaluronic acid.

Hyaluronic acid is a linear anionic polysaccharide with high molecular weight that consists of β-(1,3)-*N*-acetyl-D-glucosamine and α (1,4)-D-glucuronic acid repeating units linked by β (1→3) bonds (Figure 6).

It is produced by bacterial fermentation of streptococcus species or by extraction processes from rooster combs, umbilical cords, synovial fluids or vitreous humour for commercial purposes. Hyaluronic acid has many applications in the medical field beeing used in ophthalmic surgery, arthritis treatment, in tissue engineering, a component of scaffolds for wound healing and implant devices [2,20,21]. Recent studies have shown that the polyelectrolyte complex between chitosan and hyaluronic acid protected hyaluronic acid against enzymatic hydrolysis, but only at pH values different from the optimal pH of the enzyme. The results from this studies revealed that the chitosan-hyaluronic acid polyelectrolyte complex unfortunately had less cell proliferation and wound healing effects compared to chitosan alone [22].

6. *Chitosan-gelatine polyelectrolyte complex*

Gelatine is a heterogeneous mixture of protein fractions consisting of single or multi-stranded polypeptides (Figure 7). The process of obtaining gelatine is by partial hydrolysis of animal collagen derived from skin, white connective tissues and bones. There are two tipes og gelatine: type A and type B. Type A gelatine is derived from pig skin by using acid hydrolysis and type B gelatine is obtained by alkaline hydrolysis of cattle hides and bones [23].

It was shown that the polyelectrolyte complex between chitosan and gelatine can only occur at a pH value above 4.7 and below 6.2. Above this value the net charge on gelatine type B is negative. A pH value of 4.7 represents the isoelectric point of gelatin. Above a pH value of 6.2 chitosan start to precipitate out of solution. [24].

Natural polymers are involved in the repair of damaged tissues and in skin regeneration by Inducing and stimulating the wound healing process. Biomaterial hydrogels are engaged in the pharmaceutical and biomedical area, due to their threedimensional cross-linked polymeric networks that are soaked with water or biological fluids, and are used especially for wound management, tissue engineering, drug delivery, and organ transplant.

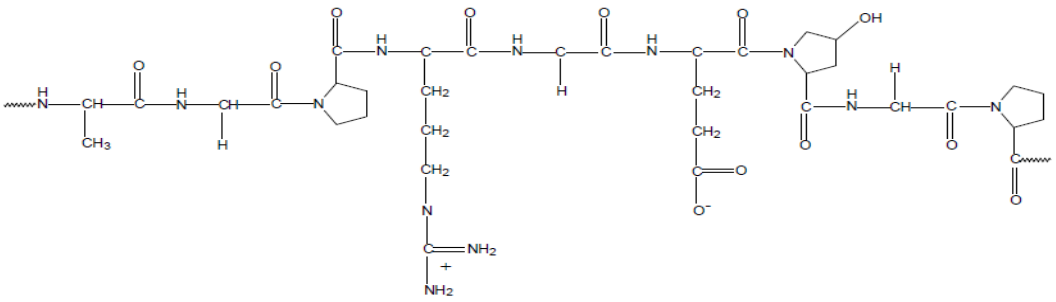

Figure 7. Typical structure of gelatin.

Hydrogels containing crosslinked natural polymers and polyelectrolyte complexes can be used for wounds and burns dressing.

Conclusion

Polyelectrolyte complexes combine unique physico-chemical properties of different polymers with the advantage of retaining high biocompatibility. It is therefore not surprising that polyelectrolyte complexes are gaining importance in modern pharmaceutical technology. In the management of wounds and burns dressings play an important role. The use of three-dimensional polymeric scaffolds for cell targeting is already a common strategy for tissue engineering. Recent studies upon the properties of natural polymers (biocompatibility and biodegradability) will lead to a substantial development of novel types of wound dressings and to outstanding applications for regenerative medicine.

In the present, the most promising materials for wounds and burns dressing are still based on natural polymers such as polysaccharides (alginates, chitin, chitosan), proteoglycans and proteins (collagen, gelatin, fibrin, keratin, silk fibroin, eggshell membrane).

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Mytilus Galloprovincialis - A Valuable Resource of the Black Sea Ecosystem

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Abstract

The Bivalvia family has been traced back to the beginning of the Paleosoic Era (The Devonian age) and have survived to this day, with very small changes. While more common in the Mediterranean, the Black Sea has been penetrated by representatives of the family, most frequently along the coastal area. Representatives of the Mytilus genus, mussels are very common in the seas and oceans of the world. Mytilus Galloprovincialis Lamarck has long been considered as being only a variety of Mytilus edulis Linne. Anatomical studies have shown that there are sufficient differences in order to accurately distinguish the two species. This paper presents a characterization of the Mytilus galloprovincialis mussel, which is a valuable resource existing in the Black Sea ecosystem.

Keywords: Molluscs, Mytilus Galloprovincialis, Mytilus Edulis Black Sea

Introduction

The Bivalvia family has been traced back to the beginning of the Paleosoic Era (The Devonian Age) and, with slight changes, has survived until today. Current representatives are very wide spread, found especially in marine waters, with the exception of a few species that can be found in fresh water. Most species of the family live in usually numerous colonies, creating genuine banks at varying depths [1]. In general, they live free or they are set in rocks through byssus. More rarely, they form galleries in limestone cliffs, through the acidic secretion of the glands located in the mantle. Several genera, which are commonly found in the Mediterranean, have entered the Black Sea and are widely spread over the Romanian coastal zone [2].

Characterization of the Mussel (*Mytilus Galloprovincialis*) [1, 3]

The Mussel - *Mytilus galloprovincialis* - (LAMARCK 1819) - according to the grading system introduced by **J. Piveteanu** order and still using the nomenclature proposed by **J. Theile** for determinations of suprafamilies and subordinate level, is a Bivalve lamelibranhiated mollusc belonging to: MOLLUSCA phylum, ord. DYSODONTA, suprafam. MYTILACEA, fam. MYTILIDAE, gen. MYTILUS. Bivalvia species representative of the genus Mytilus, mussels are widespread in seas and oceans. They are spread from tropical to polar seas, usually at depths of 10 to 15 m, but can be found at higher depths, reaching up to 60-70 m, sometimes forming large areas referred to as "mussel banks" or "true facies with mussels".

a b

Fig. 1 - *Mytilus edulis* LINNE' (a) and *Mytilus galloprovincialis* LAMARCK(b)

There are many species of *Mytilus* but only two of them are the object of exploitation in Europe: *Mytilus edulis* and *Mytilus galloprovincialis* Lamarck Linn. Fig. 1. [4]. *Mytilus edulis* lives in the northern regions and is found in the Baltic. North Sea and Atlantic Ocean all the way to Portugal, while *Mytilus galloprovincialis* is common in Mediterranean and Atlantic coasts by the Western Channel.

Mytilus galloprovincialis Lamarck has long been considered only as a variety of *Mytilus edulis* Linn. Anatomical research has established that there are enough differences that clearly separate these species. The main issue to solve is the convergence due to the same factors – mainly salinity and temperature – which make the valves of these animals to exhibit numerous similiarities and make both forms of *M. Galloprovincialis* to resemble *M. Edulis*. This has made numerous researchers, not only those studying the Mediterranean and Black Sea, to reconsider the existence of *M. Edulis* in these seas. What can still distinguish the two species are: biometric characters (in case the two species coexist in the same conditions), the height / length ratio for the species *M. edulis*, mantle edge color (yellow-brown for *M. edulis* and black-purple for *M. galloprovincialis*) and the presence for *M. edulis* or absence in *M. galloprovincialis* of longitudinal purple bands on the shell visible until the periostracum is removed. Lubert (1969) managed to hybrid the two species, obtaining viable larvae perfectly normal. He observed a number of identical chromosomes in the two species *M. edulis* and *M. galloprovincialis* but a different behaviour regarding sexual cycle. The author concluded that certain biological features (growth in height, sexual cycle) allow separating the two species - *galloprovincialis* - and - ¬ *edulis* - hampered by high variability identification of individuals (as valves). However likely, the same number of chromosomes and the possibility of hybridization emphasize the possibility of two distinct genetic races of the same species, differentiated by ecological conditions.

The rock mussels are found in rocky areas with shallow waters - less than 20 m and although they can be found in relatively large quantities they do not have a direct economic interest yet for at least two reasons: strong attachment to the hard substrate (cliffs, rock platforms, articulated concrete blocks) which means they cannot be harvested mechanically (harvesting by autonomous divers only) and by the fact they are heavily covered by a rich epibiosies and contain a high percentage of foreign bodies inside them (sand, small fragments of valves, pearls, etc.) that do not give them a commercial aspect (or require a great deal of work for sale). However, rock mussels have an important role in maintaining a healthy quality of water near tourist beaches by strong water biofiltration action.

Deep mussels form a ring around the Black Sea, located on the continental shelf between izobates of 25 ÷ 55 m deep on the muddy bottoms. - The presence of mussels on the muddy bottoms of Black Sea - an exception in this world, is one of the basic features of the basin. - Mussels living in the muddy areas in 'nests' consisting of a few specimens caught with the byssus between them and the substrate, which forms a real "condensation" core, isolated specimens are rarely met. – The epibiosies attached to mussels at higher depths is generally poor and the valves have a small percentage of foreign bodies.

The Main Nutrients in Mussels [1,3]

It is well known that, certain mussels, such as midia, represent an appreciated food product in numerous European, Asian, and North American countries. Due to its nutritious qualities, this species has been the object of research for numerous marine laboratories around the world, where important attention is given to ecological, physiological, and biochemical research and analysis [1,3].

Biochemical analysis has shown that this species has superior nutritive qualities, comparable to food products obtained from terrestrial animals, while in what regards certain biochemical compounds, midia are undeniably superior – vitamins and amino acids.

In the Romanian Black Sea sector, besides deep mussels banks that can be exploited there are specific opportunities on intensive mariculture installations. To determine the optimal period to harvest mussels for industrialization it is necessary to determine the seasonal dynamics of physiological parameters and nutritive value of major compounds in mussels. Meat is 30 ÷ 31% by weight, and valves 40 ÷ 41% , 24.0 ÷ 26.3% interstitial juice, so a full harvest would contribute to efficient cultivation of mussels. Biochemical composition of biologically active points attribute support - protein, carbohydrates, lipids and their distribution in different extracts (aqueous and organic solvents) and enzymatic events adjutant with anti-inflammatory qualities. Processing this raw material will bring changes in the microbial load and the relationship between biochemical constituents detected in meaning, amplify the effects of using products to achieve desired bioproducts. Concerns and recommends analysis results for mineral and organic component recovery shell - and juice recovery by appropriate processing procedures. The Romanian Black Sea coast is characterized by wide variations of the main environmental factors that have direct impact on physiological behaviour of organisms and the accumulation of biochemical components with nutritional value. Key dynamics biochemical compounds were correlated with environmental factors and annual ontogenetic stages of an organism. (Table 1 and 2). The ultimate aim of these biochemical tests was to specify the annual biological cycle of major quantitative changes in biochemical components of food value and optimal timing of harvesting industry without jeopardizing the current stocks in the Romanian Black Sea coast. (Table 2).

Table 1. Physical and biological characteristics of aqueous extract of shellfish meat

pH	6,3 – 7,4
Electrical conductivity µS	8,3 – 8,5
Salinity ‰	2,1 – 2,5
Proteins mg/ml	7,5 – 7,8
Carbohydrates mg/ml	2,8 – 3,0
Uronic acid mg/ml	0,5
Enzyme activity and enzyme inhibition	
Superoxiddismutasa U/mg protein	2,20 – 2,24
Alkaline phosphatase m U/mg protein	3,9 – 5,7
Alkaline catalase µ mol H_2O_2/sec	0,65 – 0,70
Hyaluronidase% inhibition compared to incubation enzyme: protein 1:1, 2:1; 3:1	40 – 15

Biochemical composition of mussels in the Black Sea has some seasonal dynamics (Table 3). These variations may be caused by: normal and abnormal seasonal physical factors - sea of chemicals, the existing density and high food quality,

depth and, in the case of mussel banks, age and annual physiological cycle of the animal. In cases with specific environmental factors, normal changes in season, area and depth of the habitat where mussels normally reside, trophic plankton elements show quantitative oscillations. These seasonal dynamic changes in mussels' biochemical composition are normal and vary according to the physiological cycle phase.

Table 2. Biochemical characterization of the lipid extract of mussels meat

Neutral Lipids	82,6 ± 0,3
Glicolipids	1,4 ± 0,1
Phospholipids of which:	17,0 ± 1,0
Fosfatidil ethanol amine and lizofosfatidil ethanol amine	31,5%
Fosfatidil colina and lizofosfatidil colina	30,2%
Fosfatidil inozitol	2,4%
Fosfatidil scrin	14,2%
Sfingomiclin	8,5%
Unidentified lipids	15,0%

Table 3. Seasonal dynamics have major biochemical components in Mytilus galloprovincialis (averages)

Quarter	Examined species	Biochemical analysis (expressed in g/100 g of fresh substance)								
		Total carbohydrate	Reducing carbohydrate	Glycogen	Total nitrogen	Total protein	Total fat	Mineral	Water %	Dry matter %
I.	Deep mussels	1,5	0,2	1,2	1,3	8,5	2,7	0,9	72,2	12,8
	Rock mussels	1,0	0,2	0,9	1,5	9,5	2,5	0,7	69,9	13,80
II.	Deep mussels	1,7	O,2	1,5	1,5	9,6	1,1	0,6	70,6	13,2
	Rock mussels	1,5	0,2	1,5	1,7	11,2	2,1	0,8	67,7	13,3
III.	Deep mussels	1,5	0,2	1,3	1,5	9,2	1,3	0,8	71,5	12,7
	Rock mussels	1,6	0,2	1,4	1,7	10,5	1,7	0,8	69	14,8
IV.	Deep mussels	1,9	0,2	1,7	1,4	8,3	2,3	0,7	70	13,5
	Rock mussels	1,8	0,2	0,5	1,3	8,5	2,5	0,6	72,9	13,2

Growth Rhythm of Midia in the Black Sea Coastal Area

In areas with a rocky aspect, the growth rhythm of new midia generations, where young species fixate on the pre-existing colony thus having to battle the laws of natural selection from the very beginning, is hampered by the limited space available for fixation [4, 5].

The domination of species under 4mm among midia colonies fixated on a hard substrate is overwhelming – 61.48% . This numeric domination is the expression of the ecological action of the substrate, which is essentially alive and incomparably larger than the denuded substrate, which is more or less smooth. For the immediate following size class, a very abrupt drop can be observed – 4.6% - which is proof of a very low survival rate. Given the age of the species with lengths between 4 and 24 mm, under 10 months, and in light of the above presented information, it can be concluded that their sum, reported to the average of the total number of population specimens – represents 77.4% . This enormous difference outlines an extremely high mortality rate between the population of the first and the second year, a mortality rate that can be explained by several factors:

- Young specimens, under 20 mm, are primarily consumed by predatory fish (*Gobiidae*);

- As they are fixed to the outer edge of the colony, young specimens are decimated primarily by the mechanic action of water (marine currents, ground swell, and so on);
- The growth of midia specimens is an allometric type of growth, as a result of the pressure created by the growth of older specimens, which completely obliterates smaller specimens, either by crushing or dislocation.

For midia which can be found at higher depths, to the lack of competition for substrate, the hierarchy of size classes among bank midia is completely different. The first and most important characteristic of these differences resides in the much reduced level of participation of the 4 mm size class – approximately 25% of the entire population.

On control surfaces – concrete tiles and chalk blocks – launched at depths of 3.5m, midia colony fixations has a particular dynamics. During one year, densities between 795-1,023/m^2 with lengths between 2.1 - 5.7 (average = 4.3 mm) can be registered. The artificial support intended for midia fixation on the mariculture installation is represented by artificial collectors, manufactured out of rhelon rope, suspended in the water mass.

Under normal hydrobiologic conditions, specific to reference seasons, the total and grouped quantity of existing organisms over one metre of natural fixation collectors – after spring reproduction – in the month of June is presented in Fig. 2. The total biomass of epibiosis organisms existing on artificial collectors 3 months after sea launch is 5.209kg/1collectorm (Fig.3).

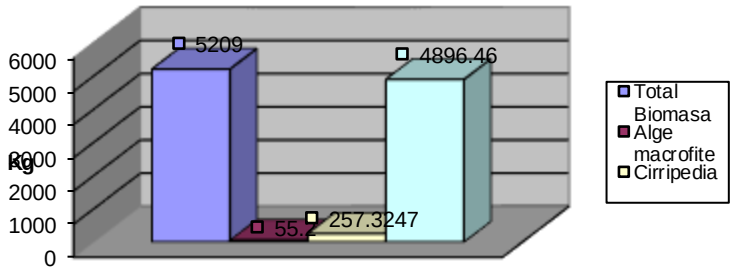

Fig. 2. Total and grouped live organism fixations on artificial collectors fixed to mariculture installations, 3 months after launch (June) [3]

Fig.3. Artificial collector, 7 months after immersion [4, 5]

Among the organisms living on collectors, the predominant population is represented by midia – which account for 94% of the total biomass – followed by cirripedia, with approximately 4,94% of the total. The remainder of the existing biomass – approximately 1% - is represented by macrophytic algae and other epibiosis invertebrates.

In what regards the collectors from mariculture installations, the predominant population is represented by midia, which account for 90% of the biomass, followed by cirripedia with about 8% of the total population. Seasonal algae represent about 1.5% , while the remainder of 0.5% of the total biomass is represented by invertebrate epibiosis organisms.

During the cold season, due to specific hydroclimatic changes characterizing the unsheltered marine areas of the Black Sea coast, high waves and particularly due to strong marine currents which influence the artificial collectors, approximately 80% of the fixated biological material is removed. In order to prevent these unwanted phenomena, the artificial collectors are picked out – 60% of the biomass is removed – and they are covered in rhelon net bags.

Conclusions

- The biochemical content of the harvested midia from the Nordic areas of the shore is higher for all analysed parameters (proteins, sugars, lipids) than in midia harvested from Southern areas. These quantitative biochemical differences come to prove, once again, that our seashore is rich in physiological species with superior nutritious qualities.
- In what regards the biochemical content of midia, alongside elements with nutritious value, it is evidenced that they also have a high content of amino acid. The concentration of some essential amino acids in midia meat is net superior to numerous similar products, currently used for human consumptions.
- The high content of nutritious biochemical compounds (proteins, sugars, and lipids), to which one can add the presence of vitamins and amino acids, outlines the high nutritious value of midia from the Romanian coast of the Black Sea, but also the possibility of using them for extracting active principles with medical uses.

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