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Long-term Effects of Kerch Strait Residual Oil-Spill: Hydrocarbon Concentration in Bottom Sediments and Biomarkers in *Mytilus galloprovincialis* (Lamarck, 1819)

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Abstract

Tamanskij Bay opens into the Kerch Strait which connects the Black and the Azov seas and houses the shallow ecosystem which is the most important in the Russian part of the Black Sea. This ecosystem is dominated by sea grasses (*Zostera marina* etc). We have been studying bottom sediment hydrocarbons distribution and biomarkers of the pollution in *Mytilus galloprovincialis* for two years after the wreck of “Volgoneft’ 139” and spilling of more than 1.5 thousand t of residual oil in the Kerch Strait in November 2007. The high level of hydrocarbons in bottom sediments was observed within the analysis of samples taken in 2008 (to 1.5 mg/g). The hydrocarbon level in bottom sediments significantly decreased by July 2009. The n-alkanes distribution analysis reveals two following pollution sources in study area: accidental pollution by residual oil after spill and local pollution sources related to the settlements and the ports. The study of biomarkers in mussels indicated the oil spill pollution effects only.

As the considered study reveals, at present the ecosystem overcomes the negative effects of the oil spill due to its self-purification sources. However, in case of the recurrent pollution, the situation may change.

Keywords: n-Alkanes, organic matter, hemocytes, micronucleus, histopathology.

Introduction

Tanker Volgoneft-139 wreckes on November 11, 2007 due to the heavy storm. The wreck resulted in more than 1.5 thousand ton residual oil spill near Ukrainian island Tuzla at the Kerch strait (Figure 1). The spill covered the areas of the Kerch strait, Tamanskii and Dinskoi bays within a few days (figure 1b, Ovsienko *et al*., 2008). These areas are unique shallow-water (<4 m) wetlands (Krivenko, 2000). The Tamanskij Bay is the only place on the Russian Black Sea coast where the eelgrass *Zostera marina* forms wide meadows and proved to be the most important structural component of the sea ecosystem and a producer of organic matter in this region (Belyaev *et al*., 2009). The ecosystems of Tamanskij and Dinskoi bays include spawning areas of mass fish species and habitats of waterfowls which stop here during the seasonal migration. In spite of uniqueness of the wetlands this area is still heavy urbanized. A number of urban, transport and military objects is located on the shore (Taman city, villages, Port Caucasus etc.). The annual input of wastes to the Kerch strait is about 45 mln t (Sovga *et al*., 2007). The bottom sediments of this region is characterized by high background concentrations of heavy metals (Zn, Ni, Co), arsenic and hydrocarbons. The background concentration of hydrocarbons in Kerch strait exceeds the European standards (100 ug/g) at least by 1.09-5.11 times (Sovga *et al*., 2003). In addition, the studies of the Russian part of Kerch strait and particular Tamanskij and Dinskoi were rear in recent 25 years. However the hydrologic, sedimentation regimes and probably ecosystems structure has significantly been changed after Tuzla spit renewal in 2003-2004 (Lomakin and Spiridonova, 2010). The main goals of the presented work were to study the distribution of the residual oil after November 2007 oil-spill and to assess its influence on the bottom ecosystems.

Chemical analysis of total hydrocarbons and aliphatic hydrocarbons concentration in the bottom sediments was performed to reveal residual oil-spill pollution and to distinguish them from the basic pollution level. The impact of pollution on bottom ecosystems was assessed by the biomarker study of the most abundant bivalve species, which are the crucial part of the local ecosystems - *Mytilus galloprovincialis* (Lamarck, 1819). Pollution effects

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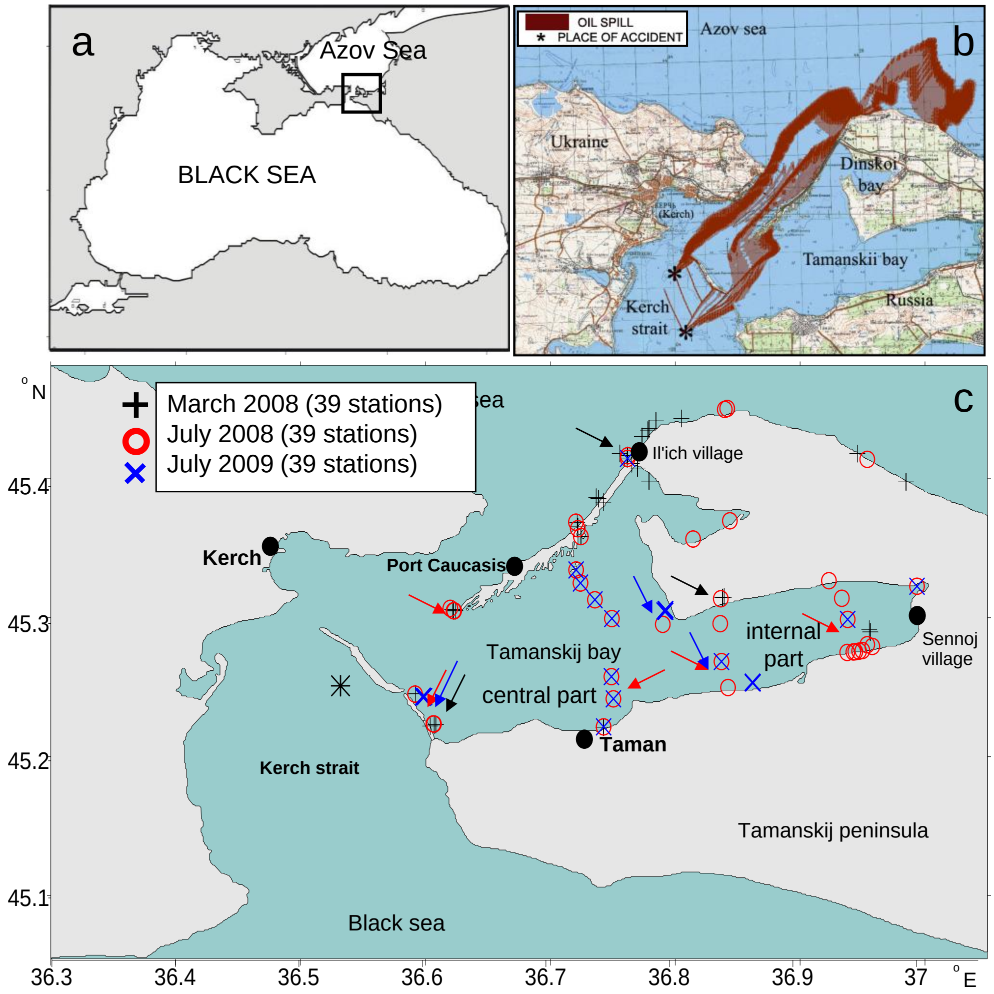

Figure 1. The region of study (a); the Volgoneft-139 wreck place and oil-spill distribution (Ovsienko *et al*., 2008) (b); and the map of stations sampled during the expeditions 2008-2009 (c). The biomarker sampling sites are marked by arrows; the color of arrows corresponds to the main stations map legend

were examined on several levels of organization (subcellular, tissue, and organismic levels).

Materials and Methods

The study area is located in Krasnodar Region of the Russian Federation. Each of three expeditions collected the samples in 39 stations (Figure 1c).

Samples of bottom sediments and mussels for biomarker analysis were collected in March 2008, July 2008 and July 2009 from the rubber boat with diving equipment. The samples of upper 30-50 mm of bottom sediments were taken manually by common plastic sampler with inner diameter 40 mm to the plastic zip-bag. Samples were stored at -20°C prior to analysis of hydrocarbon concentration, and other samples were dried at a room temperature grain size structure analysis of the sediments.

The grain size structure analysis was performed by separation of the samples with different size sieves.

Organic Carbon Analysis

100–150 g of sediment sample taken from each station was dried at 60°C, ground and homogenized. Total organic carbon (TOC) and carbonates contents were determined in dry sediment samples on the GC-2010 High Performance Capillary Gas Chromatograph (Shimudzu Co.) in the Laboratory of Ocean Chemistry of P. P. Shirshov Institute of Oceanology of RAS.

Hydrocarbons (HC) concentration was assessed in the bottom sediments with gravimertrical method.

Normal Alkanes Analysis

The normal alkanes (n-alkanes) were isolated from dry powdered sample by 45 min hexane extraction in Branson 1210 ultrasonic bath preceded by 15 min degassing. The extract obtained was filtered through GF/F glass fiber filters under vacuum. The solvent was evaporated at 35°C in Yamato RE-52 vacuum rotor vaporizer. To remove dissolved sulfur, the obtained extract was passed through an activated metallic copper column, evaporated in a nitrogen flow and stored in a refrigerator at +5°C until instrumental analysis. Further determination of n-alkanes contents in the extracts was carried out on Shimadzu GCMS-QP 5050 chromatographic mass spectrometer (Shimudzu Co.) in the Ocean Chemistry Laboratory of P. P. Shirshov Institute of Oceanology of RAS.

Biomarker Study

Mytilus galloprovincialis, shell length 12–18 mm, for analysis were sampled by scuba divers and processed during two hours after sampling.

The histological examination (30 mussels taken at each site) was performed as described in Kolyuchkina and Ismailov, 2007, 2011.

Micronucleus Assay: Aliquots of 0.1 ml hemolymph from each of 10 mussels per site, were placed on slides and left for 15 min in a humidified chamber at a room temperature allowing hemocytes to settle down, next the slides were fixed in 2.5% glutaraldehyde for 5 min, rinsing with PBS, and stained with Gimza dye. The slides were scored under the Olympus microscope at 900X magnification. On each slide, 500 cells were counted (i.e. 5000 cells per site).

Shell Abnormalities: The shells of all mussels sampled from each site (from 21-150 animals) were studied visually to assess some deformation or disturbances in calcification process.

Statistical Data Processing

The software package Statistica 7.0 was used. To assess the pairwise similarity/dissimilarity of the data (compared to the control and neighboring values), the Mann–Whitney nonparametric U-test ($P<0.05$) was used.

Results and Discussion

The main results of the sediment grain size analysis, the organic carbon (Corg) analysis, and also hydrocarbon concentration and n-alkanes distribution analysis were presented in Table 1.

The Sediment Grain Size Analysis

The main fraction in the samples taken during all three expeditions is fine sand with mean grain size 0.1-0.25 mm, and alevropelit (AP) with grain size lower than 0.01 mm (Table 1). The AP level varied from 0.50 to 98.64%. The high level of AP (5-50%) was found in shallow regions with slow hydrodynamic environment: overflow land of Taman and Dinskoi bays, a number of stations in Tamanskij bay in the middle depth in *Zostera marina* belt (Belyaev *et al*., 2009) and also sandy beaches of Kerch strait and Azov sea (Figures 2c, 2d). The maximal AP levels were found in internal and central parts of Tamanskij bay deeper than 3.5 m, and in one shallow water station in the apex part of Dinskoi bay. (Figures 2c, 2d). The significant positive correlation of AP level and sampling depth was found from depth 1.5 m and deeper (R^2=0.63; $P<0.05$). The second maximum of AP level was found on depth 0.5-1.5 m.

The similar distribution of AP was found in 1979 by Shnyukov and Palanskij (Shnyukov and Palanskij, 1979). They also outlined alevritic and silt bottom sediments in the central and internal parts of Tamanskij bay. However they observed the sandy sediments in the northern part of central part of Tamanskij bay and in the north-western part of

Table 1. Organic carbon, alevropelit, hydrocarbons and n-alkanes distribution in bottom sediments, and the main characteristics of n-alkanes

Expedi-tion date	Item	C org, %	Alevropelit, %	HC, mg/g	n-alkanes, ug/g	□□C_{10}-C_{22})/ □□C_{23}-C_{40})	CPI	i-C19/ i-C20
29 February–8 March 2008	Mean	0.63	15.13	0.161	3.46	2.91	1.52	1.26
	Max	3.28	84.82	0.111	11.60	9.36	3.28	3.44
	Min	0.05	0.50	0.009	0.80	0.27	0.91	0.38
	SD	0.82	21.73	0.256	2.81	2.32	0.71	0.45
	Med	0.17	7.84	0.063	2.70	2.28	1.16	1.27
7-29 July 2008	Mean	2.09	29.04	0.269	3.12	0.94	1.74	1.20
	Max	5.01	96.08	0.156	17.31	2.95	5.01	3.50
	Min	0.02	0.77	0.240	0.17	0.28	0.76	0.38
	SD	1.36	31.68	0.332	4.11	0.58	0.92	0.56
	Med	2.08	20.58	0.117	1.30	0.98	1.39	1.22
03-10 July 2009	Mean	1.25	32.73	0.290	1.51	0.87	3.30	0.84
	Max	3.01	98.64	3.760	10.77	2.12	5.93	2.05
	Min	0.02	7.43	0.010	0.17	0.04	1.03	0.07
	SD	0.80	27.35	0.910	2.02	0.48	1.28	0.48
	Med	1.01	22.09	0.040	0.83	0.87	3.21	0.84

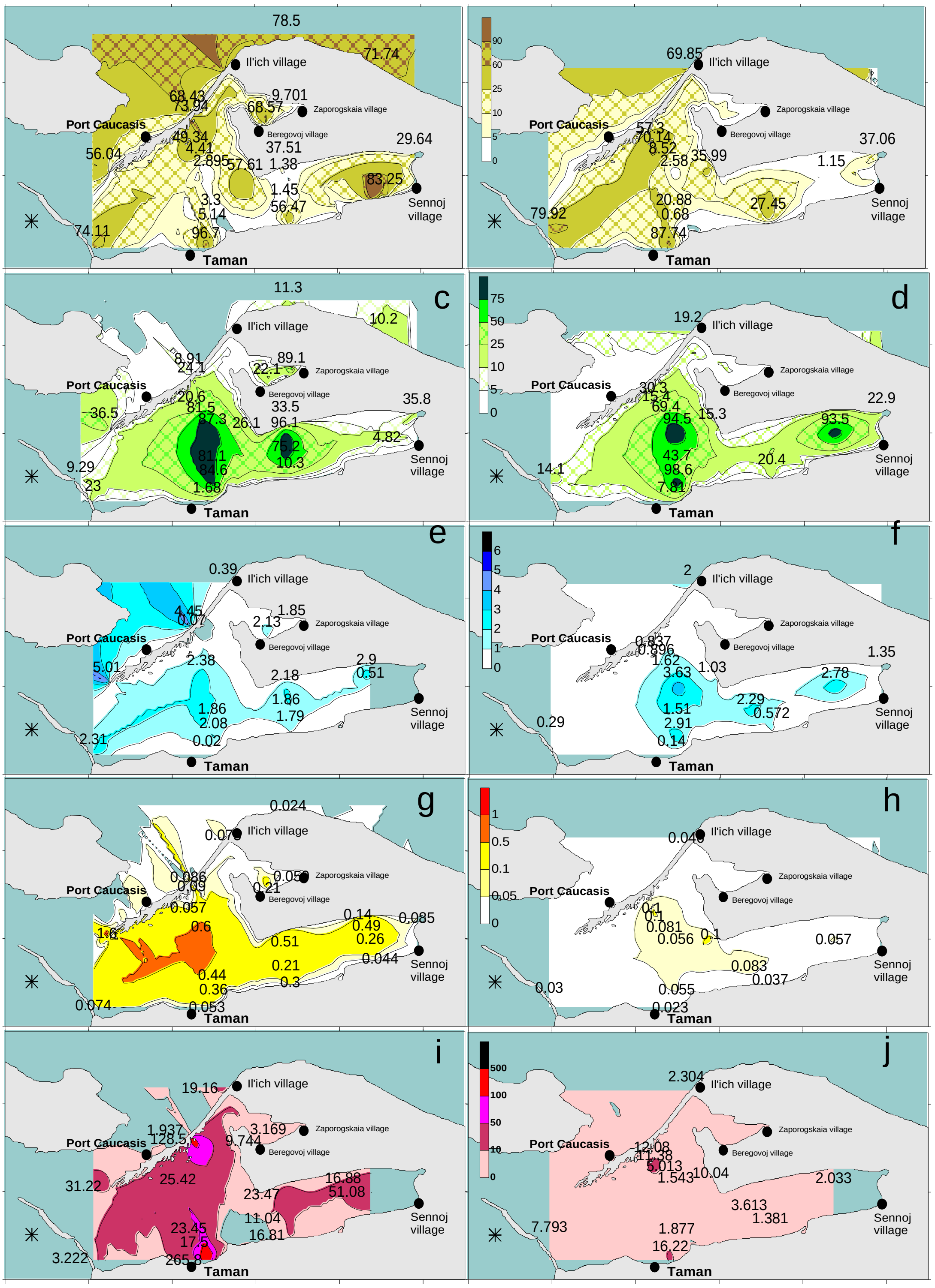

Figure 2. The bottom sediments characteristics spatial distribution in July 2008 (a, c, e, g, i) and July 2009 (b, d, f, h, j): the percent of sand (a, b);the percent of alevropelit (c, d); the percent of organic carbon (e, f); the hydrocarbons level, mg/g (g, h); and the relative enrichment of organic matter by hydrocarbons, mgHC/gCorg (i, j). The Volgoneft-139 shipwreck marked by arrow

Dinskoi bay (figure 2a, 2b). Currently the sediments of these areas consist of silt sand with low alevropelit level and the high level of detritus, and the sandy bottom sediments (60% grains with 0.05-2.00 mm size) were found to have sparse distribution and associated only with coastal zones (in the zone of connection of Tamanskij bay at the end of Tuzla spit and in one place in the south internal part of Tamanskij bay, figure 2c, 2d). Such considerable decreasing of sand in bottom sediments could be owing to change of the hydrophysical and lithodynamic processes in the Kerch strait and Tamanskij bay after Tuzla spit renewal (Brjancev, 2005; Lomakin and Spiridonova, 2010). Thus, under the south wind directions the Black Sea waters were found to come to Tamanskij bay not from Tuzla gully, but from Pavlovskaja narrowness. As a result in such periods the cyclonic type of water circulation in the bay changed to anticyclonic, which promoted the suspension accumulation in the bay and correspondingly its silting (Ovsienko *et al*., 2008). The silting could lead to increasing in organic matter (OM) accumulation and preventing of aerobic OM bacterial destruction due to reducing conditions (Shkapenko *et al*., 2007).

Organic Carbon (C_{org}): The organic carbon (C_{org}) level in the 2008-2009 bottom sediment samples varied from 0.02 to 5.01%, the mean value was equal to 1.17±1.07% (table 1). Such level corresponded to Corg values at the 30-50 m depth in Black sea North Caucasian coast, the mean value of C_{org} level at this region in 2001-2006 was 0.42±0.3% (Chikina, 2009). It indicates the difference of the organic carbon concentration in the study area. The maximal concentrations of organic carbon (3-5%) were found in the shallow coastal marshy with bottom sediments consisted of plants debris, and also for the internal and central part of Taman bay (figure 2e, 2f), associated with high AP level (figure 2c-f,). Generally, the organic carbon level increases with depth (R^2=0.45, P<0.05) and AP level (R^2=0.56, P<0.05; figure 3a). But the maximal values of organic carbon were associated with relatively low AP level and different depths and corresponded to samples enriched by HC, originated presumably from oil-spill (see n-alkanes analysis, figure 4g).

The organic carbon content in the sediment samples decrease from 2.09±1.36% in July 2008 to 1.28±0.85% in July 2009. However, generally the character of spatial distribution of the Corg was not changed (figure 2e, 2f).

Hydrocarbon (HC) Concentration: The mean HC concentration in the bottom sediments of Kerch strait in 2005 before the catastrophic residual oil-spill was equal to high values: from 0.110 mg/g (min value 0, max value 0.551 mg/g; Sovga *et al*., 2009) to 0.398 mg/g (Eremeev *et al*., 2003). This concentration exceeds European standard (0,1 mg/g).

HC concentration observed in the bottom sediments samples in March 2008 varied from 0.009 mg/g to 1.106 mg/g. The maximal concentration of HC, what exceeds background concentration three-tenfold, was found in the sandy coast of the Chushka spit on the side of the Kerch Strait (0.311 mg/g), in the shallow water meadows with silty sand bottom near end (0.729 mg/g) and in the middle part of spit (1.106 mg/g), and in the inner part of Tamanskij bay near Sennoi village in silty sand bottom (0.888 mg/g).

During the second expedition the values of HC bottom sediments concentration were equal to 0.269±0.333 mg/g, and maximal came to 1.564 mg/g, detected also near end of Chushka spit in samples of silty bottom sediments enriched in organic matter (figure 2g, 2h). The high concentration of HC, what exceeds background concentration at least twofold, was found also in the same zone as during previous observation in silty sand bottom sediments: in the meadows near Chushka spit (0.769 mg/g), in the inner part of Tamanskij bays near Sennoi village (0.489 mg/g). However the high level of HC (0.300-0.600 mg/g) was observed also in the central part of Taman bay adjacent to Kerch strait in silty bottom sediments, presumably affected by oil-spill (figure 2g).and was not studied during previous expedition owing to storm

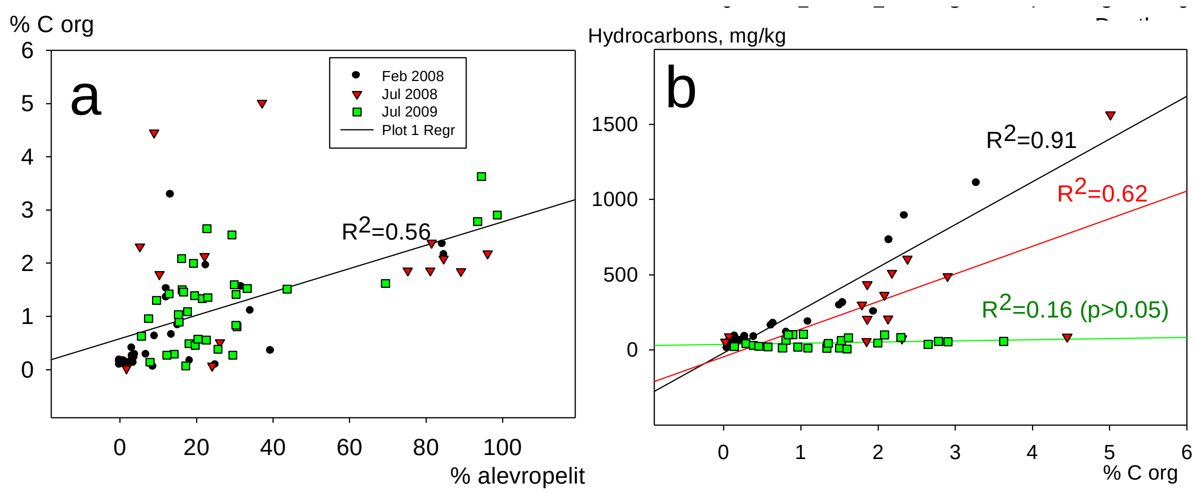

Figure 3. Correlation of the organic carbon level with alevropelit (a) and correlation hydrocarbons concentration with organic carbon level (b) in all studied bottom sediment samples. Each expedition samples were marked by corresponding colour: the black circles – March 2008 samples; the red triangles – July 2008; the green squares – July 2009.

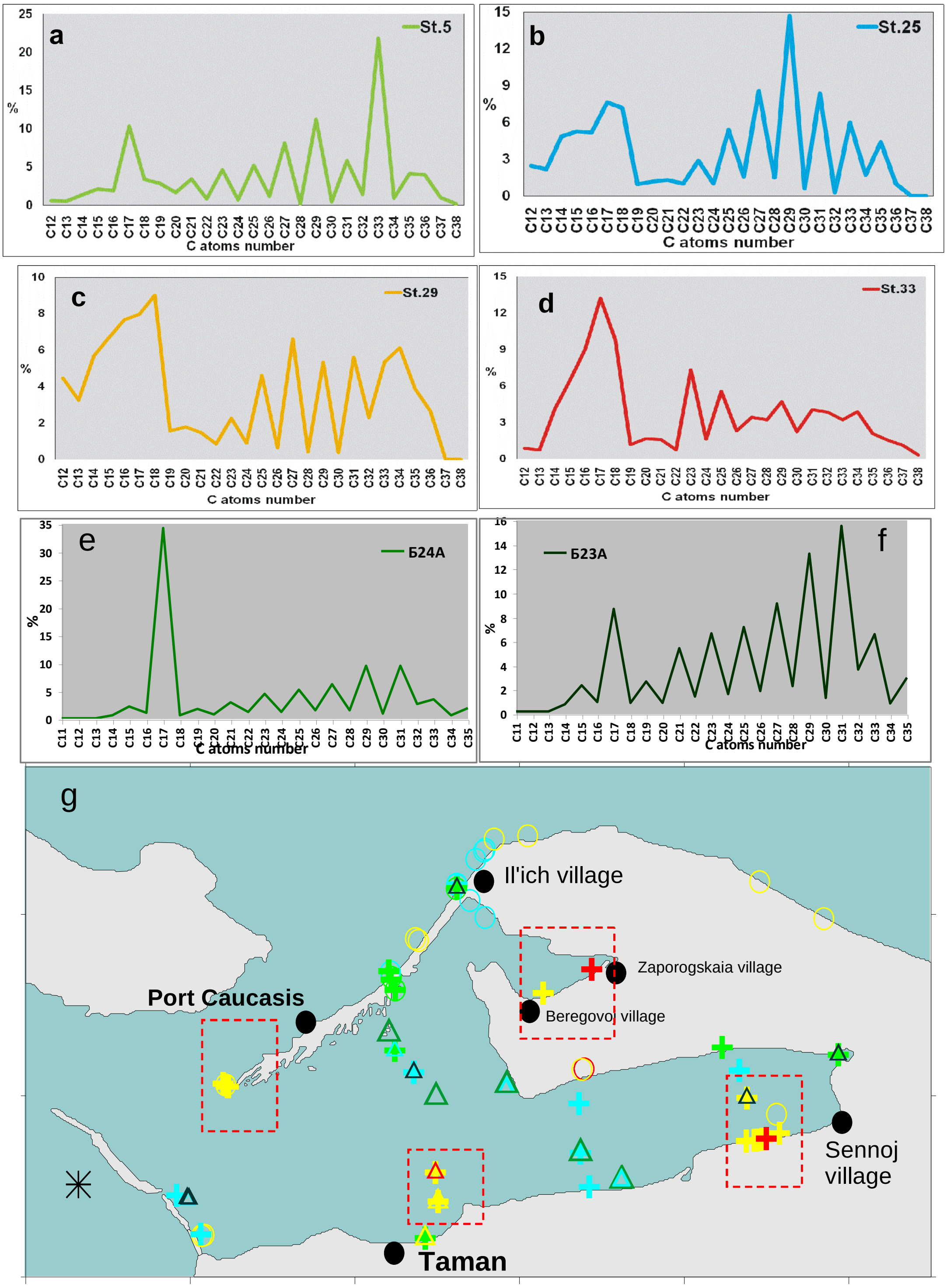

Figure 4. Nomal alkanes distribution types (a-f) in 2008 (a-d) -2009 (b-f) samples and spatial distribution pattern of them (g). (a) – I type, (b) – II type, (c) – III type, (d) – IV type; (e) – I a type, (f) – I b type. The circles marked stations of March 2008 expeditions, crosses – July 2008, triangles – July 2009. The n-alkanes distribution type marked by color, corresponding the distribution graphs color: bright green – I (2008), green – I a, dark green – I b, blue -II, yellow – III, red – IV. Regions revealed polluted marked by red squares.

weather.

During the third expedition concentration of HC in bottom sediments was reduced to 0.055±0.034 mg/g, and did not exceed 0.104 mg/g (figure 2h), except two stations. The first station was located in the central part of Tamanskij bay (the silty bottom sediments) and HC concentration was equal to 3.38 mg/g. The second one was from apex part of Tamanskij bay with silty bottom sediments and HC level equal to 3.76 mg/g. The high level of HC in the first station could be due to oil-spill, but second station was isolated from spilled area and its pollution could be attributing to local source pollution. Generally, the spatial distribution of HC in July 2009 was mosaic and did not correspond to the oil-spill. In the whole, the decrease of HC concentration to level lower than Kerch strait background values and European standards were observed by July 2009.

The hydrocarbons concentrations in bottom sediments positively correlated with organic carbon level only in 2008 samples (figure 3b). At the same time the mean relative enrichness of organic matter by hydrocarbons significantly decreased in 2009 as against to concentration in March and July 2008, and was equal to 6.27±4.86 mg HC/g C_{org}, 38.20±37.69 mg HC/g C_{org} and 40.53±65.05 mg HC/g C_{org} correspondingly (figure 2i, 2j). The maximal values of relative enrichness of organic matter by hydrocarbons were observed in silty sand bottom sediments in the central and internal part of Taman bay near the settlements and in March 2008 in the sandy bottom sediments of the Kerch strait and Azov Sea coastal zones, exposed to the residual oil-spill. These fasts seem to be an evidence of significant decreasing of samples with oil-spilled sediments in 2009, the detailed analysis of origin of organic matter was made by investigation of the n-alkanes distribution.

n-Alkanes Distribution

The content and structure of n-alkanes was assessed in 103 bottom sediments samples taken during three expeditions. The main data was presented in Table 1. The n-alkanes distribution relative to hydrocarbon chain length showed the maximum in C_{12} in some samples. This effect is obviously related to the pollution. This pollution most probably occurs during sampling, transporting or conservation of samples. It is unlikely that the pollution occurs during sample examination, as the simultaneous nalysis of the samples taken in another region did not reveal any pollution traces. In some samples the maximums of C_{11}-C_{14} were corrected to facilitate the significant results of n-alkanes distribution.

The mean concentration of n-alkanes in samples, taken in 2009, was equal to 1.5 ug/g, it was lower than one, observed in previous year (2.3 мкг/г ug/g). In addition the frequency of samples with anomalous high n-alkanes level decreased from five in July 2008 to one in 2009. The short-chain HC to long-chain HC ratio and CPI index were revealed the mainly terrigenous origin of organic matter of Taman and Dinskoi bays. The pristine/phytane ratio pointed on oxidative environment of organic matter formation. In the whole, the n-alkanes samples spectrum was more regular in 2009 than 2008 samples.

Analysis of aliphatic hydrocarbons in the bottom sediments samples taken in 2008 indicates the presence of four types of hydrocarbons in organic matter (Belyaev *et al*., 2009; Figure 4):

I. mixed terrigenous-planktonogenous origin without pollution OM

II. the main part of aliphatic hydrocarbons was generated by received OM destruction

III. mixed matter of natural origin with the traces of oil pollution

IV. highly oil-products polluted OM.

During 2009 study only one sample could be rating to *fourth type*, located in the central part of Taman bay with silty sand bottom sediments and characterize by anomalous high HC level and. Presumably it could be through residual oil-spill. The *third sediment type* almost was not observed during third expedition. Only two stations, the first one at 4,2 m depth with silty sediments and second one at 1 m depth with sandy bottom sediments, near Taman city in the central part of Taman bay with could be suppositively related to this type. Consequently the distinct appearance this type in 2008 was connected only to the residual oil-spill. Detailed analysis of aliphatic hydrocarbons revealed that hydrocarbon pollution originates mainly from long-term anthropogenic impact on this area, not from the oil-spill. Therefore in spite of the background chronic anthropogenic pollution, the self-purification processes take place in the study areas.

In 2009 the main type of organic matter was also the *second one* – bacterially destructed matter. The sediments of such type were presented in all regions of study basin on shelly and sandy bottom sediments. The main characteristics of this type were: (a) the relatively decreased n-alkanes level in bottom sediment (0.89 ug/g), (b) the prevalence of short-chain HC ((C_{10}-C_{22})/C_{23}-C_{40}) – 1.26), (c) reductive environment of organic matter formation (the mean pristine/phytane – 1.20), (d) the presence, but not prevalence, of terrigenous component of in organic matter. Presumably such type was formed in local zones of accumulation of organic debris in zostera meadows. Where the stagnant anoxic conditions formed owing to debris accumulation, and such conditions were favorable for bacterial destruction processes.

In the *first* type of organic matter there were revealed two subtypes: the first one (I.I) was organic matter with distinct C_{17} peak sampled in silty sand bottom, and second one – with prevalence of terrigenous HC and also presented C_{17} peak. The alkane C_{17} is a typical marker of sapropel in brackish water basins and possibly may appear as the result of bottom vegetation decay (e.g. zostera which is common in the study area). The second subtype (I.II), which was observer either in silty or in sandy bottoms, characterized by oxidative environment of organic matter formation (the mean pristane/phytane –0.45), predominance of odd HC and absolute dominating by odd homologues (CPI – 4). Thus, the three types of n-alkanes distribution were revealed in

Tamanskij and Dinskoi bays ecosystems: terrigenous, autochthonous and microbial genesis. The main part of observed n-alkanes was presented by homologous C17, which incident for some macrophytes.

The spatial distribution of n-alkanes types is presented in Figure 4. The significant correlation of pollution level in samples and settlements was observed and confirmed the previous work results (Belyaev *et al*., 2009). The more polluted samples were taken from coastal zones near Taman city, Sennoj village, Port Caucasis, Zaporogskaja village (in the Dinskoi bay apex) during all three expeditions. The internal part of Tamanskij bay and apex part of both bays were observed heavy polluted by local pollution sources.

Biomarkers Assay

Histological Examination: During the first expedition mussels were found stressed and emaciated: a lot of brownish lipofuscin-like grain was found in digestive epithelium. The mollusks from stations near North end of Chushka spit had vacuolization of basophilic cell from digestive gland acinuses in 100%. The inflammation in the connective tissue was observed. Increasing by the first order of magnitude in granulocytes number in connective tissue and oocytes degeneration (in 67% case) was observed of these animals. Mussels collected from the internal part of Tuzla spit were found to have oocytes vacuolization in 72% case. But mussels sampled from internal part of Taman bay were not found to have any histopathology.

However, during the second expedition histological analysis indicated good health state of mollusks from all control and polluted stations (as was assessed by chemical assay).

Micronucleus Assay: The micronucleus test was made only during the third expedition, and micronucleus count in molluscan hemocytes (0.33±0.08%) was equal to control values, normal or mussel from clean environment (Dailianis *et al*., 2003) (Figure 5).

Shell Abnormalities: The background level of shell abnormalities of *Mytilus* sp. was found to equal to 3% (Sunila, 1987).

Shells abnormalities were found increased and was equal to 21.0% (N=98) of mussels sampled in July 2008 (curvatures in nonrelevant shell parts, exfoliations, etc). Only 12.0% animals had exfoliations of latest shell layer formed in last year. During the third expedition in the Tuzla spit region 154 mussels (40-50 mm shell length) were observed, and 14.3% of animals were found to have abnormalities. 112 mussels from the internal part of Taman bay were studied, and 10.7% shell abnormalities were found. 28 mussels from the central part of Taman bay were study, and there was found only 7.14% mussels with shell abnormalities. The shells violations could be consequences of toxic oil hydrocarbons influence.

Conclusions

Within recent 20 years the ecosystems of Kerch strait and adjacent Tamanskij and Dinskoi bays suffered at least two catastrophic events: the Tuzla spit renewal and the Kerch strait oil spill. The consequences of the first event were reduction of sandy bottom sediments and enhancing silting and debris accumulation in study areas. The second accident led to high level pollution of shoreline of Kerch strait and Azov Sea and central stagnated regions of Tamanskij and Dinskoj bays. However the studied ecosystems have the high potential of self-purification. Thus, active biodegradation processes cope with hydrocarbons impact now. The samples with oil pollution traces, clearly revealed in 2008, were not found during 2009 observation. The general n-alkaned decrease and declining of samples with

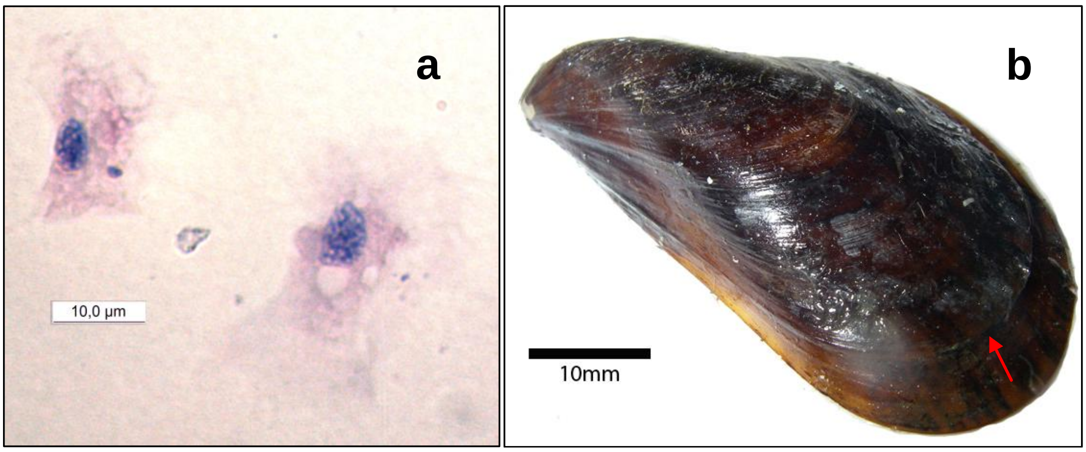

Figure 5. Microphotographs of hemocytes with micronucleus and control hemocytes (a) and photo of *Mythilus galloprovincialis* shells with abnormalities.

anomalous high n-alkanes values were found in 2009. The bottom invertebrates were heavy affected during oil spill, but recovery of physiological state was occurred already next summer season.

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