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ORIGINAL PAPERS

Fatty-Acid and Stable-Isotope Compositions in Shallow-Water Bivalve Mollusks and their Food

V. I. Kharlamenko[a, *] and S. I. Kiyashko[a, b]

[a] *Zhirmunsky Institute of Marine Biology, National Scientific Center of Marine Biology, Far Eastern Branch, Russian Academy of Sciences, Vladivostok, 690041 Russia*

[b] *Far Eastern Federal University, Vladivostok, 690090 Russia*

**e-mail: kharlvi@mail.ru*

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Abstract—We conducted a comparative analysis of the fatty acid (FA) composition and the ratios of stable isotopes of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) in soft tissues of ten species of bivalve mollusks collected simultaneously on adjacent biotopes in shallow Vostok Bay (the Sea of Japan). Comparison of the FA composition of the lipids of digestive gland and all soft tissues showed that the percentages of C_{16} and C_{18} marker FAs were greater in the digestive gland and the levels of marker C_{20} and C_{22} FAs were, in most cases, higher in soft tissues. According to the results of cluster analysis and principal component analysis, four groups of samples were identified with a similarity of the FA composition of more than 80% within groups. The carbon stable-isotope ratios varied within very wide limits in the studied species of bivalves; the range of $\delta^{13}C$ variations was 8.1‰. The range of $\delta^{15}N$ variations was much smaller, 2.5‰. Two pairs of species of mollusks (*Saxidomus purpurata–Protothaca euglypta* and *P. jedoensis–Diplodonta semiasperoides*) did not differ in the values of both $\delta^{15}N$ and $\delta^{13}C$, the remaining species differed in at least one of these parameters. The greatest similarity of the FA composition and stable-isotope ratios was found in species that inhabit similar substrates, except *Macoma irus* and *D. semiasperoides*. Particularly marked differences in the FA composition and stable-isotope ratios were found between a filter-/surface deposit-feeder *M. irus* and filter-feeders *Arca boucardi* and *Mytilus coruscus* that live next to this species.

Keywords: bivalves, fatty acids, stable isotopes, sestonophages, Vostok Bay, Sea of Japan

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INTRODUCTION

The trophic position of bivalve mollusks that participate in the transformation of tremendous amounts of suspended organic matter (SOM) in coastal marine ecosystems brings particular attention to the study of their food sources and potential food selectivity. There are three main approaches to analyzing the food of bivalves: study of the contents of digestive tract (microscopic or metagenomic analysis), determination of the stable-isotope ratios in soft tissues, and analysis of the marker fatty acid (FA) composition in different organs of a mollusk [24]. In recent years, studies of the feeding of bivalves have more often used a combination of the two last approaches as they complement each other well [5, 25, 32, 33].

When analyzing the marker FA composition, the different distribution of lipid classes among organs of bivalves may be of much importance [8]. In trophic studies, the lipid extract of entire soft tissues or only of the digestive gland of bivalves is used for analysis of the marker FA composition, assuming that the composition of digestive gland better reflects the composition of freshly assimilated food [22]. When using the marker FA analysis in the comparative study of the trophodynamics of communities of invertebrates of different systematic groups, it would be logically most feasible to analyze the marker FA composition of soft tissues of an entire animal. It is also desirable to know in what manner the FA composition of digestive gland differs from that of soft tissues. The single comparison that is known to us of the FA composition of digestive gland and soft tissues in the bivalve *Patinopecten yessoensis* demonstrated the similarity of their composition [3]. If there are differences in the FA composition of digestive gland and soft tissues, it is important to determine which of the types of tissue sample is more adequate to reflect the food composition. To this end, other methods of analysis should be employed. The most appropriate method for revealing the food sources of bivalves is considered to be the analysis of stable-isotope ratios. Microscopic data are less informative, because the main component of the digestive tract contents in most bivalves is an unidentifiable mass of detritus [7].

The aims of the present study was to search for differences in the FA and stable-isotope composition in

Table 1. The characteristics of habitats and samples of bivalves collected in Vostok Bay, the Sea of Japan

Species	Substrate	Depth, m	Shell length, mm	Number of samples	
				FAs* (DG/ST)	stable isotopes
Family Mytilidae *Mytilus coruscus*	Gravel	0.5	28–92	5/5	5
Family Ostreidae *Crassostrea gigas*	Silty sand	3	110–300	5/5	5
Family Arcidae *Arca boucardi*	Gravel	1	38–60	5/5	5
Family Tellinidae					
Macoma incongrua	Silty sand	1	22–30	4/5	5
M. irus	Pebbles, sand	1	18–45	5/5	5
Family Veneridae					
Protothaca euglypta	Boulders, pebbles, sand	1	37–44	5/5	5
P. jedoensis	Boulders, pebbles, sand	1	18–35	4/5	4
Saxidomus purpurata	Boulders, pebbles, sand	1	70–86	4/5	5
Family Ungulinidae *Diplodonta semiasperoides*	Boulders, pebbles, sand	1	14–18	3/4	4
Family Myidae					
Mya japonica	Silty sand	3	87–108	5/5	5

*FA, fatty acid; DG, digestive gland; ST, all soft tissues.

marine bivalves; these data can be useful in characterizing the food sources and the tropic position of the investigated species of invertebrates. A comparative analysis of the FA composition revealed differences in the percentages of marker FAs in soft tissues and digestive gland among ten species of bivalve mollusks that were collected simultaneously and represent a significant part of the taxonomic diversity of this group on adjacent biotopes within a limited area in the open shallow-water Vostok Bay (the Sea of Japan). Based on the marker FA composition, the samples were separated into groups using multiple-factor analysis; the differences between the groups in the FA composition were shown. The ratios of stable isotopes of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) in the tissues of the investigated species of bivalves were analyzed; the data on the FA composition and stable-isotope ratios were compared. The results we obtained allowed us to suggest that a diversity of food sources are consumed by species of bivalves that coexist in a shallow-water habitat.

MATERIALS AND METHODS

Ten species of bivalves from seven families were investigated: *Arca boucardi* Jousseaume, 1894; *Mytilus coruscus* Gould, 1861; *Crassostrea gigas* (Thunberg, 1793); *Saxidomus purpurata* (Sowerby II, 1852); *Protothaca euglypta* (Sowerby III, 1914); *Protothaca jedoensis* (Lischke, 1874); *Mya japonica* Jay, 1857; *Macoma incongrua* (Martens, 1865); *Macoma irus* (Hanley, 1844); and *Diplodonta semiasperoides* Nomura, 1932. Bivalves were collected using scuba on October 20–23, 2011, in Vostok Bay (the Sea of Japan) at depths of 0.5 to 3.0 m (Table 1). Each sample was taken from one individual.

For isotope analysis, muscle tissues (foot and mantle) were oven dried at a temperature of 60°C and ground with a pestle in an agate mortar. The isotope analysis was carried out at the Laboratory of Stable Isotopes (Pacific Institute of Geology, Far Eastern Branch, Russian Academy of Sciences), using a system coupling a FlashEA 1112 element analyzer, a ConFlo-IV interface, and a MAT 253 isotope mass-spectrometer (ThermoQuest, Germany). The relative content of heavy isotopes ^{13}C and ^{15}N in samples were determined in promille as the values of deviations $\delta^{13}C$ or $\delta^{15}N$ from a reference isotope composition. All values of $\delta^{13}C$ and $\delta^{15}N$ are given relative to international standards PDB (carbon) and AIR (nitrogen), respectively. Reference materials distributed by the International Atomic Energy Agency (Vienna, Austria) were used for calibration: IAEA CH-6, NBS-22, IAEA N-1, and IAEA N-2. For this series of samples, the replicability of results was checked by regular measurement of a running standard (one measurement of standard after each sixth measurement of samples). The running standard was a sample of homogenized dry mantle of the Pacific squid. The replicability was ±0.10‰ for $\delta^{15}N$ and ±0.06‰ for $\delta^{13}C$.

For analysis of the FA composition, the digestive gland or whole soft tissues of bivalves were sampled;

Table 2. Fatty acids that were used as markers of the food sources of the bivalves studied

FAs:	Main source of FAs*:
ΣC_{15}, C_{17} FA and 18:1n-7	Bacteria [26]
16:1n-7	Diatoms [9]
18:1n-9	Brown algae, dinoflagellates [17]
Σ18:2n-6 and18:3n-3	Green algae, seagrasses [17]; terrestrial organic matter [12]
18:4n-3	Brown algae [17]; dinoflagellates [23]; crypromonads, raphydophytes [14]
ΣNMI FA	Bacteria and subsequent own biosynthesis [16]
20:4n-6	Red and brown algae [17]; protozoans [29]
20:5n-3	Diatoms [9], red and brown algae [17]
22:4n-6	Benthic diatoms [19]
22:6n-3	Dinoflagellates [17]; zooplankton [14, 17]

* Sources of FAs are indicated according to the Reference numbers.

three to five samples of each type were taken (Table 1). A sample represented the entire digestive gland or 1–3 g of soft tissues. Samples were stored at a temperature of –20°C for 1–3 months. Lipids were extracted according to the method of Bligh and Dyer [11], using a mixture of chloroform : methanol (2 : 1). Fatty acid methyl esters were obtained by the method of Carreau and Dubacq [13] and then purified by preparative thin-layer chromatography in benzene. The 4,4-dimethyloxazoline (DMOX) derivatives of FAs were prepared according to the method of Svetashev [30]. FA methyl esters were analyzed on a Shimadzu GC 2010 Plus chromatograph (Japan) with a quartz capillary column (length, 30 m; inner diameter, 0.25 mm; Supelcowax 10; Supelco, United States). The column temperature was 205°C; the injector and detector temperature was 250°C; the carrier gas was helium. The analysis was run for 60 min. Mass spectrometry of methyl esters and DMOX was carried out on a Shimadzu GC-MS QP5050A mass spectrometer with an MDN-5S column (length, 30 m; inner diameter, 0.25 mm; Supelco). The initial column temperature (160°C) was elevated to 260°C at a rate of 2°C/min, and the column was analyzed at this temperature for another 25 min. The FA spectra were compared to those in the NIsoft tissues 2.0 library and the FA mass-spectral archive [31]. The data are given in percent of the total content of FAs (as a percentage).

Statistical analysis was performed using the Statistica 8 and Primer 6 program packages. The one-way analysis of the variance (ANOVA) with the Tukey test was used to analyze the significance of the differences ($p < 0.05$) of the mean values of parameters of FA composition and stable-isotope ratios between species and between groups of samples. A multivariate analysis was carried out on marker FAs with a concentration of more than 3% in at least one bivalve (Table 2). A cluster analysis of the similarity of bivalves by the marker FA composition and a principal component analysis were performed using the Bray–Curtis similarity index; the data were pre-log-transformed. The contribution of FAs in different groups of bivalves to similarity and dissimilarity between groups was estimated using the SIMPER program.

RESULTS

Fatty Acids

In all bivalves, except *D. semiasperoides* and *M. irus*, the total percentage of polyunsaturated fatty acids (PUFAs) exceeded the total percentage of saturated fatty acids (SFAs) or monounsaturated fatty acids (MUFAs). It was 27.3–47.6%, with the minimum value found in *D. semiasperoides* and the maximum in *C. gigas* (Table 3). Among PUFAs, 20:5n-3 was the predominant acid. The ratio of n-3/n-6 PUFAs ranged from 1.1 to 8.7, with the minimum in *D. semiasperoides* and the maximum in *C. gigas*. The percentage of SFAs was 24.8–32.9%, it was smallest in *C. gigas* and highest in *D. semiasperoides*. Among saturated acids, the percentage of 16:0 was greatest in all species. The percentage of MUFAs was 17.8–32.7%; the lowest value was noted in *P. euglypta* and the highest in *M. incongrua*. Among MUFAs, 16:1n-7 was predominant in all bivalves. The total percentage of bacterial FAs (15:0, *iso*-17:0, *anteiso*-17:0, 17:0, and 17:1n-8) varied from 1.9 to 7.3%. The minimum percentage of bacterial FAs was found in *C. gigas*, the maximum was found in *D. semiasperoides*. Among bacterial FAs, the percentages of *iso*-17:0, 17:0 or *anteiso*-17:0 FAs were highest. The percentage of nonmethylene-interrupted (NMI) fatty acids varied from 0.1 to 8.4%. 22:2(7,15) was the predominant NMI FA in most bivalves, 22:2(7,13) predominated only in *D. semiasperoides*. In bivalves of the genus *Macoma,* the percentage of NMI FAs was very low.

The main FAs (more than 10%) in digestive gland of *C. gigas* were 20:5n-3, 16:0, and 22:6n-3. These FAs in the same order dominated in *M. japonica* and *S. purpurata*; in *P. euglypta,* the percentage of 22:6n-3

Table 3. The percentage of fatty acids* (of the sum of fatty acids) in the digestive gland of bivalves (mean ± standard deviation; *n*, number of samples)

FA	*Mytilus coruscus*	*Crassostrea gigas*	*Arca boucardi*	*Macoma incongrua*	*Macoma irus*	*Protothaca euglypta*	*Protothaca jedoensis*	*Saxidomus purpurata*	*Diplodonta semiasperoides*	*Mya japonica*
	n = 5	*n* = 5	*n* = 5	*n* = 4	*n* = 5	*n* = 5	*n* = 4	*n* = 4	*n* = 3	*n* = 4
ΣSFAs	$29.2^{a, b} \pm 1.8$	$24.8^{b} \pm 1.6$	$27.7^{a, b} \pm 1.6$	$27.9^{a, b} \pm 3.3$	$31.5^{a} \pm 2.3$	$27.2^{a, b} \pm 3.0$	$31.5^{a} \pm 4.6$	$28.6^{a, b} \pm 3.2$	$32.9^{a} \pm 0.8$	$27.6^{a,b} \pm 1.4$
14:0 + TMTD**	$8.2^{a, b} \pm 2.3$	$6.3^{a, b} \pm 1.9$	$5.8^{a,b} \pm 0.4$	$6.4^{a, b} \pm 1.8$	$9.2^{a} \pm 0.9$	$6.1^{b} \pm 1.5$	$4.8^{b} \pm 1.5$	$7.3^{a, b} \pm 1.4$	$5.7^{a, b} \pm 0.7$	$6.8^{a, b} \pm 0.5$
16:0	18.3 ± 0.9	15.3 ± 2.0	17.7 ± 1.0	15.5 ± 2.2	17.1 ± 1.4	15.4 ± 1.9	18.5 ± 2.8	17.6 ± 1.1	19.5 ± 0.8	16.3 ± 0.9
18:0	$2.8^{d} \pm 0.6$	$3.2^{d} \pm 0.7$	$4.2^{d} \pm 0.7$	$6.1^{b, c} \pm 0.5$	$5.1^{c, d} \pm 0.5$	$5.7^{c} \pm 1.2$	$8.3^{a} \pm 1.0$	$3.6^{d} \pm 0.7$	$7.6^{a, b} \pm 0.6$	$4.6^{c, d} \pm 0.3$
ΣMUFAs	$23.8^{b} \pm 1.4$	$20.8^{b} \pm 1.0$	$22.4^{b} \pm 1.0$	$32.7^{a} \pm 2.1$	$31.7^{a} \pm 1.0$	$17.8^{c} \pm 3.2$	$20.7^{b} \pm 1.0$	$22.0^{b} \pm 1.6$	$24.1^{b} \pm 1.4$	$24.4^{b} \pm 1.2$
16:1n-7	$13.2^{a} \pm 2.1$	3.9c ± 1.3	$10.6^{a, b} \pm 1.3$	$9.0^{b} \pm 1.2$	$12.3^{a,b} \pm 1.6$	4.6c ± 1.5	4.0c ± 0.4	$10.8^{a, b} \pm 1.2$	4.5c ± 0.5	$8.9^{b} \pm 0.7$
18:1n-9	$2.9^{d} \pm 0.5$	$4.0^{c, d} \pm 1.2$	$2.9^{d} \pm 0.2$	$6.8^{b} \pm 0.4$	$6.9^{b} \pm 1.3$	$2.5^{d} \pm 0.2$	$5.3^{b, c} \pm 0.5$	$2.9^{d} \pm 0.3$	$9.3^{a} \pm 0.3$	$3.2^{d} \pm 1.0$
18:1n-7	$3.7^{b} \pm 0.4$	$5.8^{a} \pm 0.9$	$3.6^{b} \pm 0.5$	$3.9^{a, b} \pm 0.6$	$3.5^{b} \pm 0.3$	$5.5^{a} \pm 2.0$	$5.9^{a} \pm 0.6$	$3.2^{b} \pm 0.1$	$4.8^{a, b} \pm 1.1$	$3.5^{b} \pm 0.4$
20:1n-13	$1.0^{c} \pm 0.2$	$1.2^{c} \pm 0.5$	$2.1^{c} \pm 0.3$	$8.4^{a} \pm 1.5$	$5.9^{b} \pm 0.7$	$2.7^{c} \pm 0.7$	$3.0^{c} \pm 0.4$	$1.8^{c} \pm 0.4$	$3.0^{c} \pm 0.1$	$5.5^{b} \pm 0.4$
20:1n-9	$1.5^{a, b} \pm 0.2$	$0.8^{c} \pm 0.1$	$0.4^{e} \pm 0.1$	$1.2^{b} \pm 0.2$	$0.7^{c,d} \pm 0.4$	$1.3^{b} \pm 0.1$	$1.5^{a,b} \pm 0.1$	$0.4^{c, d} \pm 0.1$	$1.7^{a} \pm 0.1$	$1.8^{a} \pm 0.1$
20:1n-7	$1.5^{c} \pm 0.2$	$5.1^{a} \pm 0.7$	$2.9^{b} \pm 0.6$	$3.5^{b} \pm 0.6.0$	$2.5^{b} \pm 0.2$	$1.3^{c} \pm 0.3$	$1.0^{c} \pm 0.1$	$2.9^{b} \pm 0.5$	$0.9^{c} \pm 0.1$	$1.6^{c} \pm 0.2$
ΣNMIFAs	$2.1^{d, e} \pm 0.5$	$4.3^{c} \pm 0.7$	$3.1^{c,d} \pm 0.9$	$0.5^{e, f} \pm 0.1$	$0.1^{f} \pm 0.2$	$6.2^{b} \pm 1.7$	$6.4^{a,b} \pm 0.5$	$3.7^{c,d} \pm 0.9$	$8.4^{a} \pm 0.1$	$1.0^{e, f} \pm 0.1$
20:2(7,13)	$0.5^{b, c} \pm 0.2$	$0.1^{c} \pm 0.1$	$0.4^{b,c} \pm 0.1$	$0.5^{b, c} \pm 0.1$	$0.1^{c} \pm 0.2$	$0.1^{c} \pm 0.2$	$0.5^{b,c} \pm 0.1$	$0.2^{c} \pm 0.2$	$3.3^{a} \pm 0.1$	$0.3^{b, c} \pm 0.0$
22:2(7,13)	$0.1^{d} \pm 0.2$	$0.8^{c, d} \pm 0.1$	$0.8^{c,d} \pm 0.3$	–	–	$1.2^{b,c} \pm 0.3$	$2.0^{b} \pm 0.3$	$0.5^{d} \pm 0.3$	$3.5^{a} \pm 0.1$	$0.4^{d} \pm 0.1$
22:2(7,15)	$1.5^{b, c} \pm 0.4$	$3.4^{a, b} \pm 0.6$	$1.9^{b, c} \pm 1.1$	–	–	$4.8^{a} \pm 1.5$	$4.0^{a,b} \pm 0.2$	$3.0^{b} \pm 0.6$	$1.5^{b,c} \pm 0.1$	$0.4^{c} \pm 0.1$
ΣPUFAs	$42.2^{a, b} \pm 2.8$	$47.6^{a} \pm 0.5$	$43.7^{a} \pm 2.5$	$32.8^{c, d} \pm 2.3$	$30.5^{c, d} \pm 1.9$	$44.0^{a} \pm 2.8$	$35.9^{b, c} \pm 5.4$	$41.2^{a, b} \pm 3.4$	$27.3^{d} \pm 3.6$	$43.9^{a} \pm 2.7$
16:4n-1	$0.7^{b} \pm 0.3$	0	$1.2^{a} \pm 0.2$	–	–	$0.1^{c} \pm 0.2$	$0.1^{c} \pm 0.1$	$0.6^{b} \pm 0.4$	$0.1^{c} \pm 0.1$	$0.1^{c} \pm 0.1$
Σn-6 PUFAs	$4.9^{e} \pm 0.6$	$4.9^{e} \pm 1.5$	$4.9^{e} \pm 0.2$	$8.3^{b, c, d} \pm 1.4$	$10.8^{a, b} \pm 0.6$	$7.3^{c, d, e} \pm 1.2$	$9.8^{a, b, c} \pm 1.5$	$5.1^{e} \pm 0.4$	$12.7^{a} \pm 1.9$	$6.5^{d,e} \pm 1.7$
18:2n-6	$2.7^{a, b} \pm 0.3$	$1.8^{b, c} \pm 0.7$	$1.9^{b, c} \pm 0.2$	$3.2^{a, b} \pm 0.5$	$3.2^{a, b} \pm 0.4$	$0.8^{c} \pm 0.2$	$1.7^{b,c} \pm 0.3$	$1.7^{b, c} \pm 0.3$	$3.8^{a} \pm 0.4$	$1.9^{b, c} \pm 1.9$
20:2n-6	$0.6^{c} \pm 0.1$	$0.3^{c} \pm 0.1$	$0.5^{c} \pm 0.1$	$0.5^{c} \pm 0.1$	$0.5 \pm {}^{c}0.1$	$1.1^{b} \pm 0.2$	$1.5^{a} \pm 0.1$	$0.5^{c} \pm 0.1$	$1.0^{b} \pm 0.2$	$0.9^{b} \pm 0.1$
20:4n-6	$1.6^{d} \pm 0.3$	$2.5^{c, d} \pm 0.7$	$2.5^{c, d} \pm 0.2$	$4.1^{b} \pm 0.9$	$6.7^{a} \pm 0.6$	$3.9^{b,c} \pm 0.9$	$4.8^{b} \pm 1.1$	$2.6^{c, d} \pm 0.5$	$3.8^{b, c, d} \pm 0.5$	$3.2^{b, c, d} \pm 0.7$

Table 3. (Contd.)

FA	*Mytilus coruscus*	*Crassostrea gigas*	*Arca boucardi*	*Macoma incongrua*	*Macoma irus*	*Protothaca euglypta*	*Protothaca jedoensis*	*Saxidomus purpurata*	*Diplodonta semiasperoides*	*Mya japonica*
	$n = 5$	$n = 5$	$n = 5$	$n = 4$	$n = 5$	$n = 5$	$n = 4$	$n = 4$	$n = 3$	$n = 4$
22:4n-6	$0.1^{c} \pm 0.1$	$0.3^{b,c} \pm 0.1$	–	$0.5^{b} \pm 0.5$	$0.4^{b,c} \pm 0.1$	$1.4^{b} \pm 0.6$	$1.8^{b} \pm 0.3$	$0.4^{b,c} \pm 0.1$	$4.0^{a} \pm 2.0$	$0.5^{b,c} \pm 0.1$
Σn-3 PUFAs	$36.6^{b} \pm 2.2$	$42.7^{a} \pm 1.8$	$37.6^{a,b} \pm 2.3$	$24.6^{c,d} \pm 1.2$	$19.7^{d,e} \pm 1.7$	$36.7^{b} \pm 1.8$	$26.1^{c} \pm 4.3$	$35.5^{b} \pm 3.6$	$14.5^{e} \pm 1.6$	$37.3^{a,b} \pm 3.5$
18:3n-3	$2.2^{a,b,c} \pm 0.2$	$2.3^{a,b} \pm 1.4$	$2.6^{a,b} \pm 0.4$	$1.2^{b,c} \pm 0.4$	$1.7^{a,b,c} \pm 0.2$	$1.0^{c} \pm 0.3$	$1.7^{a,b,c} \pm 0.2$	$1.2^{b,c} \pm 0.3$	$1.7^{a,b,c} \pm 0.1$	$0.7^{c} \pm 0.2$
18:4n-3	$3.9^{b} \pm 0.5$	$3.9^{b} \pm 0.8$	$5.3^{a} \pm 0.8$	$0.9^{d} \pm 0.1$	$1.4^{d} \pm 0.4$	$2.1^{c,d} \pm 0.7$	$2.2^{c,d} \pm 0.4$	$2.8^{b,c} \pm 0.3$	$1.0^{d} \pm 0.1$	$1.9^{c,d} \pm 0.2$
20:5n-3	$16.2^{b,c} \pm 1.1$	$21.3^{a} \pm 2.8$	$17.9^{a,b} \pm 0.8$	$14.5^{c,d} \pm 0.8$	$10.8^{d,e} \pm 1.6$	$15.0^{c} \pm 1.5$	$9.9^{e} \pm 2.3$	$17.8^{a,b} \pm 2.6$	$4.0^{f} \pm 0.4$	$19.4^{a,b} \pm 1.6$
21:5n-3	$0.6^{c} \pm 0.2$	$0.9^{b,c} \pm 0.1$	$0.9^{b,c} \pm 0.1$	$0.6^{c,d} \pm 0.1$	$0.2^{d} \pm 0.2$	$1.2^{b} \pm 0.1$	$0.6^{c,d} \pm 0.1$	$0.9^{b,c} \pm 0.1$	$1.9^{a} \pm 0.3$	$1.0^{b,c} \pm 0.1$
22:5n-3	$0.7^{d} \pm 0.1$	$1.2^{b} \pm 0.1$	$0.7^{c,d} \pm 0.1$	$1.7^{a,b} \pm 0.5$	$1.0^{c} \pm 0.1$	$1.9^{a} \pm 0.5$	$1.4^{a,b,c} \pm 0.2$	$1.0^{c,d} \pm 0.1$	$1.1^{b,c,d} \pm 0.2$	$1.3^{a,b,c,d} \pm 0.1$
22:6n-3	$13.0^{a,b} \pm 1.2$	$13.2^{a,b} \pm 1.4$	$10.1^{b} \pm 1.6$	$5.7^{c} \pm 0.5$	$4.5^{c} \pm 0.5$	$15.6^{a} \pm 2.8$	$10.1^{b} \pm 1.5$	$11.9^{b} \pm 0.7$	$4.8^{c} \pm 1.3$	$13.1^{a,b} \pm 2.0$
ΣC_{15} and C_{17} FAs	$2.6^{c} \pm 0.2$	$2.5^{c} \pm 0.5$	$3.1^{c} \pm 0.3$	$6.2^{a,b} \pm 0.2$	$6.2^{a,b} \pm 0.6$	$4.9^{b} \pm 0.7$	$5.4^{a,b} \pm 0.2$	$4.7^{b,c} \pm 1.7$	$7.3^{a} \pm 1.4$	$3.2^{b,c} \pm 0.2$
15:0	$0.6^{c,d} \pm 0.1$	$0.6^{c,d} \pm 0.1$	$0.4^{d} \pm 0.1$	$1.7^{a} \pm 0.2$	$1.6^{a} \pm 0.2$	$0.7^{b,c} \pm 0.1$	$0.6^{b,c,d} \pm 0.2$	$0.7^{b,c} \pm 0.1$	$1.0^{b} \pm 0.1$	$0.6^{c,d} \pm 0.1$
iso-17:0	$0.5^{c} \pm 0.1$	$0.4^{c} \pm 0.1$	$0.4^{c} \pm 0.1$	$1.6^{b} \pm 0.1$	$1.9^{b} \pm 0.2$	$1.3^{b} \pm 0.2$	$1.6^{b} \pm 0.2$	$1.4^{b} \pm 0.3$	$2.9^{a} \pm 0.8$	$0.6^{c} \pm 0.2$
anteiso-17:0	$0.3^{c} \pm 0.2$	$0.1^{c} \pm 0.1$	$0.3^{c} \pm 0.2$	$1.0^{b} \pm 0.1$	$1.0^{b} \pm 0.1$	$1.3^{a,b} \pm 0.4$	$1.0^{b} \pm 0.1$	$0.7^{b,c} \pm 0.3$	$2.1^{a} \pm 0.3$	$0.3^{c} \pm 0.1$
17:0	$0.7^{c} \pm 0.1$	$0.8^{b,c} \pm 0.1$	$1.0^{b,c} \pm 0.1$	$0.9^{b,c} \pm 0.1$	$0.9^{b,c} \pm 0.2$	$1.2^{b} \pm 0.2$	$1.8^{a} \pm 0.2$	$1.2^{b,c} \pm 0.7$	$1.1^{b,c} \pm 0.1$	$1.2^{b} \pm 0.1$
17:1n-8	0.6 ± 0.3	0.6 ± 0.4	1.0 ± 0.3	1.0 ± 0.2	0.8 ± 0.2	0.4 ± 0.3	0.3 ± 0.3	0.7 ± 0.5	0.2 ± 0.4	0.5 ± 0.1

* Fatty acids with a percentage of greater than 1% in at least one sample are given. In addition to them, the following acids were found in bivalves: *iso*-16:0, 16:1n-10, 16:2, 16:3; 18:1n-11; 19:1; 20:1n-5; 21:0; 22:1n-13; 22:5n-6; and 24:1n-9.

** TMTD, 4,8,12-trimethyltridecanoic acid.

Mean values marked with at least one letter, which is the same, were not significantly different (Tukey test, $p < 0.05$).

Table 4. The ratio of the percentage of marker fatty acids in digestive gland to that in soft tissues of bivalves

Species	ΣC_{15}, C_{17} FA and 18:1n-7	16:1n-7	18:1n-9	Σ18:2n-6 and 18:3n-3	18:4n-3	20:1n-13	ΣNMI FA	20:4n-6	20:5n-3	22:4n-6	22:6n-3
Mytilus coruscus	1.1	1.2	1.4	1.0	1.3	1.1	0.9	**0.6**	0.9	–	0.9
Crassostrea gigas	0.9	1.2	0.9	0.9	0.9	1.1	1.0	1.0	1.2	0.8	0.9
Arca boucardi	1.1	**1.3**	1.1	1.1	**1.5**	1.0	**0.5**	**0.6**	0.9	–	**0.7**
Macoma incongrua	**1.4**	**1.5**	1.0	1.0	1.1	**0.7**	0.5	**0.6**	0.9	0.6	0.7
M. irus	1.1	1.2	0.9	**1.3**	1.3	**0.7**	0.5	**0.6**	1.0	**0.4**	**0.8**
Protothaca euglypta	1.2	1.4	1.0	**1.5**	**1.9**	**0.6**	**0.7**	**0.6**	**1.4**	**0.5**	**0.7**
P. jedoensis	**1.4**	**2.4**	1.1	1.5	**3.7**	**0.6**	**0.7**	**0.6**	1.2	**0.4**	**0.6**
Saxidomus purpurata	1.1	**2.2**	0.9	**1.6**	**2.3**	**0.5**	**0.5**	**0.4**	**1.6**	**0.2**	**0.6**
Diplodonta semiasperoides	1.1	**1.7**	**1.9**	0.9	**1.4**	0.9	1.0	**0.6**	0.7	0.6	0.6
Mya japonica	0.8	**1.1**	0.8	1.7	**1.3**	**0.7**	1.2	**0.7**	1.1	**0.7**	1.0

The values that are significantly different (Tukey's test $p < 0.05$) are shown in bold face.

was higher than that of 20:5n-3; in *A. boucardi* and *M.coruscus,* the main FAs also included16:1n-7 along with the above acids. Among the main FAs, there was no 22:6n-3 in *M. irus* and *M. incongrua*, no 20:5n-3 in *P. jedoensis,* and no either 20:5n-3 and 22:6n-3a in *D. semiasperoides*. Among FAs with a percentage of more than 3%, 20:4n-6 was found in digestive gland of *P. jedoensis*, *P. euglypta*, *M. irus*, *M. incongrua,* and *D. semiasperoides*. The percentage of some FAs in the digestive gland and soft tissues of bivalves differed significantly (Table 4). The general tendency was the following: the percentage of C_{16} and C_{18} FAs was higher in digestive gland, while the percentage of C_{20} and C_{22} FAs was, in most cases, higher in soft tissues. Thus, in all mollusks the percentage of 16:1n-7 in digestive gland exceeded its percentage in soft tissues. Among C_{20} and C_{22} FAs, an exception was 20:5n-3, which was predominant in the digestive gland of most of bivalves (Table 4). The lipids of digestive gland and soft tissues in *S. purpurata*, *P. euglypta,* and *P. jedoensis* differed by many FAs.

The data on the percentages of marker FAs in the digestive gland and soft tissues were used for cluster analysis and principal component analysis. Four groups of samples with a similarity of more than 80% within groups were identified (Fig. 1; Table 5). The first and second principal components together explained 77.3% of the variance. The largest contribution to the separation along the first principal component was made by 20:5n-3 and 22:6n-3, as well as by 18:1n-9, the sum of C_{15}, C_{17} FAs and 18:1n-7. The major contributors to the separation along the second principal component were 22:6n-3 and the sum of NMI FAs, as well as 16:1n-7. Almost all samples of the digestive gland and soft tissues of each species of bivalves were placed in the same group; the exception was samples of *S. purpurata*. Group I was made up of digestive gland and soft tissues samples of the bivalves *M. japonica*, *M. coruscus*, *A. boucardi*, *C. gigas*, as well as samples of the digestive gland of *S. purpurata*. According to the SIMPER analysis, the similarity in Group I was up to 86.7%; the major contributors (more than 70%) to the similarity of the FA composition of bivalves in this group were 20:5n-3, 22:6n-3, and 16:1n-7, as well as the sum of C_{15}, C_{17} FAs and 18:1n-7. Samples of soft tissues of *S. purpurata*, soft tissues and digestive gland of *P. euglypta* and *P. jedoensis* were combined into Group II. The similarity level in this group was 83.3%; more than 75% of this was accounted for by 22:6n-3, 20:5n-3, the sum of C_{15}, C_{17} FAs and 18:1n-7, 16:1n-7, the sum of NMI FAs, and 20:4n-6. Group III was constituted by two species of the genus *Macoma*; *D. semiasperoides* was separated into Group IV. The similarity in Group III was up to 86.9%; the main contributors were 20:5n-3, the sum of C_{15}, C_{17} FAs and 18:1n-7, 16:1n-7, 20:1n-13, as well as 18:1n-9. The main contribution to similarity in Group IV (84.7%) was made by the sum of C_{15}, C_{17} FAs and 18:1n-7, the sum of NMI FAs, the sum of 18:2n-6 and 18:3n-3, 18:1n-9, and 22:4n-6.

Group I differed from Group II by higher levels of 20:5n-3 and 16:1n-7, as well as by a lower percentage of 20:4n-6; from Group III by higher percentages of 22:6n-3 and 20:5n-3, as well as by lower values of content of 20:1n-13, 20:4n-6, and 18:1n-9. Group II differed from Group III by higher levels of 22:6n-3 and the sum of NMI FAs and by lower percentages of 16:1n-7, 20:1n-13, and 18:1n-9. Group IV differed from other groups by a low percentage of 20:5n-3 and a notable concentration of 22:4n-6.

Stable Isotopes

The isotope composition of carbon in the investigated species of bivalves varied very widely (Fig. 2). The variation range of $\delta^{13}C$ values was 8.1‰ (from −20.7‰ in *M. coruscus* to −12.6‰ in *M. incongrua*). The range of $\delta^{15}N$ values was substantially smaller (2.5‰):

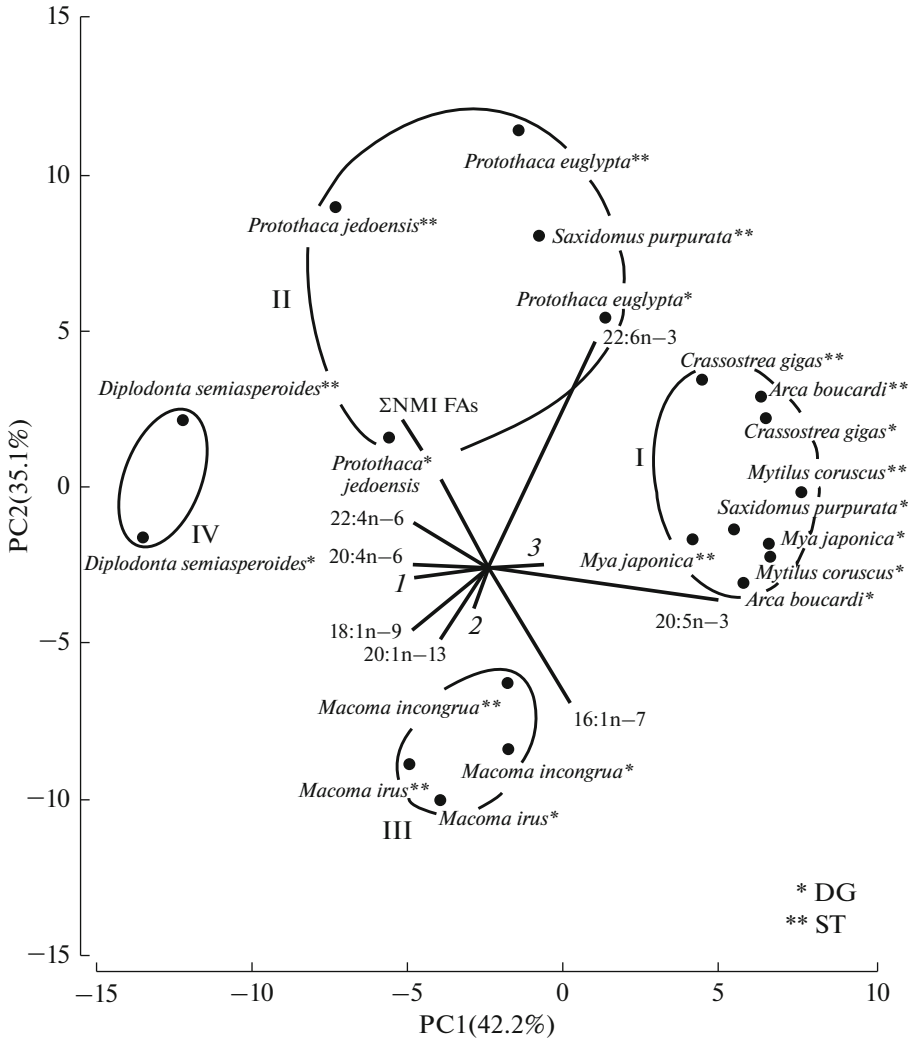

Fig. 1. The position of bivalves in the space of two first principal components based on the principal component analysis of marker FA composition. I–IV, groups of samples, in which the similarity of marker FA composition is more than 80%. DG, digestive gland; ST, all soft tissues. (*1*) ΣC_{15}, C_{17} FAs and 18:1n-7; (*2*) Σ18:2n-6 and 18:3n-3; (*3*) 18:4n-3.

from 6.6‰ in *M. irus* to 9.1‰ in *P. euglypta*. Most species significantly differed from each other by at least one of the parameters ($\delta^{13}C$ or $\delta^{15}N$) of the isotope composition (ANOVA, Tukey test, $p < 0.05$). An exception was two pairs of species: *S. purpurata*–*P. euglypta* and *P. jedoensis*–*D. semiasperoides*, which did not differ in the isotope composition of both nitrogen and carbon (Fig. 2).

Species of the genus *Macoma,* which were separated into a distinct Group III based on the marker FA composition, were characterized by very high $\delta^{13}C$ values, compared to other species of bivalves. At the same time, species from Group I showed the lowest $\delta^{13}C$ values and obviously became "heavier" in the series from *M. coruscus* towards *M. japonica* (Fig. 2). The values of $\delta^{15}N$ were higher in Group II than in Group I. Remarkably, *D. semiasperoides* (Group IV), with the most specific marker FA composition, was close in terms of the isotope composition to bivalves of Group II and virtually did not differ from one of them (*P. jedoensis*).

DISCUSSION

Among marine invertebrates, bivalve mollusks have been best studied with respect to the FA composition. Many studies have dealt with the effect of food on the FA composition of cultivated species, as well as with the relationship between the food composition and growth of commercially important species of bivalves. A number of studies have been devoted to the seasonal dynamics of the FA composition of bivalves in correlation to the change in planktonic community composition. One of the best-studied species in this respect is the oyster *C. gigas*. In terms of FA percentages, digestive gland samples of *C. gigas* from Vostok

Table 5. Differences in the marker fatty acid composition between Groups I–IV of bivalves (Fig. 1) (SIMPER analysis)

Fatty acid	Mean, %		Percent contribution	Cumulative contribution
	of first group compared	of second group compared		
Group I – Group II (dissimilarity 27.3%)				
20:5n-3	18.43	11.11	19.92	19.92
16:1n-7	8.61	3.74	14.02	33.94
NMI FAs	2.98	7.65	12.7	46.64
22:6n-3	13.2	16.52	12.4	59.04
20:4n-6	2.93	6.09	8.69	67.73
Group I – Group III (dissimilarity 28.5%)				
22:6n-3	13.2	5.91	19.22	19.22
20:1n-13	2.57	8.45	15.67	34.89
20:5n-3	18.43	13.02	14.27	49.16
20:4n-6	2.93	6.82	10.33	59.49
18:1n-9	3.25	7.06	10.04	69.53
Group II – Group III (dissimilarity 32.0%)				
22:6n-3	16.52	5.91	24.62	24.62
NMI FAs	7.65	0.39	16.88	41.5
16:1n-7	3.74	9.49	13.43	54.93
20:1n-13	3.82	8.45	10.78	65.71
18:1n-9	3.66	7.06	7.91	73.62
Group I – Group IV (dissimilarity 41.5%)				
20:5n-3	18.43	4.89	25.29	25.29
22:6n-3	13.2	6.21	13.05	38.35
NMI FAs	2.98	8.42	10.17	48.51
16:1n-7	8.61	3.56	9.82	58.33
22:4n-6	0.35	5.47	9.52	67.85
Group II – Group IV (dissimilarity 27.3%)				
22:6n-3	16.52	6.21	28.91	28.91
20:5n-3	11.11	4.89	17.65	46.56
18:2n-6 + 18:3n-3	2.06	5.77	10.4	56.96
18:1n-9	3.66	7.04	9.91	66.87
22:4n-6	2.53	5.47	8.78	75.65
Group III – Group IV (dissimilarity 32.9%)				
20:5n-3	13.02	4.89	19.22	19.22
NMI FAs	0.39	8.42	18.97	38.19
16:1n-7	9.49	3.56	14.04	52.23
20:1n-13	8.45	3.18	12.4	64.63
22:4n-6	0.69	5.47	11.25	75.88

The table represents fatty acids that in sum give a percent contribution of no less than 65% to the dissimilarity between groups.

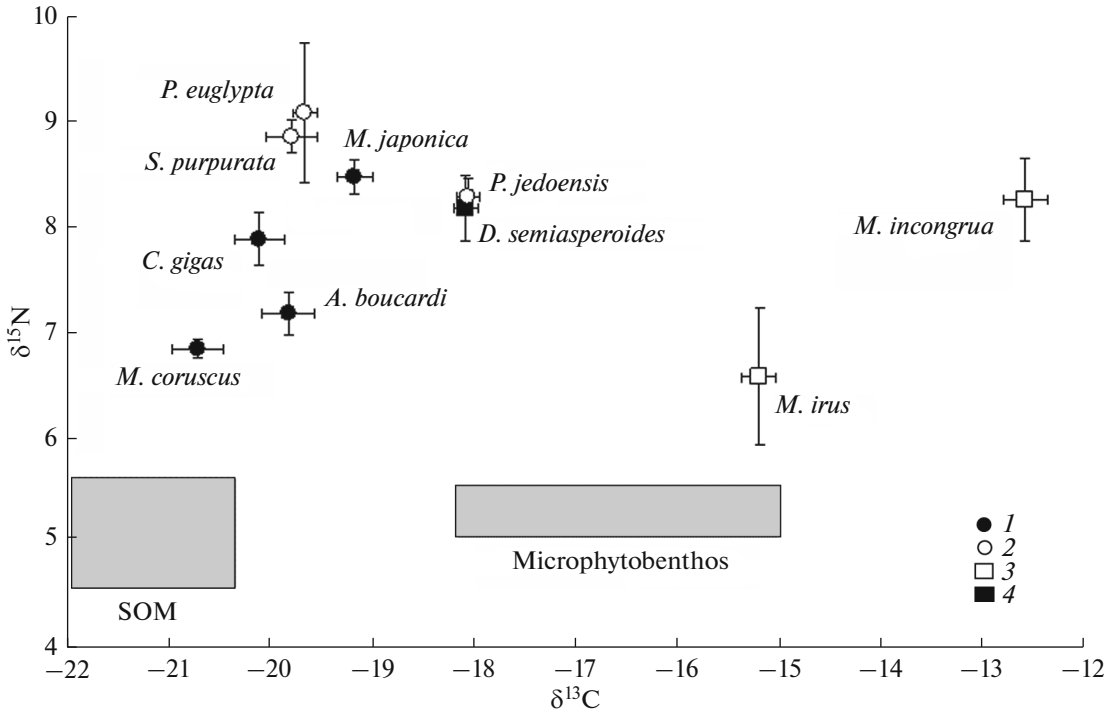

Fig. 2. The isotope composition of carbon and nitrogen in 10 species of bivalves (mean ± standard deviation). Symbols indicate groups of species that were separated based on the principal component analysis of marker FAs (see Fig. 1): (*1*) Group I; (*2*) Group II; (*3*) Group III; (*4*) Group IV. The data on $\delta^{13}C$ and $\delta^{15}N$ values in suspended organic matter (SOM) and in microphytobenthos, which are characteristic for Vostok Bay, are given according to the published data [4].

Bay much resembled samples of *C. gigas* collected in Japanese and French waters [28]. The main feature of their composition was a high percentage of 20:5n-3 and 22:6n-3. Apart from nine FAs reported in these studies (14:0, 16:0, 18:0, 16:1n-7, 18:1n-9, 18:1n-7, 18:4n-3, 20:5n-3, and 22:6n-3), another two FAs, 20:1n-7 and 22:2(7,15), with a percentage greater than 3% were found in our samples of *C. gigas*. We note that in other bivalves the content of the above-listed unsaturated FAs (MUFAs 16:1n-7 and 18:1n-7, PUFAs 20:5n-3 and 22:6n-3) also exceeded 3%, the content of 18:1n-9 was greater than 3% or close to this value, and only the percentage of 18:4n-3 was far lower in some bivalves. Despite the seeming uniformity of the FA composition, the cluster analysis and principal component analysis revealed four groups of samples based on the percentages of marker FAs.

The reason for the interspecies differences in the marker FA composition among invertebrates from closely related taxa with a similar type of feeding may be the differences in the metabolism (accumulation and synthesis) of FAs or differences (spatial and/or temporal) in the FA composition of their food sources. Significant differences, when found, in the marker FA composition among coexisting species of filter-feeding invertebrates that use the same pool of suspended organic matter are usually attributed to the difference in FA metabolism [27]. In such interspecies comparisons, much depends on the selection of organs for analysis. The results we obtained suggest that the bulk of the interspecies differences in the marker FA composition of digestive gland and, to a lesser degree, of soft tissues in the investigated species of bivalves are likely to be due to the specific features of the extraction and assimilation of different components from suspended organic matter, which is a common food resource for the given species.

The comparative study of the marker FA composition of the lipid extracts from digestive gland and soft tissues in ten species of bivalves has shown that the marker FA composition of these tissues similarly reflects the feeding habits of a species and can be used for characterizing the trophic niche of species of bivalves. This is primarily corroborated by substantial interspecies differences in the isotope composition of the bivalves studied, which, in most cases, coincided with the differences in the marker FA composition. At the same time, we found significant differences in the percentage of some FAs in digestive gland and soft tissues in most of the investigated species of Bivalvia (Table 4). Compared to the digestive gland, in soft tissues there was a general tendency towards a higher percentage of FAs with a chain length of more than C_{20} (both PUFAs and marker FAs) and towards a lower percentage of FAs with a chain length of less than or equal to C_{18}. This tendency probably reflects the effect

of the peculiarities of metabolism of different FAs in bivalve mollusks on the FA composition, which should be taken into account in trophic researches.

Mollusks that filter SOM from the water column were placed in Groups I and II according to the marker FAs (Fig. 1) and because of the significant interspecies differences in the isotope composition (Fig. 2), suggesting considerable species-specific peculiarities of feeding. Analysis of marker FAs showed that diatoms were not an overwhelming component of the food of these species. Invertebrates that feed predominantly on diatoms are generally characterized by a very high content of 20:5n-3 (> 20%) and a ratio of 16:1n-7/16:0 > 1, as well as by a considerable percentage of the 16:4n-1 FA [6]. Among PUFAs, the marker FA 20:5n-3 dominated in most samples of Groups I and II; however, its content exceeded 20% only in digestive gland samples of *C. gigas*. The 16:1n-7/16:0 ratio in bivalves of these groups was much less than 1 (from 0.16 to 0.73), and a notable percentage of 16:4n-1 was only found in some samples of digestive gland in Group I. The results we obtained are in agreement with the data on the low abundance of diatoms in the plankton of Vostok Bay in October during the sampling of mollusks for the study [2]. Most samples of bivalves in Groups I and II had an increased percentage of 18:4n-3 and a high percentage of 22:6n-3. The source of the acids may be dinoflagellates [17].

Samples from Group II, compared to Group I, were characterized by a lower percentage of 18:4n-3 and a higher percentage of 22:6n-3. In addition, samples of Group II had a high percentage of the FA 20:4n-6, as well as a marked percentage of NMI FAs, which were previously found in many invertebrates and can be synthesized by mollusks themselves [10]. For this reason, NMI FAs are clearly not always appropriate as trophic markers. Nevertheless, the substantial content of NMI FAs in lipids, as well as the high content of 22:6n-3 and 20:4n-6 may be indicative of a larger contribution of heterotrophic components to the food of these primary consumers. This suggestion is confirmed by the higher $\delta^{15}N$ values and, correspondingly, higher tropic position of most bivalves from Group II compared to those of mollusks in Group I.

Based on the marker FA composition, all samples of *M. irus* and *M. incongrua* formed a separate Group III. Like other representatives of the family Tellinidae, these bivalves are able to pull in particles from the sediment surface via a flow of water through the siphon. The intake of ^{13}C-enriched particles of bottom detritus and microphytobenthos is probably reflected in the high $\delta^{13}C$ values in species of the genus *Macoma*, compared to those of other species of bivalves and of SOM (Fig. 2). These mollusks were characterized by the high levels of 20:1n-13 and 16:1n-7, increased percentage of 20:4n-6, and the lowest percentage of 22:6n-3. It was previously assumed that the main source of the very rare FA 20:1n-13 may be detritus-feeding ophiurans [21]; however, the FA was later found in the detritus-feeding mollusk *Acila insignis*. It was suggested that the high percentage of this acid coupled with the high percentage of 20:4n-6 in the same animal may indicate that components of a benthic microbial loop are used as food sources [5]. In our study, all bivalves contained the FA 20:1n-13 but substantially differed in its content (Table 3). Species, which, unlike *Macoma*, were closest to SOM in terms of the carbon isotope composition (*M. coruscus*, *A. boucardi*, and *C. gigas*), had the lowest percentage of 20:1n-13 in both digestive gland and soft tissues samples (from 0.9 to 2.1%). The isotope and marker FA compositions in *Macoma* species indicate a substantial contribution of detritus from bottom sediment to the food of these tellinids. Thus, *M. incongrua* from the *Zostera marina* community in Novgorodskaya Bay was reported to have similar values of $\delta^{13}C$, which points to a considerable contribution of seagrass detritus to the food of the mollusk [18]. In the latter species, *M. irus*, which lives outside of seagrass thickets but is able to collect detritus from the substrate surface, the $\delta^{13}C$ value substantially differed from that in *M. incongrua* and in other species of bivalves (Fig. 2). The FA composition of the digestive gland of *M. irus* and *M. incongrua* virtually did not differ, which is possible when obtaining food from different sources of detritus via the same intermediate links of the microbial food web. The substantial contribution of these microbial food web components can be judged from the increased percentages of 20:1n-13 and 20:4n-6 [5].

All samples of *D. semiasperoides* (Fig. 1, Group IV) stood out most with respect to the marker FA composition. Compared to other bivalves, this species had the lowest levels of PUFAs 20:5n-3 and 22:6n-3 and the highest levels of FAs of bacterial origin, as well as of 18:2n-6. The bivalve *D. semiasperoides* differed from another ungulinid, *Felaniella usta,* which inhabits the subtidal sand community [4] by the increased content of 22:4n-6. This acid is found in microalgae, among them benthic ones, in which its content may sometimes exceed 19% [19]. In bivalves, this FA can be formed as a result of the elongation of 20:4n-6 [15]. However, we propose that the peculiarities of the marker FA composition in *D. semiasperoides* are connected with the specific sources of food of this species. Like other ungulinids, despite the absence of a siphon, *D. semiasperoides* is able to burrow deep into the sediment and filter particles from the interstitial water. This is indicated by the substantial enrichment in ^{13}C isotope in tissues of *D. semiasperoides*, compared to tissues of most bivalves from Groups I and II that filter particles from the water column. The FA analysis suggests a considerable contribution of microbial loop and a notable contribution of benthic diatoms to the food of this species.

Few comparative studies of bivalves have been carried out using FA and stable isotope analyses. Thus, among six species of intertidal bivalves from Zhuanghe Bay (the Yellow Sea), no significant differences were found in the isotope composition of carbon [33]. With respect to this finding and the supposedly marked contribution of organic matter of a terrestrial origin, the results of the above study disagree with the data we obtained. A study performed on three species of freshwater mollusks by Makhutova et al. [20] revealed differences in the FA composition and in possible food sources between an invasive species *Dreissena polymorpha* and *Unio tumidus*, on shell valves of which it settled. The authors suggested a trophic specialization of coexisting species of bivalves: *D. polymorpha* feeds on plankton, while *U. tumidus* feeds on detritus and phytobenthos. The mollusk *U. tumidus* was distinguished by the notable percentage of n-6 PUFAs, as was the case with the bivalve *D. semiasperoides* we studied; the putative food sources of these species were also similar.

In summary, it can be concluded from the data on the FA and stable isotope compositions that in the period preceding the sampling of bivalves there was no apparently predominant food resource for seston-feeders in the water column of Vostok Bay. Group I mainly fed on suspended organic matter from the water column; detritus had a marked part in the food of mollusks from other groups, particularly in bivalves of the genus *Macoma*. Some similarity in the FA and stable isotope compositions was observed in species inhabiting similar substrate type; however, the exceptions were *D. semiasperoides* and *M. irus*. Tellinids are capable of selective detritus-feeding along with seston-feeding, which helps them withstand considerable alterations in ecosystems better than other species [1]. The detritivorous feeding of tellinids is reflected in the composition of FAs, and particularly the composition of carbon stable isotopes. Considerable differences in the composition of FAs and stable isotopes of carbon and sulfur were previously found between the detritus-feeder *M. incongrua* and the filter-feeder *Ruditapes philippinarum* inhabiting *Z. marina* beds [18]. The differences in the FA and stable isotopic compositions between the tellinid *M. irus* and *A. boucardi* and *M. coruscus* that live close to it were no less pronounced. In this case, the differences in the isotope composition of carbon were particularly indicative, because the difference in the fatty acid composition is more difficult to interpret.

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