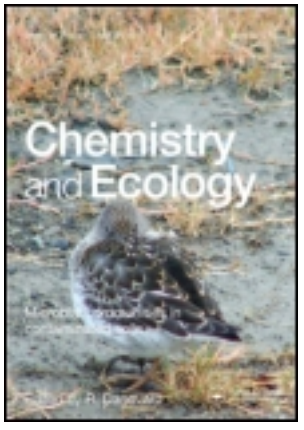

Chemistry and Ecology
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/gche20

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Available online: 16 Feb 2011

To cite this article: Snejana Moncheva, Jacek Namiesnik, Resat Apak, Patricia Arancibia-Avila, Fernando Toledo, Seong-Gook Kang, Soon-Teck Jung & Shela Gorinstein (2011): *Rapana venosa* as a bioindicator of environmental pollution, Chemistry and Ecology, 27:1, 31-41

To link to this article: http://dx.doi.org/10.1080/02757540.2010.522996

Chemistry and Ecology
Vol. 27, No. 1, February 2011, 31–41

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Rapana venosa as a bioindicator of environmental pollution

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(*Received 16 February 2010; final version received 20 July 2010*)

The aim of this investigation was to examine biochemical differences in the gastropod *Rapana venosa* (*R. venosa*) from polluted (*Rv*P) and nonpolluted (*Rv*N) sites of the Black Sea's Bulgarian coast that may serve as bioindicators of environmental quality. Mussels *Mytilus galloprovincialis* (M*Mg*) were collected from polluted (M*Mg*P) and nonpolluted (M*Mg*N) sites at the same time for comparison. Bioactive compounds and the antioxidant activity of dry matter (DM) methanol extracts from *Rv*P and *Rv*N were determined. Three-dimensional fluorometry (3D-FL) and Fourier transform infrared (FTIR) analyses were used to evaluate polyphenols and proteins. DM methanol extracts from *Rv*P, and to lesser extent *Rv*N, showed high amounts of total phenolics (23.22 ± 2.9 and 15.60 ± 1.8 mg GAE $\cdot$ g^{-1} DW) and exhibited high levels of antioxidant activity in some radical scavenging assays (μMTE $\cdot$ g^{-1} DW): 56.38 ± 5.2 and 33.79 ± 3.3 by 2,2azino-bis (3-ethyl-benzothiazoline-6-sulfonic acid) diamonium salt (ABTS$^{\cdot+}$); 54.79 ± 5.6 and 33.7 ± 2.6 by cupric reducing antioxidant capacity (CUPRAC); 62.34 ± 6.8 and 30.31 ± 3.9 by the 1-diphenyl-2-picrylhydrazyl method (DPPH). A correlation in the above indices was found between *R. venosa* and *M. galloprovincialis*, but all results were relatively higher *for M. galloprovincialis* than for *R. venosa*. The obtained indices of *R. venosa* may serve as a bioindicator of environmental ecological quality.

Keywords: *Rapana venosa*; mussels *Mytilus galloprovincialis*; biomonitoring; chemical indices; bioindicators; Black Sea

1. Introduction

Study of the physiological behaviour of marine organisms has been shown to be a valuable approach for the assessment of biological responses to environmental stress [1–4]. Different animals and their chemical components, or molecular, biochemical and/or physiological properties have been used as bioindicators of marine pollution [5–8].

In their environmental policy agenda, European maritime states have outlined the protection of species and habitats and the need to maintain quality standards in coastal and offshore waters

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ISSN 0275-7540 print/ISSN 1029-0370 online

DOI: 10.1080/02757540.2010.522996
http://www.informaworld.com

as a priority [2]. The Water Framework Directive and Marine Strategy Directive call for the definition and application of biological quality elements and the development of early-warning approaches for environmental health assessment and monitoring. Although there have been numerous investigations in mussels, *Mytilus galloprovincialis* [3,4,8], there are few available studies of *Rapana venosa*, mainly due to its capacity to accumulate heavy metals and mass mortality related to toxic phytoplankton blooms [9–15].

R. venosa is a marine snail of high ecological fitness due to its high fertility, fast growth rate and tolerance to low salinity, high and low temperatures, water pollution and oxygen deficiency, which have a documented impact on both natural and cultivated populations of oysters, mussels and other molluscs, and significant negative changes in the ecosystem [1]. *R. venosa* is a very voracious predator; introduced into the Black Sea in the early 1940s, it is blamed for the decline in the native, edible bivalve fauna. Its populations have caused significant changes in the ecology of bottom-dwelling organisms and serious deterioration of benthic communities [1]. Since the 1980s, *R. venosa* has become a valuable commercial resource: its meat is exported to Japan for food and it has recently also been included in the diet of those native to the Black Sea area. Extensive harvesting by trawling (although officially forbidden in the Black Sea) adds further to the deterioration of the marine environment [10]. According to some recent reports, annual Rapa whelk catches from Turkey and Bulgaria exceed 13,000 $t \cdot year^{-1}$ [14].

In this study, chemical differences between *R. venosa* from polluted (*Rv*P) and nonpolluted (*Rv*N) sites of the Bulgarian Black Sea were studied to establish whether the results could be used in the diagnosis of environmental contamination. Three-dimensional fluorometry (3D-FL) and Fourier transform infrared (FTIR) analyses were used to evaluate polyphenols and proteins [3,4,15,16]. In order to obtain reliable antioxidant activity data, four complementary assays were used: cupric-reducing antioxidant capacity (CUPRAC), 2,2azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diamonium salt (ABTS), ferric reducing antioxidant power (FRAP) and 1-diphenyl-2-picrylhydrazyl method (DPPH) [3,4,17–20]. Furthermore, the aim of this investigation was to analyse the antioxidant content of the test organisms as bioindicators in an attempt to optimise environment monitoring.

2. Materials and methods

2.1. *Study area*

Varna Bay, the second largest bay along the Bulgarian Black Sea coast, is subjected to multiple anthropogenic stresses (chemical industry, shipping, tourism, fishing, port activities, urban pressures, etc.), resulting in severe deterioration of the ecological quality of the area [12,13,21]. Stations for the collection of *R. venosa* and *M. galloprovincialis* samples (Figure 1) were selected based on data of the level of contamination and eutrophication. *R. venosa* and *M. galloprovincialis* were collected in two regions of Bulgarian Black Sea: (i) a non-impacted area (Galata station, 3 mile offshore Varna city) and (ii) polluted (Varna Bay) sites at a depth of 3–4 m.

2.2. *Sample collection*

Sampling was carried out in late July to early August 2006. The snails and mussels were collected by divers, and *R. venosa* specimens 60–70 mm in length were selected for analysis. Mussels were processed as previously described [3,4]. The size of *R. venosa* and *M. galloprovincialis* from both polluted and nonpolluted sites corresponded to 75–85% of the maximum size reached within each population in the area. This approach guaranteed that the compared *R. venosa* and mussels

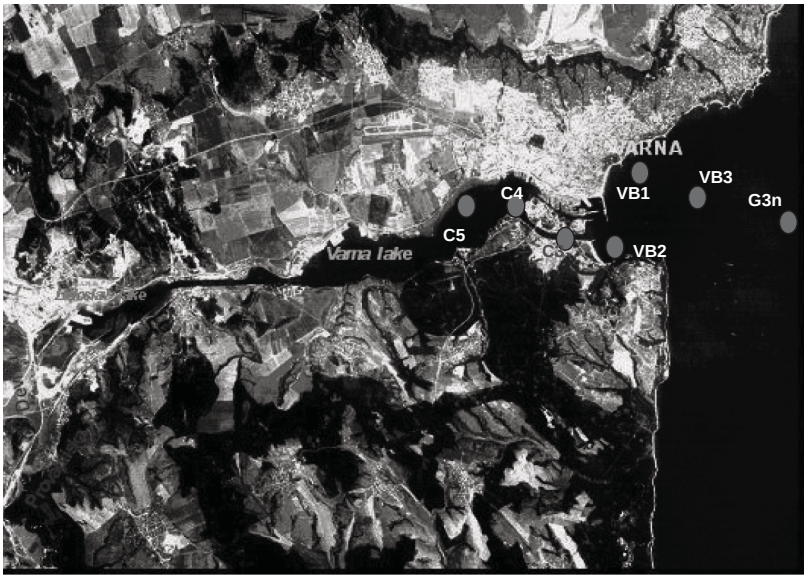

Figure 1. Map of sampling area along the Bulgarian Black Sea coast: VB2, polluted site and G-3nm, relatively nonpolluted site.

had similar metabolic conditions, and the influence of physiological differences between the two populations was less pronounced [20].

After immediate transportation to the laboratory, the shells of the organisms were measured for appropriate size selection, then carefully removed and the whole of the soft tissue from 30 specimens of each species was rapidly frozen in liquid nitrogen and stored at $-80\,^{\circ}C$ [4,17]. Samples were then freeze dried in glass flasks on Finn- Aqua, Lyovac GT-2 equipment for 36 h.

2.3. *Determination of the contents of the studied bioactive compounds*

The following chemicals were used in the applied methods: 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), ABTS, DPPH, Folin–Ciocalteu reagent (FCR), lanthanum (III) chloride heptahydrate, $FeCl_3 \cdot 6H_2O$, $CuCl_2 \cdot 2H_2O$, 2,9-dimethyl-1,10-phenanthroline (neocuproine) and butylated hydroxyanisole (BHA) all purchased from Sigma Chemical Co. (St. Louis, MO, USA). 2,4,6-Tripyridyl-*s*-triazine (TPTZ) was purchased from Fluka Chemie (Buchs, Switzerland).

Most of the bioactive compounds (polyphenols, tannins, flavonoids and flavanols) were determined as previously described [3,4,17].

The presence of polyphenol and protein compounds in the DM of the investigated *R. venosa* and mussel samples was studied using FTIR spectroscopy and 3D-FL. A Bruker Optic GMBH Vector FTIR spectrometer (Bruker Optic GMBH, Attingen, Germany) was used to record IR spectra. A potassium bromide microdisk was prepared from finely ground lyophilised *R. venosa* powder (2 mg) with 100 mg of KBr [16].

2.4. *Fluorescence measurements*

Fluorescence spectra of methanol extracts of *R. venosa* and *M. galloprovincialis* at a concentration of $0.01\ mg \cdot mL^{-1}$ were recorded on a model FP-6500, Jasco spectrofluorometer (serial N261332, Japan), equipped with 1.0 cm quartz cells and a thermostat bath. The widths of the excitation and

emission slits were set to 10.0 and 5.0 nm, respectively. Three-dimensional spectra were collected with subsequent scanning emission spectra from 330 to 740 nm in 1.0 nm increments by varying the excitation wavelength from 265 to 695 nm in 10 nm increments. The scanning speed was set at 1000 nm $\cdot$ min^{-1} for all measurements [22,23].

Unconjugated plus conjugated (total) polyphenols (TP) and tannins were extracted from dried defatted samples (50 mg) with 5 mL of 1.2 M HCl in 50% methanol/water and heated at 90 °C for 3 h. FCR was used to determine the total amount of polyphenols in the studied extracts and the measurement was performed at 765 nm with gallic acid as the standard. Results were expressed as mg of gallic acid equivalent (GAE).

Spectrophotometric determination of flavonoids was carried out with 5% $NaNO_2$, 10% $AlCl_3 \cdot 6H_2O$ and 1 M NaOH, and was measured at 510 nm. The extracts of condensed tannins (procyanidins) with 4% methanol vanillin solution were measured at 500 nm. The total flavanols were estimated using the *p*-dimethylaminocinnamaldehyde (DMACA) method, and the absorbance at 640 nm was then read. (+)-Catechin served as a standard for flavonoids, flavanols and tannins, and the results were expressed as catechin equivalents (CE).

2.5. *Determination of the antioxidant activity*

The following radical scavenging tests were used: (i) 2,2-azino-bis (3-ethyl-benzothiazoline-6-sulfonic acid) diamonium salt (ABTS$^{\cdot+}$) was generated by interacting ABTS (7 mmol $\cdot$ L^{-1}) and $K_2S_2O_8$ (2.45 mmol $\cdot$ L^{-1}). This solution was diluted with methanol until the absorbance reached 0.7 at 734 nm. (ii) CUPRAC assay, which is based on utilising the copper (II)-neocuproine [Cu(II)-Nc] reagent as the chromogenic oxidising agent. The absorbance at 450 nm was recorded against a reagent blank. (iii) The FRAP assay measures the ability of the antioxidants in the investigated samples to reduce ferric-tripiridyltriazine (Fe^{3+}TPTZ) to a ferrous form (Fe^{2+}), which absorbs light at 593 nm. (iv) DPPH solution (3.9 mL, 25 mg $\cdot$ L^{-1}) in methanol was mixed with the sample extracts (0.1 mL). The progress of the reaction was monitored at 515 nm until the absorbance was stable [3,17–19].

2.6. *Statistical analyses*

The results of this investigation are given as the means $\pm$ SD of three measurements. Differences between groups were tested by two-way ANOVA. To assess the antioxidant activity, Spearman's correlation coefficient (R) was used. Linear regressions were also calculated. Values of $p < 0.05$ were considered significant.

3. Results and discussion

As mentioned in the Introduction, mussels are recognised worldwide as pollution bioindicators, because they accumulate pollutants in their tissues at elevated levels in relation to the biological availability of the pollutant in the marine environment. This study deals with the use of *R. venosa* and comparison with *M. galloprovincialis* as a local bioindicator of organic compounds. It is necessary to have bioindicators of ecological quality, in general, and of seas, in particular, and to find their chemical and biochemical responses. Therefore, in this investigation, chemical differences between *R. venosa* from polluted (*Rv*P) and nonpolluted (*Rv*N) sites of the Bulgarian Black Sea coast were studied in order to use them as additional bioindicators of environmental ecological quality. The results were compared with data from mussels, *M. galloprovincialis*.

Table 1. General characteristics of the sampling stations [21].

Season	Station	T (°C)	S (ppm)	O_2 ($mL \cdot L^{-1}$)	N total ($\mu g \cdot mL^{-1}$)	P total ($\mu g \cdot mL^{-1}$)	Si ($mg \cdot L^{-1}$)	TRIX	Total biomass ($mg \cdot m^3$)
Spring	NP	15.8	17.3	7.46	82.18	13.68	104.88	5.55	1451
	P	15.5	16.4	8.21	522.65	56.88	167.70	6.79	3930
Summer	NP	25.0	17.1	2.82	55.14	18.63	25.15	4.55	1636
	P	25.0	15.9	1.27	275.78	107.61	377.33	7.61	1291

Notes: P, polluted sites; NP, nonpolluted sites.

Table 2. Concentration of contaminants in the sampling stations.

	Metals	P ($mg \cdot kg^{-1}$ DWD)	NP ($mg \cdot kg^{-1}$ DW)
1	Cu	16.5–44.0	16
2	Zn	6.4-17.2–95	6.4
3	Pb	15.3–33	18
4	Cd	0.5–1.0	0.5
5	As	1.9–6.8	ND
6	Cr	9.0[a]–15.4–19.0	ND
7	Mn	245–283	231
8	Hg	0.06–0.3	ND
9	Fe	0.2–1.2	ND
10	Ni	1.8–18	11
11	Co	5	0.2
12[a]	Li	60	ND
13[a]	TPH	2500–6300	ND
14	Oil	120	3
	Radioactive elements	P ($Bq \cdot kg^{-1}$)	NP ($Bq \cdot kg^{-1}$)
15	U	238–22	ND
16	Ra	276–18	ND
17	Th	23–16	ND
18	K	567–40	ND
19	Cs	138–34	ND

Notes: P, polluted areas; NP, nonpolluted areas; DW, dry weight; ND, not detected; TPH, total petroleum hydrocarbons. [a]Results taken from previous studies [12,31].

The environmental parameters for the two stations are presented in Tables 1 and 2. The data demonstrate a persistent gradient between the stations, both in the level of nutrients, phytoplankton biomass, the eutrophication index TRIX and the degree of contamination. As can be seen in Table 1, there was a significant difference between the seasons (spring and summer): the polluted area was lower in oxygen, but higher in total N and total phytoplankton biomass than the nonpolluted area. In summer, when the samples were collected, a significant increase in the polluted area in comparison with the nonpolluted area was seen in terms of the amounts of total N (5×), total P (6×), Si (15×) and TRIX (2×). Metal concentrations at the sampling stations also differed (Table 2). In the nonpolluted area, radioactive elements were not detected, nor were As, Cr, Hg, Fe and Li. TPH was not found and the amount of oil was ∼40× lower than in the polluted area.

3.1. *FTIR measurements*

FTIR spectra wavenumbers for catechin at 827, 1039, 1115, 1143, 1286, 1478, 1511 and 1610 cm^{-1} were assigned to C−H alkenes, –C−O alcohols, C−OH alcohols, –OH aromatic, C−O alcohols, C−H alkanes, C=C aromatic ring and C=C alkenes. Gallic acid showed the following wavenumbers (cm^{-1}): 866, 1026, 1238, 1450, 1542 and 1618. The polyphenols region in the investigated samples (Figure 2, lower and upper lines) showed bands that were slightly

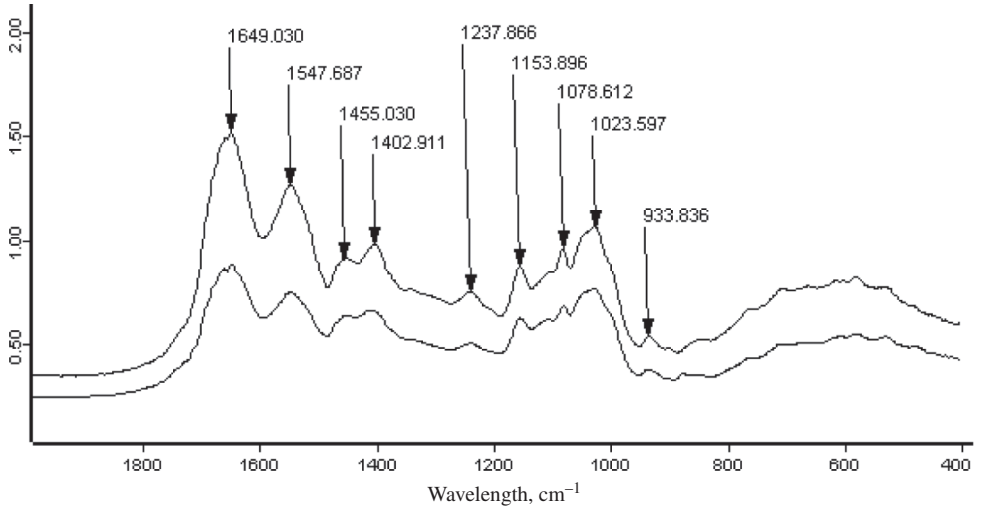

Figure 2. FTIR spectra for *Rapana venosa* polluted (upper curve) and nonpolluted (lower curve) samples.

different from the standards: 934, 1024, 1079, 1154, 1238, and 1649 cm^{-1}, but the wavelengths of the bands were similar in all *R. venosa* samples (Figure 2). Other additional bands in the *R. venosa* samples in the polyphenols region were shifted slightly in comparison with the standards. FTIR was used for mussel samples only [6,24,25], because an interaction of fibrinogen and foot protein 1 (Mefp-1), the adhesive protein of the common blue mussel *Mytilus edulis*, with methyl- and oligo-(ethylene oxide) (OEG) terminated self-assembled monolayers (SAMs). We obtained FTIR results only for proteins in mussels containing the redox-functional amino acid 3,4-dihydroxyphenylalanine (DOPA), which is a typical feature of most Mefp proteins [24,25]. The spectrum for mussel proteins was carried out in aqueous solution and displays absorption bands at 1570, 1472, 1260, and 973 cm^{-1} [24,25]. The band at 1260 cm^{-1} is attributed to the C−O stretching vibration of the side chain hydroxyl groups, which changed during cross-linking and did not appear in our spectra data. Therefore, the results for *R. venosa* can be compared with the spectra for *M. galloprovincialis* [4]. The two mussel samples showed broad amide I (AI) bands at 1648 cm^{-1}, typical for proteins with a high α-helical content [3,4]. In *R. venosa*, the peak was relatively sharp and narrow (Figure 2), and a slight shift of 1 cm^{-1} was observed. The high-frequency components in the AI band at 1669 cm^{-1} which can be assigned to turns and elements of β-sheet [26] was not found in *R. venosa*. An amide II (AII) band was seen at 1556 and 1540 cm^{-1} for *M. galloprovincialis*. Differences in the FTIR data between the nonpolluted and polluted mussel samples indicated only in a small shift in the AI and AII bands, characteristic of a decrease in α-helix content [4]. Similar bands were obtained for *R. venosa* (Figure 2; 1548 cm^{-1}). It can be concluded that the region of the proteins in *Mytilus edulis*, *M. galloprovincialis* and *R. venosa* showed similar bands for amide I (AI), amide II (AII), and amide III (AIII) (in the range of 1650, 1530 and 1300–1250 cm^{-1}). FTIR can be used as an additional tool in determining changes in different environmental conditions.

3.2. *Fluorimetric measurements*

The 3D-FL spectra (Figure 3) highlight the elliptical shape of the contours. The x-axis represents the excitation spectra from 265 to 695 nm, and the y-axis is the emission spectra from 330 to 740 nm: of *Rv*P (A), *Rv*N (B), M*Mg*N (C) and M*Mg*P (D) in methanol extract. In 3D-FL spectra, the excitation and emission wavelengths and the fluorescence intensity were used as

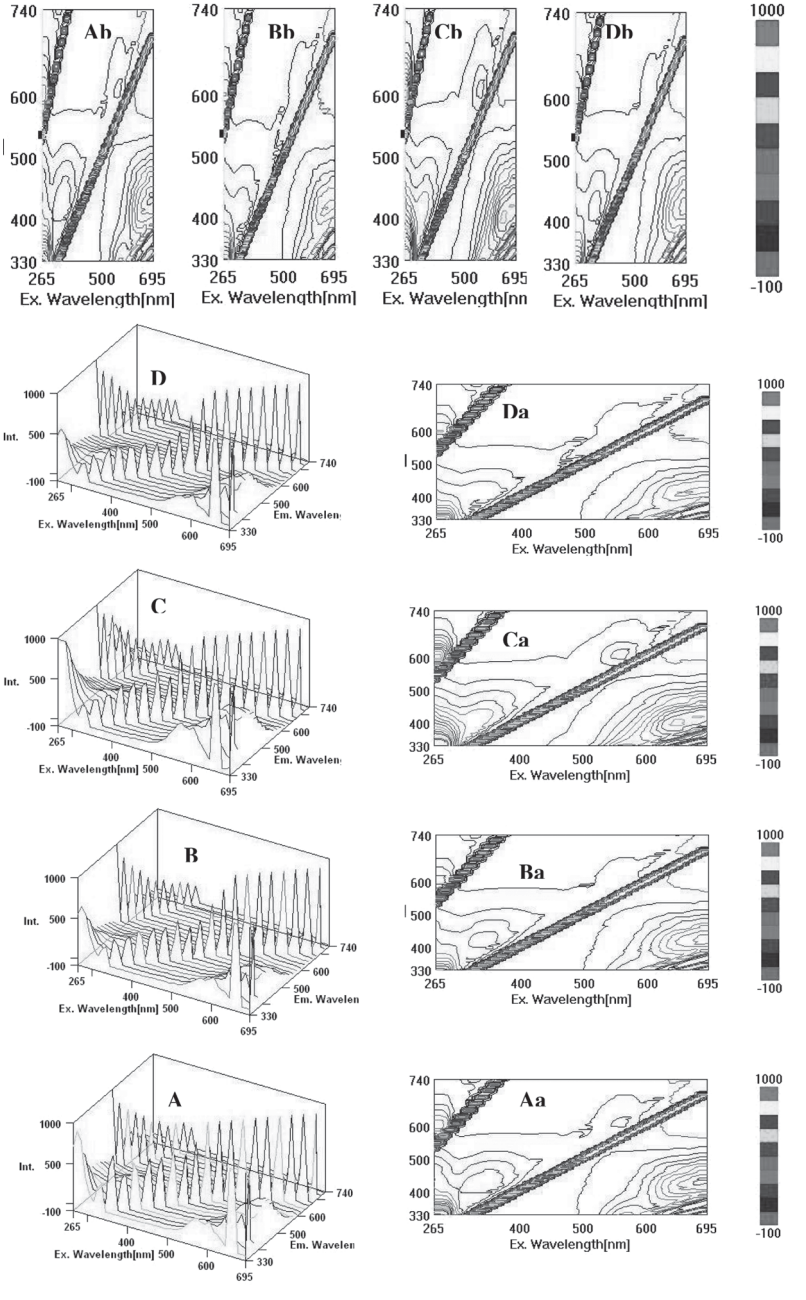

Figure 3. Three-dimensional fluorescence map of methanol extracts of *Rv*P (A), *Rv*N (B), M*Mg*N (C) and M*Mg*P (D), respectively. The contour and cross-maps (Aa, Ab, Ba, Bb, Ca, Cb, Da, Db) displayed a view of the corresponding fluorescence spectra. The three-dimensional spectra were for emission wavelengths from 330 to 740 nm and excitation wavelengths from 265 to 695 nm, scanning speed was 1000 nm · min^{-1}, excitation mode and fluorescence intensity 1000. A–D on z-axis: Int, fluorescence intensity; on x-axis: Ex. Wavelength, excitation wavelength; on y-axis, Em. Wavelen, emission wavelength; Aa, Ab, Ba, Bb, Ca, Cb, Da, Db on x-axis: Em Wavelength, emission wavelength; on y-axis, excitation wavelength. All fluorescence intensity values from -100 to 1000 are shopwn. *Rv*P, *Rapana venosa* from polluted sites; *Rv*N, *Rapana venosa* from nonpolluted sites; M*Mg*P, *Mytilus galloprovincialis* from polluted sites; MMgN, *Mytilus galloprovincialis* from nonpolluted sites (colour online).

Table 3. Bioactivity of methanol extracts of *Rapana venosa* and *Mytilus galloprovincialis*.

Indices	*Rv*P	*Rv*N	M*Mg*N	M*Mg*P
POL, mg GAE $\cdot g^{-1}$	23.22 ± 2.9^{b}	15.60 ± 1.8^{a}	32.17 ± 3.7^{c}	38.29 ± 4.1^{d}
FRAP, μM TE $\cdot g^{-1}$	4.75 ± 0.5^{a}	5.61 ± 0.5^{b}	8.46 ± 0.8^{c}	11.42 ± 1.3^{d}
ABTS, μM TE $\cdot g^{-1}$	56.38 ± 5.2^{b}	33.79 ± 3.3^{a}	87.76 ± 8.2^{d}	79.38 ± 7.9^{c}
CUPRAC, μM TE $\cdot g^{-1}$	54.79 ± 5.6^{b}	33.7 ± 2.6^{a}	60.7 ± 6.9^{b}	111.28 ± 10.9^{c}
DPPH, μM TE $\cdot g^{-1}$	62.34 ± 6.8^{c}	30.31 ± 3.9^{a}	49.09 ± 4.7^{b}	47.92 ± 4.5^{b}
Tannins, mg CE $\cdot g^{-1}$	2.08 ± 0.2^{b}	0.90 ± 0.1^{a}	2.96 ± 0.3^{c}	3.44 ± 0.3^{c}
Flavonoids, mg CE $\cdot g^{-1}$	4.91 ± 0.4^{c}	0.92 ± 0.1^{a}	1.31 ± 0.1^{a}	3.44 ± 0.3^{b}
Flavanols, mg CE $\cdot 100\ g^{-1}$	4.10 ± 0.4^{b}	2.03 ± 0.2^{a}	ND	ND

Notes: Values are means $\pm$ SD of five measurements. Values in rows with different superscript letters are significantly different ($p < 0.05$). *Rv*P, *Rapana venosa* from polluted sites; *Rv*N, *Rapana venosa* from nonpolluted sites; M*Mg*N, *Mytilus galloprovincialis* from nonpolluted sites; M*Mg*P, *Mytilus galloprovincialis* from polluted sites; FRAP, ferric-reducing/antioxidant power; ABTS, 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diamonium salt; CUPRAC, cupric reducing antioxidant capacity; DPPH, 1-diphenyl-2-picrylhydrazyl method; GAE, gallic acid equivalent; CE, catechin equivalent; TE, trolox equivalent; ND, not detected.

axes to investigate the extracted bioactive compounds in the samples, and the contour spectra provided more information about these samples. The appearance of main peaks can be displayed in two ways: contour maps (Aa, Ba, Ca and Da), which correspond to samples of *R. venosa* and *M. galloprovincialis* from polluted and nonpolluted sites and cross-maps (Ab, Bb, Cb and Db) corresponding to the same samples of *R. venosa* and *M. galloprovincialis* from polluted and non polluted sites.

The contour maps of the methanol extracts (Figure 3) showed one main peak for *Rv*P and M*Mg*P at $\lambda_{ex/em}$ 300/440 nm and a small peak at $\lambda_{ex/em}$ 550/600 nm. The main peaks were similar between *R. venosa* and *M. galloprovincialis*, but differ in fluorescence intensity in samples from polluted and nonpolluted sites. The fluorescence intensity of the peaks for polluted areas was higher than that for nonpolluted areas for *M. galloprovincialis*, but lower for *R. venosa*. Our results can be compared only with the recent reports [22,23], in which protein-like fluorescence was composed of tyrosine-like fluorescence and tryptophan-like fluorescence. The main protein-like fluorescent peak was at $\lambda_{ex/em}$ 270/290–310 nm. The data obtained in this study showed slightly different peak locations than those cited [22,23]. The three-dimesnional fluorescence results can be used as an additional tool for studying changes in comparison of *R. venosa* and *M. galloprovincialis* samples.

3.3. *Bioactive compounds*

Methanol (1.2 M HCl in 50% methanol/water) was used for unconjugated plus conjugated ('total') polyphenol extraction and showed variation in the amounts of bioactive compounds, depending on the animals used and the collection site. Polyphenols (mg GAE $\cdot g^{-1}$ DW) and tannins (mg CE $\cdot g^{-1}$ DW) were lower in *R. venosa* samples than in mussels, flavonoids (mg CE $\cdot g^{-1}$ DW) were comparable and flavanols (mg CE $\cdot 100\ g^{-1}$ DW) were found only in *R. venosa* (Table 3; $p < 0.05$). All bioactive compounds in mussels and *R. venosa* from polluted sites were significantly higher than in samples from nonpolluted sites ($p < 0.05$).

3.4. *Antioxidant activity*

Antioxidant activity, determined using several antioxidant scavenging methods in methanol extract, was significantly higher in mussel samples than in *R. venosa* (FRAP, ABTS and CUPRAC), showing approximately the same relationship as for bioactive compounds (polyphenols and tannins, $p < 0.05$). A direct relationship between polyphenols and antioxidant activity was

obtained in mussel samples, with correlation coefficients (R^2) determined using FRAP and CUPRAC radical scavenging assays of 0.87–0.96. A correlation was found between the polyphenols and ABTS and CUPRAC radical scavenging results in *R. venosa* samples.

Antioxidant values for mussels from polluted area were significantly higher than for nonpolluted samples, as found in a number of previous reports [3,4,17,20].

The same data were obtained for three molluscs species from the Black Sea: *M. galloprovincialis*, *Mya arenaria* and *Rapana besoar*. Studies found the highest catalase activity in the mid-gland of *Mya arenaria*, probably because of their evolution in a very polluted environment [27]. *R. venosa* samples react in the same way: the antioxidant characteristics in polluted samples (polyphenols, tannins, flavonoids, flavanols and overall antioxidant activities by ABTS, CUPRAC and DPPH) were significantly higher than in nonpolluted samples ($p < 0.05$). Comparison of the changes seen in two animals from polluted and nonpolluted areas showed that mussels were more sensitive to pollution.

Differences in the sensitivity of various organisms are expected because no single species could be the most suitable for detecting all possible pollutants [5]. Some organisms demonstrate a range of compensatory mechanisms that may mask the expected biological response to exposure [11]. Our results are in agreement with other reports in which two green algae (*Ulva rigida* and *Cladophora coelothrix*), the mussel, *M. galloprovincialis*, and the snail, *R. venosa*, from the Bulgarian Black Sea shore were treated with diesel fuel [28]. Changes appeared to be greater in the evolutionary less-advanced species from both groups of marine organisms, algae and invertebrates (*U. rigida* and *M. galloprovincialis*, respectively) than in *R. venosa*. These results may be also explained by differences in the feeding behaviour of the two types of marine animal (*R. venosa* is a carnivore, whereas *Mytilus* is a filtrator). According to others [11], soft tissue may not clearly reflect environmental concentrations because the active excretion of metals prevents excessive bioaccumulation, and in some freshwater snails the shell demonstrates a higher affinity for metal accumulation than the soft tissue. Our results can also be explained by the ability of *R. venosa* to avoid pollutants by retracting into their shell, which might act as a defence mechanism. Therefore, organisms may not give the expected biological response when exposed to chronically polluted environments [29,30].

It has been reported that during a toxic bloom, *R. venosa* stopped feeding as dissolved oxygen and chlorophyll concentrations increased with development of the bloom. *R. venosa* mortality was preceded, over a period of 24–48 h, by external signs of stress including reduced ventilation, inability to attach to hard substrates, periodic pumping of the opercular plate and increased mucus production [13,14]. As properties of living organisms, bioindicators could be affected by periodic variations in environmental factors (such as light, temperature, dissolved O_2, and nutrient and contaminant input) and by changes in biological functions (e.g. rate of metabolic processes or the reproductive cycles usually connected to environmental changes). The ecological plasticity of the different organisms may also play a significant role.

In conclusion, dry matter extracts from polluted (*Rv*P) and to a lesser degree nonpolluted (*Rv*N) areas possess high amounts of total phenolics, and show high levels of antioxidant activity. The highest significant content of the above-mentioned indices in *Rv*P may serve as a bioindicator of environmental ecological quality. Although less sensitive, *R. venosa* and mussels can be used as bioindicators, representing an important tool for the biomonitoring of environmental pollution in coastal areas. Our results demonstrated that alterations in antioxidant enzymes reflected the gradient of contamination, confirming the rational use of biomarkers of oxidative stress in biomonitoring aquatic metal pollution. Despite its widespread use, the lack of detailed knowledge about variability in species-specific responses to different pollutants is still a limitation of the biomarker approach. Standardisation of toxicology databases would assist with accurate toxicological prediction and its effective practical implementation.

Acknowledgements

The authors are thankful to Dr Elena Katrich (Hebrew University of Jerusalem, School of Pharmacy) for her technical assistance in the determination of antioxidant activity and 3D-FL, Dr Ivan Goshev and Mrs Boryana Mihaylova (Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, Sofia, Bulgaria) for the transportation and lyophilisation of the samples. The study has been partly supported by SESAME FP6, EC Project, No. 036949.

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