Effect of Diesel Fuel Pollution on the Lipid Composition of Some Wide-Spread Black Sea Algae and Invertebrates

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Z. Naturforsch. **57c,** 339–343 (2002); received September 24/November 27, 2001

Diesel Fuel, Lipids, Marine Organisms

Two green algae (*Ulva rigida* and *Cladophora coelothrix*), the mussel *Mytilus galloprovincialis* and the snail *Rapana thomasiana* from the Bulgarian Black Sea shore have been treated with diesel fuel (100 mg l^{-1}) in an aquarium with sea-water for three days. The lipids and their fatty acid changes have been examined. Significant changes have been observed mainly in the polar lipids and in the saturation of the fatty acids. These changes appeared to be bigger in the evolutionary less advanced species from both groups of marine organisms – algae and invertebrates (*Ulva rigida* and *Mytilus galloprovincialis* respectively). The data obtained could be used for a biomonitoring of the pollution.

Introduction

Petroleum contamination of the environment has been recognised as a serious pollution problem. As a result of increasing urbanization and industrialization Black Sea is now heavily polluted. Part of the pollution is due to petroleum, coming from partially treated industrial wastewater and from ships.

The effect of the petroleum pollution on the marine microalgae is well studied. As a rule, low concentrations of petrol and its derivatives stimulate photosynthesis, while high concentrations inhibit this process (Patin, 1985). In some freshwater microalgae (Petkov *et al.*, 1992) petroleum has a significant effect on the lipids from the cell membrane. There were small changes in the fatty acid composition of lipids, but the total sterol concentrations and their composition markedly changed. This will change the cell membrane permeability and might have an adaptive value. An investigation on the volatile constituents from aquatic higher plants (*Zostera marina* and *Z. nana*) collected at same location in Black Sea revealed significant amounts of alkylated benzenes, tetraethyl lead and triethyl-methyl lead only in *Z. nana* (Milkova *et al.*, 1995). These compounds probably originate from gasoline pollution and are an indication that in some organisms petroleum could penetrate at least partially into the cells, instead of covering only the outer surface of the marine organism. We did not find any data on the effect of petroleum on lipids of marine invertebrates. Probably an addition of petroleum to the water, simulating the ecological conditions in the polluted sea may cause some changes in the cell membrane composition and functions, which in some cases might have an adaptive value. To check this hypothesis we investigated the lipid composition of some of the main Black Sea organisms: two green algae and two molluscs, before and after the addition of diesel fuel to their aquarium.

Materials and Methods

Collection and incubation

Two wide-spread green algae – *Ulva rigida* and *Cladophora coelothrix*, as well as the mussel *Mytilus galloprovincialis* and the snail *Rapana thomasi-*

0939–5075/2002/0300–0339 $ 06.00

ana were used for experiments. The algae and invertebrates were collected in August 1998 near the village of Ravda, southern part of the Bulgarian Black Sea coast. The samples of algae and invertebrates were kept for three days in an aquarium with sea-water, containing diesel fuel (100 mg. l^{-1}). Diesel fuel was manifactured in "Luckoil – Neftochim", Burgas. The distillation range was 215 °C – 360 °C. It contains mainly alkanes and cycloalkanes, with 24–30 carbon atoms, together with about 10% aromatic hydrocarbons. Constant aeration, irradiance of 100 μmol m^{-2} s^{-1}, 12-h photoperiod, day/night temperature of 26/24 °C were kept during the experiment. Control samples were kept at the same conditions without diesel fuel.

Analysis of lipid classes and their FA composition

The samples were carefully separated from the shells or dead algal parts, washed with tap water containing few drops of detergent to remove the remaining diesel fuel, homogenized with chloroform – methanol (1:1 v/v) and refluxed for few min in order to inactivate the phospholipases. The extraction of the lipids and purification of the extracts were performed according to Bligh and Dyer (1959).

The total lipid extracts were chromatographed on a silica gel S (Merck) column according to Privett *et al.* (1973). The hydrocarbons were eluted with hexane, while the triacylglycerols (TAG) were eluded with chloroform-acetone (4:1 v/v), glycolipids (GL) – with acetone and finally the phospholipids (PL) with methanol. The amount of each fraction was determined gravimetrically. Since the amount of glycolipids in the invertebrates was less than 1% of total lipids, they were not investigated.

After addition of internal standard (heptadecanoic acid) the different lipid classes were esterified with 5% HCl in absolute methanol according Christie (1973). Analysis of the obtained fatty acids methyl esters (FAME) was carried out by a Hewlett Packard 5890 gas chromatograph equipped with an FID-glass capillary column (20 m, 0.25 mm i.d., coated with INNOWAX). The column temperature was increased from 165 °C to 220 °C (4 °C min^{-1}) and held for 10 min at 220 °C. Carrier gas was nitrogen. To determine the amounts of each lipid class the weights of FAME were multiplied by a *K*-factor. The conversion factors used were $K = 1.0$ for TAG, $K = 1.4$ for PL and $K = 1.6$ for GL (Elenkov *et al.*, 1993).

Results and Discussion

In Table I we summarised the data on the effect of the diesel fuel treatment on the amounts of the

Table I. Effect of diesel fuel treatment on lipid composition of algae and invertebrates from the Black Sea.

Sample	Lipid content	
	mg g $^{-1}$ DW*	% of total lipids
Ulva rigida		
Control	37.5 ± 4.5	
Hydrocarbons	1.7 ± 0.2	25.0
TAG	2.6 ± 0.5	37.5
GL	2.6 ± 0.5	37.5
PL**		
After diesel fuel treatment		
Hydrocarbons	120.7 ± 9.6	
TAG	1.0 ± 0.1	9.3
GL	6.6 ± 0.7	58.6
PL	3.6 ± 0.4	32.1
Cladophora coelothrix		
Control	23.7 ± 4.2	
Hydrocarbons	1.8 ± 0.2	20.0
TAG	5.4 ± 0.6	60.2
GL	1.8 ± 0.2	19.8
PL		
After diesel fuel treatment		
Hydrocarbons	24.0 ± 4.2	
TAG	3.6 ± 0.4	25.0
GL	5.4 ± 0.6	37.5
PL	5.4 ± 0.6	37.5
Mytilus galloprovincialis		
Control	85.8 ± 6.8	
Hydrocarbons	8.2 ± 0.9	89.1
TAG	1.0 ± 0.1	10.9
PL		
After diesel fuel treatment		
Hydrocarbons	81.3 ± 6.7	
TAG	16.5 ± 1.3	88.2
PL	2.2 ±0.4	11.8
Rapana thomasiana		
Control	13.3 ± 1.2	
Hydrocarbons	10.2 ± 0.9	89.5
TAG	1.2 ± 0.1	10.5
PL		
After diesel fuel treatment		
Hydrocarbons	44.7 ± 5.0	
TAG	35.0 ± 4.8	94.6
PL	2.0 ± 0.3	5.4

* Values obtained from three parallel measurements ± SD.
** TAG – triacylglycerols; GL – glycolipids; PL – phospholipids.

main lipid classes in the investigated algae and invertebrates.

In *Ulva rigida* diesel fuel treatment caused a sharp increase of the hydrocarbon concentrations. The relative concentrations of the triacyl glycerols (TAG) significantly decreased, while the concentrations of glycolipids (GL) and phospholipids (PL) increased. The increase of the polar lipids concentrations indicated a stronger control on the cell membrane permeability, which might have some adaptive value.

The changes in the lipid composition of *Cladophora coelothrix* significantly differed from those in *Ulva rigida.* There was no increase in the hydrocarbon concentrations, while the relative concentrations of TAG and especially of PL increased. The amount of GL did not change. The differences found, probably, were due to different lipid composition of the cell membranes in both algae, which led to different reactions towards pollution.

It is known that the petroleum treatment leads to an increase of the amount of total lipophylic extracts, due to its uptake of into cells. An investigation on the effect of petroleum on some freshwater microalgae also shows a penetration of hydrocarbons into the algal cells (Petkov *et al.*, 1992). Now, all investigated organisms were treated with the same concentrations of diesel fuel for three days and were subjected to the same cleaning procedure, so the differences in hydrocarbon concentrations obtained must be due to different penetration of diesel fuel through cell membranes and not to the adsorption of diesel fuel on the surface of the investigated organisms. The same was observed in two species of *Zostera* seagrass from the Black Sea. They grow mixed near Ravda village, but only one of them (*Zostera nana*) contains aromatic hydrocarbons and other products, characteristic for petroleum derived products (Milkova *et al.*, 1995).

The lower hydrocarbon content in *C coelothrix* in comparison with the *Ulva rigida* could be due to some bacteria associated with *C. coelothrix*. Marine algae are often associated with bacterial strains (Berland *et al.*, 1970). Recently, we found that *Cladophora coelothrix* serves as a habitat for a bacterial strain (*Vibrio alginolyticus*), capable of sequestering linear alkylbenzene hydrocarbons from the sea water and oxidizing them to corresponding acids (Carballeira *et al.*, 1997). In such a case part of the alkylbenzenes from the diesel fuel, which penetrated into the *C. coelothrix* cells, might be oxidized to the corresponding acids and the amount of hydrocarbons in this alga will be lower.

Diesel fuel treatment of algae also strongly changes the fatty acid composition of different lipid classes in *U. rigida* (Table II). Main acid in this alga appeared to be trienoic acid – 18:3, a high concentration of 16:4 was also found, while palmitic acid and polyunsaturated acids (PUFA) were present in low concentrations. In all three lipid classes investigated the concentrations of saturated acids decreased after the treatment with diesel fuel. A decrease of all PUFA of (n-3) series was also noted in TAG. In PL the amounts of 16:4 and 20:5 acids decreased, whereas the content of (n-6) PUFA – 18:2 and 20:4 acids increased. Also diesel fuel treatment led to a some increase of the monoenoic acids concentrations, especially of cis-vaccenic acid in TAG and palmitooleic acid in PL. Some increase of octadecatetraenoic acid was also observed, especially in the polar lipids. In addition, the content of 20:5 and especially 22:5 acids increased in GL. The increase of the unsaturation might increase the permeability of the cell membrane and stimulate the influx of diesel fuel in the cells and probably the accumulation of a high quantity of hydrocarbons.

Fatty acid composition of *C. coelothrix* and its changes strongly differed from that of *U. rigida* (Table II). The main fatty acid in *C. coelothrix* appeared to be palmitic acid, followed by oleic acid, contrary to the trienoic and tetraenoic acids in *U. rigida*. Unlike *U. rigida* the diesel fuel treatment changed the fatty acid composition of *C. coelothrics* insignificantly. There was an increase of 16:3 acid concentration and a decrease of 16:4 one. The concentrations of the saturated fatty acids decreased only in glycolipids (GL). In *U. rigida* the content of these FA decreased in all lipid groups, especially in GL. The increase of the saturated acids, especially in PL, might be a reason for a decreased permeability of the cell membranes, which could reduce the influx of diesel fuel into the cells. Both investigated algae belong to Chlorophyta, but *Ulva rigida* is evolutionary less advanced than *Cladophora coelothrix* and evidently the effect of the diesel fuel on the lipid composition is more significant in the first algae

Table II. Effect of diesel fuel treatment on fatty acid composition (wt % of total)* of lipids in two green algae from the Black Sea.

Fatty acid	Control			After diesel fuel treatment		
	TAG	GL	PL	TAG	GL	PL
Ulva rigida						
14:0	5.8	8.0	1.8	1.0	3.0	0.8
16:0	10.0	7.0	16.0	8.0	0.8	5.5
16:1	3.4	2.2	3.4	0.4	7.6	8.6
16:4 n-3	10.1	15.5	10.0	6.0	5.0	5.0
18:0	7.1	1.8	5.7	0.9	0.7	0.7
18:1	1.2	4.0	0.9	27.0	8.0	1.9
18:2 n-6	1.4	6.0	3.7	8.2	2.1	5.1
18:3 n-3	48.0	40.0	40.2	40.4	40.0	48.0
18:4 n-3	4.0	5.0	5.0	1.2	10.4	14.0
20:4 n-6	2.1	2.3	1.8	3.3	2.6	2.6
20:5 n-3	5.8	5.0	11.3	0.3	12.4	7.8
22:5 n-3	0.9	3.2	–	3.1	7.5	–
Cladophora coelothrix						
14:0	8.2	4.8	8.2	5.7	8.1	5.3
16:0	35.1	45.6	40.8	41.6	34.1	51.4
16:1	3.2	4.1	3.9	3.2	1.7	2.9
16:4 n-3	5.4	6.8	10.0	8.8	10.1	10.6
18:0	8.5	5.7	1.7	9.4	7.4	2.4
18:1	20.6	22.0	19.9	19.2	27.6	15.6
18:2 n-6	5.2	0.6	3.6	2.0	0.3	0.4
18:3 n-3	6.6	5.4	5.8	5.3	6.0	6.0
18:4 n-3	0.1	–	–	0.2	–	–
20:4 n-6	2.0	2.5	1.9	2.6	2.7	3.3
20:5 n-3	5.1	2.5	4.2	2.0	2.0	2.1

*) Values, obtained from three parallel measurements. The standard deviations (related to peak proportion on the chromatograms) are as follows:
± 1.0 for C16:0 and C18:1 and ± 0.3 for the others.

The diesel fuel treatment of the investigated marine invertebrates led to an increase of the absolute amounts of TAG and PL, but their ratio did not change in *M. galloprovincialis* whereas it showed small changes in *R. thomasiana* (Table I). The main components of the total lipids were TAG. Only the snail, *Rapana thomasiana,* accumulated hydrocarbons. This was in agreement with the observation that some marine gastropods could accumulate short-chain aliphatic hydrocarbons in their tissues (Walsh *et al.*, 1995).

In *M. galloprovincialis* (Table III) 16:0 and 16:1 acids were the main FA, followed by 20:5 acid. In *R. thomasiana* we found 16:0, 18:3, 20:1 and 22:6 acids in high concentrations. The changes in the fatty acid composition, due to diesel fuel pollution, were more significant in *M. galloprovincialis* (Table III). There was some decrease of the saturated fatty acids concentrations and increase of PUFA content, especially in phospholipids. Only in PL we observed a sharp decrease of 18:1 acid concentrations and an increase of 16:1 acid concentrations. The absence of similar changes in fatty acid composition of TAG was an indication, that the changes observed were connected with the cell membrane functions after a diesel fuel treatment. In *R. thomasiana* the diesel fuel treatment caused insignificant changes in the fatty acid composition. This invertebrate is evolutionary more advanced than the mussel *M. galloprovincialis* and the effect of diesel fuel on lipid composition was smaller. The same was observed for the investigated algae. Therefore, we can assume that the effect of diesel fuel on lipid metabolism is stronger in the evolutionary less advanced organisms.

Acknowledgements

The authors are grateful to the National Council for Scientific Research (Contract X-1006) for the partial support of this work.

Table III. Effect of diesel fuel treatment on fatty acid composition of lipids in *Mytilus galloprovincialis* and *Rapana thomasiana* (wt% of total).*

Fatty acid	*M. galloprovincialis*				*R. thomasiana*			
	Control		After diesel fuel treatment		Control		After diesel fuel treatment	
	TAG	PL	TAG	PL	TAG	PL	TAG	PL
14:0	2.0	2.9	3.7	1.6	4.3	2.0	6.5	2.1
16:0	22.3	26.1	17.3	16.1	22.1	19.1	20.7	20.2
16:1	25.5	20.9	27.9	30.4	4.7	1.5	3.5	1.5
16:3	1.4	0.5	1.8	0.4	2.0	2.9	2.5	1.8
16:4	0.4	0.7	0.5	0.4	1.0	0.8	0.2	0.2
18:0	2.8	5.0	0.5	1.4	4.3	8.7	4.0	7.6
18:1	4.1	9.0	3.4	1.6	2.3	1.4	1.6	1.8
18:2	2.1	0.6	3.3	1.8	1.1	2.1	1.2	1.7
18:3	4.3	1.9	9.2	5.9	12.3	10.8	15.3	14.6
18:4	1.8	0.5	2.7	8.0	4.3	0.6	2.6	0.4
20:1	5.7	5.0	1.1	1.1	10.7	18.2	11.3	18.0
20:2	1.0	1.1	0.3	0.5	0.2	0.3	0.2	0.3
20:4	3.0	4.0	7.9	12.8	6.3	8.3	8.1	8.2
20:5	18.5	13.5	10.7	11.3	8.0	6.2	6.9	4.5
22:5	0.7	1.2	1.0	0.7	3.4	8.8	5.0	8.9
22:6	4.4	7.1	8.7	6.0	13.0	8.3	10.4	8.2

* For details see Tables I and II.

Berland B. R., Bonin D. J. and Maestrini S. Y. (1970), Study of bacteria associated with marine algae in culture. III. Organic substrate supporting growth. Mar. Biol. (Berlin) **5**, 68–76.

Bligh E. G. and Dyer W. J. (1959), A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. **37**, 911–917.

Carballeira N. M., Sostre A., Stefanov K., Popov S., Kujumgiev A., Dimitrova-Konaklieva S., Tosteson K. and Tosteson T. (1997), The fatty acid composition of a *Vibrio alginolyticus* associated with the alga *Cladophora coelothrix*. Identification of the novel 9-methyl-10-hexadecenoic acid. Lipids **32**, 1271–1275.

Christie W., Lipid Analysis. Pergamon Press, Oxford, 1973.

Elenkov I. J., Ivanova A. P., Stefanov K. L., Seizova K. A. and Popov S. S. (1993), A quantitative determination of lipid classes in higher plants and algae by a gas-chromatographic procedure. Bulg. Chem. Commun. **26**, 98–103.

Milkova T., Petkova R., Christov R., Popov S. and Dimitrova-Konaklieva S. (1995), Comparative study of the chemical composition of *Zostera marina L.* and *Zostera nana* [Roth] from the Black Sea. Botan. Marina **38**, 99–101.

Patin S. A. (1985), Ecologic and Toxicologic Aspects of Marine Pollution. Vol. **5**, Gidrometeoisdat, Leningrad [Russ.]

Petkov G., Furnadzieva S. and Popov S. (1992), Petrol-induced changes in the lipid and sterol composition of three microalgae. Phytochemistry **31**, 1165–1166.

Privett O. S., Dougherty K. A., Erdahl W. L. and Stolyhwo A. (1973), Studies on the lipid composition of developing soybeans. J. Amer. Oil Chem. Soc. **50**, 516–520.

Stefanov K., Budevska B., Ivanov O., Genov N., Andreev St. and Popov S. (1993), Lipid and amino acid changes in the mussel *Pseudoanodonta complanata* caused by water pollution. Comp. Biochem. Physiol. **105C**, 39–42.

Walsh K., Dunstan R. H. and Murdoch R. N. (1995), Differential bioaccumulation of heavy metals and organopollutants in the soft tissue and shell of the marine gastropod, *Austrocochlea constricta*. Arch. Environ. Contamin. Toxicol. **28**, 35–39.