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Antioxidants in the black mussel (*Mytilus galloprovincialis*) as an indicator of black sea coastal pollution

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Abstract

The biologically active compounds, antioxidant activities, and free radical scavenging effects of black mussel dry matter (*Mytilus galloprovincialis*)—(BMDM) were investigated. The extract from BMDM with absolute methanol (BMDMAMet) showed the strongest inhibition of lipid peroxidation as a function of its concentration, and was comparable to the antioxidant activity of butylated hydroxyanisole, at the same concentration of 0.2 mg/ml. The extract with 50%methanol/water (BMDM50%Met) had the weakest antioxidant activity, whereas other extracts such as 1.2 M HCl in 50%methanol/water (BMDM50%Met/HCl) exhibited an average inhibition of lipid peroxidation. BMDMAMet extract showed marked activities in free radical scavenging determined by β -carotene bleaching (β -carotene), nitric oxide (NO \cdot) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate) radical cation (ABTS $^{+}$) methods. The antioxidant activities of BMDMAMet extract determined β -carotene, NO \cdot and ABTS $^{+}$ methods showed the highest correlation coefficients (R^2) such as 0.998, 0.9976 and 0.6103, respectively, with the presence of total polyphenols. BMDM50%Met had lower antioxidant ability than BMDM50%Met/HCl in different scavenging methods, indicating that the major antioxidant components in these extracts must be derived from the polyphenols. Correlation between proteins and antioxidants was very low (0.0318 and 0.0433). The mussel extracts from polluted areas have shown by all using methods higher antioxidant activity than from the clean ones. The results presented in this report indicate that the antioxidant activities of mussel extracts could possibly be an additional index of pollution characterization.

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Keywords: Mussels; Scavengers; Polyphenols; Temperature; Proteins; Antioxidants; Pollution

1. Introduction

Several classes of environmental pollutants are known to enhance the intracellular formation of reactive oxygen species in marine invertebrates with different consequences on their antioxidant system. Despite variations in the endogenous levels of antioxidants may reveal biological effects induced by pollutants, the overall efficiency of antioxidant system is not evaluated from such data. Some work has been done in the com-

parison of mussels. Digestive glands of the mangrove mussel *Mytella guyanensis*, collected at one non-polluted site and two polluted sites, were analyzed for different antioxidant defences (Torres et al., 2002). The apparent lack of correlation between trace organic pollutants and some of the enzymatic antioxidants may be due to the inhibitory effects caused by these chemicals (Regoli, 2000; Cheung et al., 2002). The antioxidant activities of mussels were determined using some scavenging methods (Winston et al., 1998; Regoli, 2000).

Antioxidative defense was studied in 3 different size groups of White Sea (Russia) blue mussels *Mytilus edulis* L. (Ribera et al., 1991; Walker et al., 2000; Livingstone et al., 2000; Cajaraville et al., 2000; Buhringer and Danischewski, 2001; Sukhotin et al., 2002). Fifteen

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specimens of adult mussels *Perna perna* (Wilhelm et al., 2001) were analyzed for antioxidant and detoxifying enzymes. Some work is done in connection with *Mytilus galloprovincialis* (Da Ros et al., 2000; Cavallo and Stabili, 2002; Orbea et al., 2002). Different biochemical and cellular markers were determined in the mussels in order to assess the effects and/or exposure to pollutants (Porte et al., 2001a,b). The efficiency of antioxidant system is generally approached by analysis of single components, which include specially adapted enzymes (superoxide dismutase, catalase, glutathione peroxidases, glutathione reductase) and smaller molecules such as vitamin E and β -carotene (as free radical scavengers in membranes), ascorbic acid, uric acid and reduced glutathione (for the aqueous phase). However, antioxidants can greatly vary for their biosynthetic pathway, intracellular localization, chemical nature and mode of action; in this respect, their response to stressors can be very different and it is not unlikely to observe the contemporary depletion of some antioxidants together with the induction of others. The aim of this work was to evaluate the relationships between free radical scavengers and lipid peroxidation in the common mussel *M. galloprovincialis*. Polyphenols and proteins were determined and the correlation was established between these classes of compounds and the antioxidant activities in mussels from a clean and a polluted area.

In our recent investigations we have determined the antioxidant capacity of different products using the total radical-trapping antioxidative potential (TRAP) test (Gorinstein et al., 2002). However, TRAP is relatively unspecific marker of free radical scavenging activity, because only water extracts can be preferably used for measurements. Therefore, in this investigation were used other more specific methods such as by β -carotene bleaching (β -carotene), nitric oxide ($\text{NO}\cdot$) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate) radical cation ($\text{ABTS}^{\cdot+}$) methods and comparison was done. As far as we know such investigation was not done.

2. Materials and methods

2.1. Collection of mussel samples

Samples of mussels (*M. galloprovincialis*) were collected in two regions of the Black Sea Coast—an ecologically clean region (Cape Galata) and polluted region (the area of Port Varna). The samples are designated as follows: Ci—clean region and Di—polluted region.

2.2. Reagents

Trolox (6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid), β -carotene, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), Greiss re-

agent, sodium nitroprusside and Folin–Ciocalteu reagent were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and 2,2'-azino-bis(3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (Fluka Chemie, Buchs, Switzerland). All reagents were of analytical grade.

2.3. Preliminary treatment of mussel samples

The samples were subdivided in three groups:

- (A) Group 1 (C1, D1)—the content of mussels was collected without any additional treatment and freeze-dried in glass flasks on Finn-Aqua, Lyovac GT-2 equipment for 36 h;
- (B) Group 2 (C2, D2)—the mussels were boiled in water for 15 min, cooled to room temperature, opened and the content was collected and freeze-dried;
- (C) Group 3 (C3, D3)—the mussels were boiled in water for 30 min, cooled to room temperature, opened and the content was collected and freeze-dried.

2.4. Extraction of mussel samples

Samples (1 g) of Ci and Di series were subjected to extraction with 0.05 M Na_2HPO_4 buffer (two portions of 20 ml each) at 4–6 °C for 48 h (2×24 h) under constant stirring. Centrifugation (K-24 D centrifuge, Janetzki, Germany) at 15,000 rpm for 45 min at 4–6 °C was applied at the end of each extraction step and the supernatants were united and freeze-dried. The dry extracts obtained were used for further analyses.

2.5. Gel filtration chromatography

Ten ml portion of phosphate buffer extracts were applied on Sephadex G-25 column (bed volume 130 ml) at flow rate 40 ml h^{-1} at 20 °C. The effluent profile was monitored at 280 nm on LKB 2510 Uvicord SD. The column was preliminarily calibrated with 0.2 M NaCl and Blue Dextran.

2.6. Determination of protein content

The chromatographic fractions were pooled, freeze-dried and 5 mg of each sample were used for determination of protein content according to the method of Bradford (1976).

2.7. Determination of total polyphenol content

Lipids were extracted from dry matter of black mussel (*M. galloprovincialis*)—(DMBM) with acetone (1:1) for 24 h at –20 °C and then air-dried. Three extracts were prepared using different solvents and temperatures.

Defatted samples (1 g) of Ci and Di series were extracted with absolute methanol (12 ml) at room temperature for 48 h (DMBMAM). The extracts were filtered and centrifuged at 8000 rpm for 20 min. Defatted samples were also extracted from a 50-mg aliquot with 5 ml of 50%methanol/water and the sample was vortexed for one minute and heated at 90 °C for 3 h with vortexing every 30 min. After the samples were cooled, they were diluted to 10 ml with methanol and centrifuged for 5 min at 5000 rpm with a benchtop centrifuge to remove solids (DMB50%M). The third extract was obtained with 5 ml of 1.2 M HCl in 50%methanol/water (DMB50%/HCl) and treated as above (Vinson et al., 2001). The clear supernatants obtained from three different extractions were used for determination of total polyphenols by the Folin–Ciocalteu method and the values were read at 675 nm (Singleton and Rossi, 1965).

2.8. Antioxidant assay using β -carotene linoleate model system

Four ml of emulsion contained β -carotene; linoleic acid and Tween-40 (polyoxyethylene sorbitan monopalmitate) were added to 0.2 ml of mussel extracts (Singh et al., 2002; Jayaprakasha and Jaganmohan Rao, 2000). The absorbance at 470 nm was taken at zero time ($t = 0$) and continued until the color of β -carotene disappeared ($t = 180$ min) at an interval of 15 min. The antioxidant activity (AA) of the extracts was evaluated in terms of bleaching of the β -carotene using the following formula, $AA = 100[1 - (A_0 - A_t)/(A_0^o - A_t^o)]$, where A_0 and A_0^o are the absorbance values measured at zero time of the incubation for test sample and control, respectively, and A_t and A_t^o are the absorbance measured in the test sample and control, respectively, after incubation for 180 min and kinetics was done. Trolox, BHT and BHA were used as standards in these methods. The kinetics was done during 180 min.

2.9. Scavenging activity against nitric oxide (NO[•] test)

0.5 ml portion of a mixture (0.4 ml of extract and 0.1 ml of sodium nitroprusside solution) was diluted with 0.3 ml of Greiss reagent. The absorbance of the chromophore formed during the diazotination of nitrite with sulfanilamide and subsequent coupling with naphthylethylenediamine dihydrochloride was immediately read at 570 nm and referred to the absorbance of standard solutions of sodium nitrite salt treated in the same way with Greiss reagent (Marcocci et al., 1994).

2.10. ABTS decolorization assay

The total antioxidant activity of extracts was measured using the Trolox equivalent antioxidant coefficient (TEAC) assay as described by Pellegrini et al. (1999)

with minor modifications. The TEAC value is based on the ability of the antioxidant to scavenge the blue–green 2,2'-azino-bis(3-ethyl-benzothiazoline-6-sulfonic acid) di-ammonium salt (ABTS⁺) radical cation relative to the ABTS⁺ scavenging ability of the water-soluble vitamin E analogue 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox). ABTS⁺ radical cation was generated by the interaction of ABTS (250 μ M) and K₂S₄O₈ (40 μ M). After addition of 990 μ l of ABTS⁺ solution to 10 μ l of different extracts (0.2 mg/ml) or Trolox standards (final concentration 0–20 μ M) in ethanol or phosphate buffered saline, the absorbance was monitored exactly 1 and 6 min after the initial mixing. The percentage decrease of the absorbance at 734 nm was calculated and plotted as a function of the concentration of the extracts and of Trolox for the standard reference data. To calculate the TEAC, the slope of the plot of the percentage inhibition of absorbance vs concentration for the antioxidant was divided by the slope of the plot of Trolox. This gives the TEAC at the specific time point (Pellegrini et al., 1999). The higher the TEAC value of the sample means the stronger the antioxidant ability.

2.11. Statistic analysis

Data were reported as mean \pm standard deviation. The analysis of variance and least significant difference tests were conducted to identify differences among means, while a Pearson correlation test was conducted to determine the correlations among means. Statistical significance was declared at $p < 0.05$.

3. Results

3.1. Gel filtration chromatography

For all samples were obtained similar profiles (data not shown). They were similar and differ to a certain extent only in the shape of the second peak, which should contain low molecular substances (salts, vitamins, phenolic compounds, short chain protein fragments, etc.). Both fractions, obtained by the samples C1 and D1 were freeze-dried and tested for antioxidant activity.

3.2. Determination of protein content

It is evident that the thermal treatment significantly affects the protein content of the sample. The protein concentrations in the main peak (μ g/mg dry weight) of C1, C2 and C3 were as 210.256; 29.376 and 22.032 and in D1, D2 and D3 87.515; 21.419 and 13.464, respectively (Table 1). The protein content of peaks 1 (protein-rich) in samples C1 and D1 is about 7–10 times higher

Table 1
Polyphenols, proteins and antioxidant activities in DMBMAMet and DMBM50%Met/HCl mussel extracts

Groups of samples	Polyphenols, $\mu\text{g}/100 \text{ g DW}^*$	Polyphenols, $\mu\text{g}/100 \text{ g DW}^{**}$	Proteins, $\mu\text{g}/\text{mg DW}$	NO, %	β -carotene, %	ABTS, TEAC
C1	649.2 \pm 51.9	363.0 \pm 34.9	210.3 \pm 17.3	41.0 \pm 3.5	59.4 \pm 5.1	2.63 \pm 0.3
C2	391.8 \pm 35.8	342.2 \pm 34.1	29.4 \pm 1.9	28.0 \pm 2.3	35.7 \pm 3.1	1.78 \pm 0.2
C3	384.4 \pm 35.1	321.0 \pm 32.0	22.0 \pm 1.7	26.0 \pm 2.1	35.0 \pm 3.0	1.19 \pm 0.1
D1	958.4 \pm 81.1	629.4 \pm 51.6	87.5 \pm 7.1	59.0 \pm 4.9	87.7 \pm 7.1	3.40 \pm 0.3
D2	892.7 \pm 76.9	489.7 \pm 41.5	21.4 \pm 1.7	55.6 \pm 4.7	80.0 \pm 6.9	1.93 \pm 0.2
D3	634.9 \pm 51.3	386.2 \pm 35.1	13.5 \pm 1.2	40.0 \pm 3.4	56.0 \pm 4.9	1.06 \pm 0.1

Abbreviations: (*) in DMBMAMet, black mussel dry matter extracted with absolute methanol; (**) in DMBM50%Met/HCl, black mussel dry matter extracted with 1.2 M HCl in 50%methanol/water; TEAC, Trolox equivalent antioxidant coefficient, $\mu\text{M}/\text{g DW}$; ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate) radical cation; β -carotene, antioxidant assay using β -carotene linoleate model system; NO, scavenging activity against nitric oxide; NO, β -carotene; ABTS, measured in DMBM 50%Met/HCl; DM, dry weight.

than that of peaks 2 (low molecular substances). After boiling of the samples a certain equalization is observed for samples C2 and C3, whereas there is a significant difference between samples D2 and D3. The reason for the latter remains unclear. The general decrease of protein content after the application of a thermal treatment might be a consequence of a certain extraction, i.e. loss of material during the boiling.

3.3. Preparation of extracts and determination of polyphenol content

The experiment was carried out using the crude dry samples. The data are represented in Table 1 as μg gallic acid according to a calibration curve. The highest values are observed in C1 and D1 samples and decreased in the heated samples (C2, C3, D2 and D3) in total polyphenols in the following order: BMDMAMet > BMDM50%Met/HCl > BMDM50%Met. This observation might be a consequence also of a certain extraction and loss of material during the thermal treatment.

3.4. Antioxidant activities

As was shown (Fig. 1A, Table 1) the NO \cdot scavenging effects of BMDM50%Met/HCl extract of D1 (59%) and D2 (55.6%) were higher than those of the other extracts at a concentration of 0.2 mg/ml, but were significantly lower than that of Trolox (66%) at the same concentration ($p < 0.05$). The NO \cdot scavenging effects of BMDM50%Met/HCl extracts of D3 and C1 (Table 1, Fig. 1A) and BMDM50%Met of D1 and D2 (Table 2, Fig. 1B) were nearly equal to that of BHA (40%). BMDM50%Met extracts of D3 and C1 (Table 2, Fig. 1B) and BMDM50%Met/HCl of C2 and C3 exhibited modest (Table 1, Fig. 1A) scavenging effects on NO \cdot (30%), whereas BMDM50%Met extracts of C3 and C2 (Table 2, Fig. 1B) had the weakest scavenging effects (<20%) and corresponded to BHT.

There are two factors in this research that may affect the antioxidant activities: temperature (groups named 2

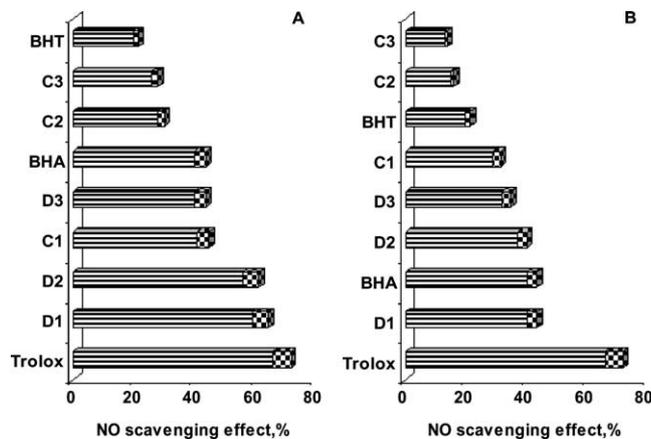


Fig. 1. NO \cdot scavenging effects of extracts (0.2 mg/ml) from: (A) BMDM50%Met/HCl; (B) BMDM50%Met. Each value is the mean \pm standard deviation ($n = 3$). BMDM50%Met/HCl, black mussel dry matter extracted with 1.2 M HCl in 50%methanol/water; BMDM50%Met, black mussel dry matter extracted with 50%methanol/water; BHA; BHT, butylated hydroxytoluene, Trolox, 6-hydroxy-2,5,7,8,-tetramethyl-chroman-2-carboxylic acid. Ci—mussels from clean region and Di—polluted region. C1, D1—without any additional treatment; C2, D2—boiled 15 min; C3, D3—boiled 30 min.

and 3: C2 and C3 and D2 and D3) and contamination status (groups named C and D: C1 and C2). In the kinetic curves (Fig. 2) are shown the changes of antioxidant activities depending on these two factors.

The antioxidant extracts D1 BMDM50%Met/HCl and BHA had similar reaction kinetics curves against β -carotene as determined by spectrophotometric measurement (Fig. 2A) and were very closed one to another. The difference in the antioxidant activity of these two samples was about 6.7%. The extracts of mussels D1 and C1 with β -carotene-linoleate model system (β -carotene) have shown 87.7% and 59.4% antioxidant activity at 2 mg/ml, respectively (Table 1). C1 BMDM50%Met was less effective in quenching free radicals in this system in comparison with D1 BMDM50%Met/HCl and the difference in their antioxidant activities was about 28.3. Kinetic curves of D samples group (Fig. 2B) showed higher scavenging effect than the kinetic curves of group

Table 2
Polyphenols and antioxidant activities in DMBM50%Met mussel extracts

Groups of samples	Polyphenols, $\mu\text{g}/100 \text{ g DW}$	Antioxidant, NO (%)	Activity, β -carotene (%)	Tests, TEAC, $\mu\text{M (g/DW)}$
C1	267.8 ± 21.9	28.9 ± 2.5	40.0 ± 3.7	0.43 ± 0.04
C2	199.3 ± 17.8	15.0 ± 1.3	20.1 ± 1.7	0.23 ± 0.02
C3	194.9 ± 17.7	13.0 ± 1.1	20.0 ± 1.7	0.27 ± 0.03
D1	305.7 ± 28.1	40.0 ± 3.7	60.5 ± 5.5	0.65 ± 0.06
D2	175.8 ± 15.9	37.0 ± 3.3	53.7 ± 5.1	0.34 ± 0.03
D3	167.5 ± 15.3	32.0 ± 2.8	32.0 ± 2.8	0.32 ± 0.03

Abbreviations: DMBM50%Met, black mussel dry matter extracted with 50%methanol/water; β -carotene, antioxidant assay using β -carotene linoleate model system; NO, scavenging activity against nitric oxide; TEAC, Trolox equivalent antioxidant coefficient; DW, dry weight.

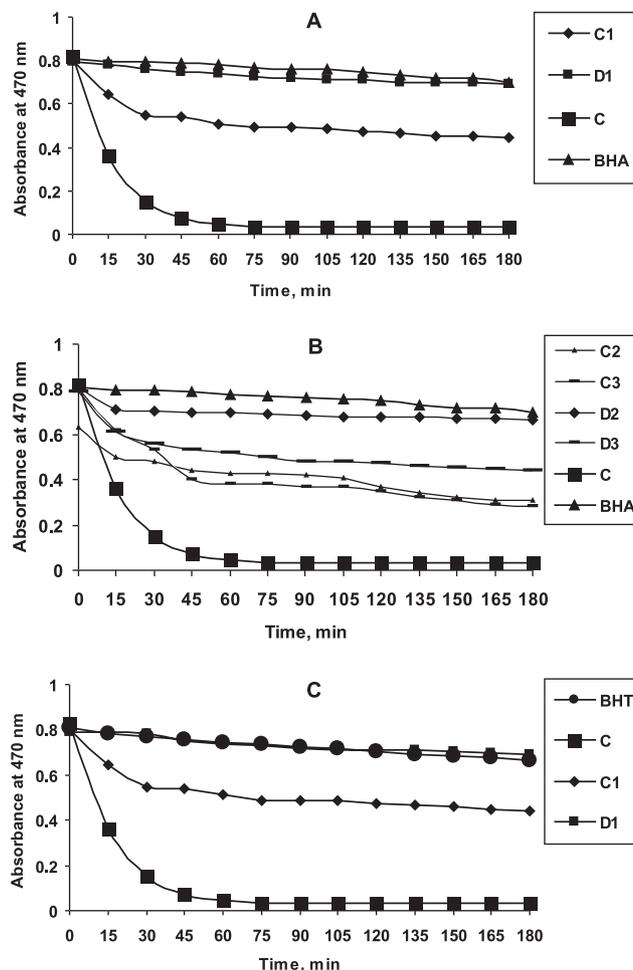


Fig. 2. Reaction kinetics of BMDM50%Met/HCl with β -carotene. The β -carotene concentration was in the reaction mixtures. (A) Reaction kinetics of C1, D1, BHA and C; (B) reaction kinetics of C2, C3, D2, D3, C and BHA; (C) reaction kinetics C1, D1, C and BHT; (C) control; C_i—mussels from clean region and D_i—polluted region. C1, D1—without any additional treatment; C2, D2—boiled 15 min; C3, D3—boiled 30 min.

C samples. It is a remarkable difference between D2 BMDM50%Met and D3 BMDM50%Met. The difference between their antioxidant activities was about 24% (Table 1). This can be explained by the longer thermal treatment of D3 sample. Oppositely between samples C2 BMDM50%Met and C3 BMDM50%Met the difference

is lower than in the previous ones and the kinetics curves are very closed one to another. This can be explained by the fact that in the mussels of clean area the thermal treatment probably less influences the polyphenol composition than in the pollution area samples. BHA in comparison with other samples was closed to sample D2 BMDM50%Met/HCl (Fig. 2B) as D1 BMDM50%Met/HCl with BHA (Fig. 2A). It is the real picture because the percentage of the antioxidant activities of D1 BMDM50%Met/HCl and D2 BMDM50%Met/HCl are 87.7% and 80.0% (Table 1) in comparison with antioxidant activity of BHA (94.4%). The comparison of investigated samples with other standards such as BHT and Trolox is shown on Fig. 2C. The kinetic curves of BHT (81.9%) and Trolox (79%) were close to that of sample D1. The results of this kinetic study have shown that the antioxidant activities of the group of samples from the polluted area were much higher (1.5–2 times) than in the clean one. It is also a difference in the same group with the increasing of the time of thermal treatment and the antioxidant activity decreases with the increasing of the time of the thermal treatment. This relationship is shown also in other methods used. The difference in the antioxidant activities between C1 BMDM50%Met and D3 BMDM50%Met (Table 2) was higher than between the samples C1 BMDM50%Met/HCl and D3 BMDM50%Met/HCl which show nearly the same value.

The highest antioxidant activities were in samples with contamination status (the antioxidant activity of D1 was about 1.5 times higher than in C1). The factor of contamination can be controlled by antioxidant activity of mussels. The temperature factor influenced the antioxidant activity as well. With the increase of thermal treatment the antioxidant activity has decreased about 1.07 times for the samples in clean area and about 1.5 times in polluted area. It means that the mussels with contamination status were less stable for thermal treatment than with clean status.

The best correlation (Fig. 3A) was between polyphenols in BMDMAMet and β -carotene ($R^2 = 0.998$), following by NO \cdot (Fig. 3B) method ($R^2 = 0.9976$), following by polyphenols (Fig. 3A) in BMDM50%Met/HCl and β -carotene ($R^2 = 0.8494$) and BMDM50%Met

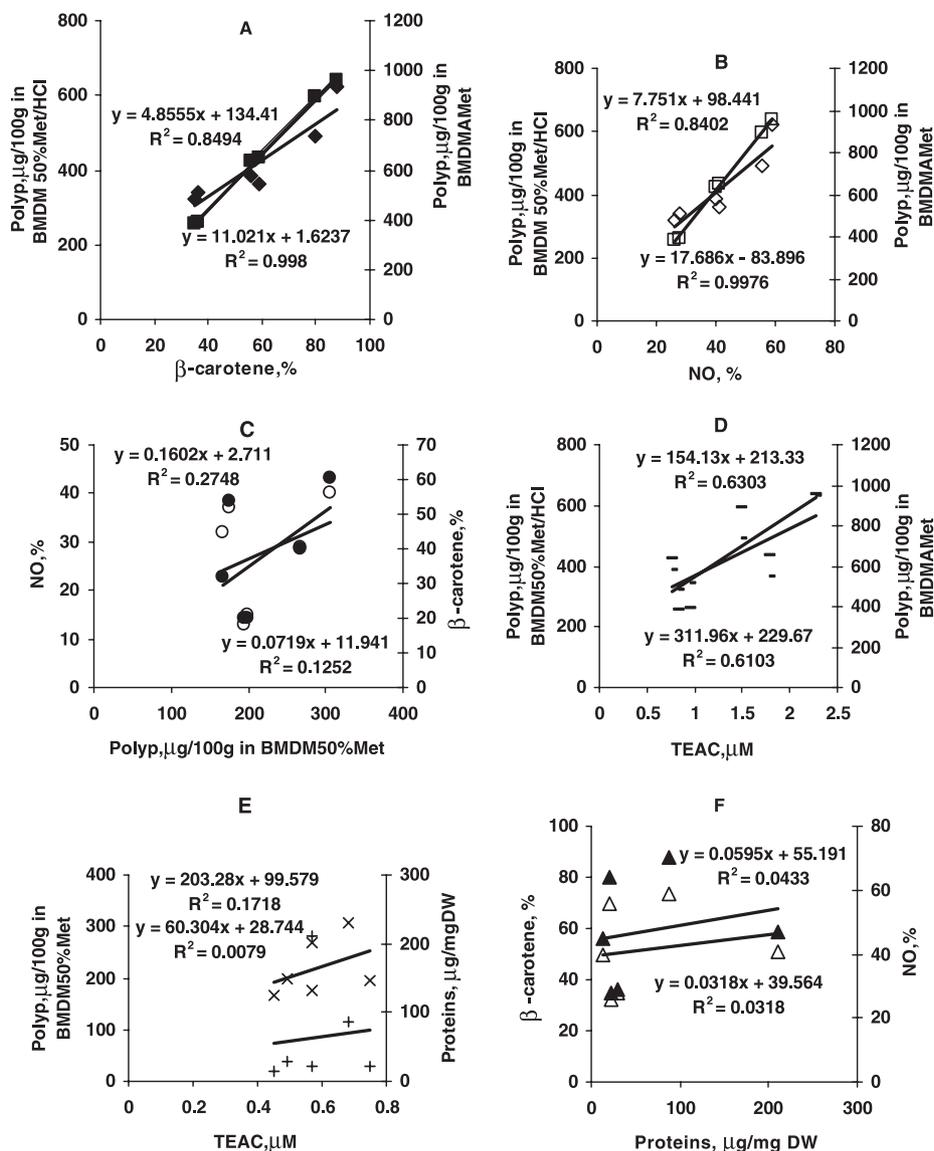


Fig. 3. Relationship, calculated by linear regression analysis for mussel extracts between: (A) (\blacklozenge) Polyp in BMDM50%Met/HCl ($\mu\text{g}/100\text{ g DW}$, Y₁) to β -carotene bleaching effect (%), X) and (\blacksquare) polyp in BMDMAMet ($\mu\text{g gallic acid}/100\text{ g DW}$, Y₂) to β -carotene bleaching effect (%), X). (B) (\diamond) Polyp in BMDM50%Met/HCl ($\mu\text{g}/100\text{ g DW}$, Y₁) to NO (%), X) and (\square) polyp in BMDMAMet ($\mu\text{g}/100\text{ g DW}$, Y₂) to NO (%), X). (C) (\circ) Polyp in BMDM50%Met ($\mu\text{g}/100\text{ g DW}$, X) to NO (%), Y₁) and (\bullet) polyp in BMDM50%Met ($\mu\text{g}/100\text{ g DW}$, X) to β -carotene bleaching effect (%), Y₂). (D) (–) Polyp in BMDM50%Met/HCl ($\mu\text{g}/100\text{ g DW}$, Y₁) to TEAC (μM , X) and (—) polyp in BMDMAMet ($\mu\text{g}/100\text{ g DW}$, Y₂) to TEAC (μM , X). (E) (\times) TEAC (μM , X) to polyp in BMDM50%Met ($\mu\text{g}/100\text{ g DW}$, Y₁) and (+) TEAC (μM , X) to proteins ($\mu\text{g}/\text{mg DW}$, Y₂). (F) (\blacktriangle) Proteins ($\mu\text{g}/\text{mg DW}$, X) to β -carotene bleaching effect (%), Y₁) and (\triangle) proteins ($\mu\text{g}/\text{mg DW}$, X) to NO (%), Y₂). BMDMAMet, black mussel dry matter extracted with absolute methanol; BMDMA50%Met, black mussel dry matter extracted with 50%methanol/water; BMDM50%Met/HCl, black mussel dry matter extracted with 1.2 M HCl in 50%methanol/water. Polyphenols expressed as $\mu\text{g gallic acid}/100\text{ g dry weight}$ (DW); TEAC, Trolox equivalent antioxidant coefficient.

(Fig. 3B) and NO \cdot ($R^2 = 0.8402$). Relatively low correlation coefficients (Fig. 3C) between antioxidant activities in BMDM50%Met extracts measured by NO \cdot and β -carotene and their polyphenols have been determined ($R^2 = 0.1253$) and ($R^2 = 0.2748$), respectively. Antioxidant activities determined by ABTS $^{+\cdot}$ method and their correlations to polyphenols in BMDM50%Met/HCl and BMDMAMet were 0.6303 and 0.6103 (Tables 1 and 2, Fig. 3D). The correlation coefficients between antioxi-

dant activities determined by ABTS $^{+\cdot}$ method (Fig. 3E) and their polyphenols in BMDM50%Met ($R^2 = 0.1718$) and to their proteins ($R^2 = 0.0079$) were low. Similar results were shown between NO \cdot ($R^2 = 0.0318$) and β -carotene ($R^2 = 0.0433$) methods (Fig. 3F) and their proteins.

We have found that the radical scavenging capacity of mussel samples varied according to the different testing radicals (NO \cdot , β -carotene and ABTS $^{+\cdot}$), but the corre-

lation between the total polyphenols and the antioxidant activities has remained in the same degree. It means that all studied methods are reliable tool to determine the changed in two factors: contamination and temperature.

4. Discussion

Marine invertebrates exposed to high environmental levels of chemical pollutants are subjected to increased intracellular flux of oxyradicals mainly produced by the Fenton reaction in the presence of transition metals or by the redox cycle of several organic compounds (Winston, 1991). Since antioxidants represent the cellular defense mechanisms to counteract toxicity of reactive oxygen species, these mechanisms, based on low molecular weight scavengers. Responsiveness of antioxidants to pollutants is difficult to predict and a high degree of variability has been reported as a function of class of chemicals, kind of exposure, phase of the biological cycle (Viarengo et al., 1991; Regoli, 1998). Variations in the levels or activities of antioxidants are potential biomarkers revealing a contaminant-mediated biological effect on the organisms (Ribera et al., 1991; Livingstone et al., 1995; Regoli and Principato, 1995; Porte et al., 2001a,b).

The ecological relevance of biomarkers is greater when, besides exposure to pollutants, they also indicate adverse effects at the organism level (Depledge, 1994). The complexity of interactions between prooxidants factors and cellular defenses, does not generally allow predicting alterations at the organism level from the response of single, specific antioxidants. This difficulty is even greater when the same organisms exhibit the depletion of some antioxidants and the contemporary induction of others (Winston and Di Giulio, 1991).

The mechanisms involved in the beneficial actions of antioxidants in biological systems include directly quenching free radicals to terminate the radical chain reaction, chelating transition metals to suppress the initiation of radical formation, acting as reducing agents, or stimulating the antioxidative defense enzyme activities. This study was conducted to evaluate free radical scavenging properties of mussels and their use as a marker for pollution area. In this study, all tested samples from the polluted area showed significant scavenging activities against NO^\bullet and β -carotene radicals. Samples from clean area had the lowest activity against both NO^\bullet and $\text{ABTS}^{+\bullet}$ radicals. The relative radical quenching activities of Ci group are 1:0.68:0.63 against NO^\bullet , β -carotene (1:0.60:0.59) and $\text{ABTS}^{+\bullet}$ (1:0.53:0.47) while the Di group showed 1:0.94:0.68 against NO^\bullet , β -carotene (1:0.91:0.64) and $\text{ABTS}^{+\bullet}$ (1:0.66:0.34).

The different relative radical scavenging capacity of individual mussel extract against different testing radicals may be explained by the different mechanisms involved in the radical-antioxidant reactions. In this study, the $\text{ABTS}^{+\bullet}$ was generated by incubating ABTS with potassium persulfate. Chemical compounds that inhibit the potassium persulfate activity may reduce the production of $\text{ABTS}^{+\bullet}$. This reduction results in a decrease of the total $\text{ABTS}^{+\bullet}$ in the system and contributes to the total $\text{ABTS}^{+\bullet}$ scavenging capacity. Other factors, such as stereoselectivity of the radicals or the solubility of mussel extracts in different testing systems, may also affect the capacity of mussel extract to react and quench different radicals.

There are very little data about these types of mussels, but some other types were investigated, such as *M. edulis* L. (Walker et al., 2000; Sukhotin et al., 2002).

This observation is supported by the study of Torres et al. (2002) that mussel extracts react differently with different radicals. Thiobarbituric acid-reactive substance (TBARS) and 8-oxo-7,8-dihydro-2'-deoxyguanosine levels were enhanced at the polluted sites. With the exception of superoxide dismutase, the activities of catalase and glutathione peroxidase were also higher at the polluted sites (Torres et al., 2002). Our results are in correspondence with others (Torres et al., 2002) that *M. galloprovincialis* is routinely exposed to an oxidative stress condition at polluted site, and considering xenobiotic bioaccumulation in bivalve molluscs, the mangrove mussel represents an excellent bioindicator for environmental monitoring studies. In their study, as well as in others (Power and Sheehan, 1996; Klobucar et al., 1997; Regoli, 2000; Wilhelm et al., 2001) the different relative activities of selected oxidants (peroxyl radicals, hydroxyl radicals, peroxynitrite, TBARS and others) were used. Our results of antioxidant activities of mussels are in correspondence with others. Seasonal changes in antioxidant activity of mussel (*Perna perna*) determined by TBARS showed higher values in May (94.6 ± 23.7 nmol/g/l) compared to March (41.0 ± 3.7 nmol/g). The observed increase in oxygen consumption is likely to have elicited an increase in cellular oxyradical generation. *P. perna* seems to seasonally compensate for these increases by increasing its antioxidant defenses (Wilhelm et al., 2001). As compared to vitamin E (Trolox), BHA and BHT, the well-known synthetic antioxidants, mussel extracts showed great NO^\bullet radical quenching capacity, although they differed to each other in their relative activities. These measurements are shown also in Cope et al., 1997. Separation of soluble antioxidants from the protein fraction, suggested a greater depletion of low molecular weight molecules during the first phase of exposure to pollutants. Conversely, stress-70 proteins were significantly induced in the most polluted locations (Porte et al., 2001b).

Our results indicate a transient response of antioxidants to pollutants, confirming similar indications reported by other authors (Kirkin et al., 1992; Livingstone, 1993). In conclusion, different radical scavenging methods such as β -carotene, NO^{\cdot} gave relatively similar results with high correlation between total polyphenols and antioxidant activities and were more suitable for the investigated samples, than ABTS⁺ method. As can be seen it was a low correlation between the antioxidant activities determined in extracts with relatively low amounts of polyphenols. Probably, in the total antioxidant activity of mussel extracts, as was determined for the first time; polyphenols play a major role in comparison with other components such as proteins. This conclusion is in accordance with other study (Regoli, 2000; Mourgaud et al., 2002) that the total scavenging capacity towards different forms of oxyradicals is confirmed a useful biomarker with predictive validity at the organism level.

Finally, the most important point of this study is the suitability of the used free radical scavenging methods (β -carotene and NO^{\cdot}) which have been used as a marker for pollution areas and an index of characterization of pollution.

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