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PII: S0013-9351(19)30582-1
DOI: https://doi.org/10.1016/j.envres.2019.108785
Reference: YENRS 108785

To appear in: *Environmental Research*

Received Date: 6 June 2019
Revised Date: 28 September 2019
Accepted Date: 28 September 2019


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Email: k.pikula@mail.ru
ABSTRACT

Air pollution caused by vehicle emissions remains a serious environmental threat in urban areas. Sedimentation of atmospheric aerosols, surface wash, drainage water, and urbane wastewater can bring vehicle particle emissions into the aquatic environment. However, the level of toxicity and mode of toxic action for this kind of particles are not fully understood. Here we explored the aquatic toxic effects of particulate matter emitted from different types of vehicles on marine microalgae *Porphyridium purpureum* and *Heterosigma akashiwo*.

We used flow cytometry to evaluate growth rate inhibition, changes in the level of esterase activity, changes in membrane potential and size changes of microalgae cells under the influence of particulate matter emitted by motorcycles, cars and specialized vehicles with different types of engines and powered by different types of fuel.

Both microalgae species were highly influenced by the particles emitted by diesel-powered vehicles. These particle samples had the highest impact on survival, esterase activity, and membrane potential of microalgae and caused the most significant increase in microalgae cell size compared to the particles produced by gasoline-powered vehicles. The results of the algae-bioassay strongly correlate with the data of laser granulometry analyses, which indicate that the most toxic samples had a significantly higher percentage of particles in the size range less than 1 µm. Visual observation with an optical microscope showed intensive agglomeration of the particles emitted by diesel-powered vehicles with microalgae cells. Moreover, within the scope of this research, we did not observe the direct influence of metal content in the particles to the level of their aquatic toxicity, and we can conclude that physical damage is the most probable mechanism of toxicity for vehicle emitted particles.

**Keywords:** Ecotoxicology; flow cytometry; microalgae; particulate matter; vehicle emitted particles.
1. Introduction

Automobile exhaust gases are the largest source of particulate matter in an urban atmosphere. Vehicle emitted particles (VEP) can have adverse impacts on living organisms from bacteria to higher animals and humans (Correa et al., 2017; Kovats et al., 2013; Veremchuk et al., 2018; Zakharenko et al., 2017). In 2013, the International Agency for Research on Cancer classified air pollution (including vehicle emissions) as a carcinogen to humans. Over the past decade, many studies have focused on the development of technological solutions and regulatory rules that would reduce the content of particulate matter in motor vehicle emissions (Johnson, 2009; Minoura et al., 2009; Rasch et al., 2013).

The composition of particulate emissions varies depending on the type of engine, mileage, type of fuel and lubricating oils (Christianson et al., 2010; Kleeman et al., 2000; Sielicki et al., 2012). Thus, the toxic effect of particles emitted by different vehicles can vary, which calls for a comprehensive assessment of VEP in ecotoxicological point of view.

The importance of phytoplankton as a bioindicator of pollution is widely accepted (Moreno-Garrido et al., 2015; Zheng et al., 2018). Phytoplankton, being on the basic trophic level, is the main producer of organic matter in the aquatic environment and it is a key component of aquatic food chains (Nyholm and Peterson, 1997). Hence, changes in phytoplankton population under the exposure of toxic substances could affect all levels of consumers, such as zooplankton, fish, and eventually humans. Thus, microalgae have become a common test-subject in ecotoxicology (Zheng et al., 2018).

In this study, we used two marine microalgae species i.e. *Porphyridium purpureum* and *Heterosigma akashiwo* to evaluate the influence of particulate matter emitted by motorcycles, cars, and specialized vehicles with different types of engines and powered by different types of fuel. We used flow cytometry to evaluate growth rate inhibition, changes in the level of esterase activity, changes in cell membrane potential, and cell size changes of both microalgae species.

2. Material and methods

2.1 Vehicle emitted particles

The vehicles used in this study were chosen according to the ON025270-66 classification. There were selected seven vehicles broadly represented in Russian cities (Table 1).

<table>
<thead>
<tr>
<th>Vehicle type</th>
<th>Coded vehicle model</th>
<th>Displacement, cc</th>
<th>Fuel (Russian standard)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Motorcycle</td>
<td>HusTE</td>
<td>300</td>
<td>AI-92 + AI-95</td>
</tr>
<tr>
<td></td>
<td>HonVT</td>
<td>1300</td>
<td>AI-95</td>
</tr>
<tr>
<td>Light-duty vehicle</td>
<td>TMar2</td>
<td>2500</td>
<td>AI-92</td>
</tr>
<tr>
<td></td>
<td>THi</td>
<td>3000</td>
<td>DIESEL</td>
</tr>
<tr>
<td></td>
<td>MiPaj</td>
<td>3000</td>
<td>AI-95</td>
</tr>
<tr>
<td></td>
<td>TLC80</td>
<td>2500</td>
<td>DIESEL</td>
</tr>
<tr>
<td>Specialized (heavy-duty)</td>
<td>KomPC</td>
<td>8300</td>
<td>DIESEL</td>
</tr>
</tbody>
</table>

AI-92 and AI-95 are the codes of Russian automobile types of gasoline according to the standard GOST 2084-77 with octane number 92 and 96, respectively (Syroezhko et al., 2004)
Vehicle emitted particles were obtained as follows: emitted particles were collected by passing exhaust gases through water, which absorbs most of the solid components (up to 80%). Prior to the collection of particle samples, the container (plastic containers with a capacity of 20 L) and the hoses (polyvinyl chloride hoses; 1 m long and 50 mm in diameter for each measurement) were washed with deionized water obtained from the ultra-clear water preparation system SGWASSER Ultra Clear TWF/EL-ION UV plus TM (Siemens, Germany). We used the VEPs at three different concentrations i.e. 1, 10, and 100 mg/l in a tetra-replicate experiment. This dosage selection is provided by the data of previous studies where the toxicity of particulate matter was assessed by the tests with microalgae or other aquatic organisms (Durga et al., 2014; Correa et al., 2016; Correa et al., 2017; Pikula et al., 2018).

Previously, we have characterized the VEPs emitted from vehicles powered by diesel and gasoline (Chernyshev et al., 2018; Golokhvast et al., 2015). We had shown that diesel vehicles tend to emit particles of lower aerodynamic diameter compared to gasoline vehicles, and particulate size-number concentration increases with increasing of engine displacement.

The metal content of the VEP samples was detected by X-ray fluorescence analyzer X-50M (Innov-X Systems, USA).

Stock solutions of each VEP sample at concentration 1000 mg/l were analyzed with laser particle sizer Analysette 22 NanoTec plus (Fritsch, Germany). All the measurements were carried out at the particle size range from 0.08 to 2000 µm. For each VEP sample, the mode of percentage in a certain particle size range was calculated on the base of five measurements.

2.2 Microalgal cultures

Microalgal cultures were provided by The Resource Collection Marine biobank of the National Scientific Center of Marine Biology, Far Eastern Branch of the Russian Academy of Sciences (NSCMB FEB RAS). The toxicity bioassay of VEP was carried out on two types of marine microalgae isolated in Peter the Great Gulf (Sea of Japan, Primorsky Krai): the red alga *P. purpureum* (Drew and Ross, 1965) and the ochrophyte *H. akashiwo* (Hara and Chihara, 1987). The choice of microalgae species was based on the successful using of these species as test organisms described in the research papers (Voznesenskiy et al., 2018; Zhao et al., 2018; Li et al., 2019).

The red planktonic alga *P. purpureum* has spherical cells with an average size of 7-8 µm (Schornstein and Scott, 1982). Each cell of the algae is surrounded by a mucous membrane with variable thickness. The cells have one nucleus with a diameter of 2 µm located inside the single star-shaped chloroplast (Oakley, Dodge, 1974; Gantt, 1980). *P. purpureum* is a very important species for the pharmaceutical industry due to its ability to produce valuable polysaccharides (Rwehumbiza et al., 2014). The algae strain used in the current research was isolated in 2012 from Amur Bay, Peter the Great Gulf, Sea of Japan (Aizdaicher et al., 2014).

The motile planktonic alga *H. akashiwo* has golden-brown pigmentation on the cell membrane. The average cells are 10-22 µm in length and 8-14 µm in width. The number of chloroplasts could vary from 10 to 25. The cells might be spherical or ovoid. Two motile flagella are responsible for the motion of the cell. Each cell has one or two teardrop nuclei (Hara and Chihara, 1987). *H. akashiwo* does not have a shell like most of the algae species but has amorphous vesicles under the cell wall. *H. akashiwo* is a mixotrophic alga and it could combine photosynthesis with the uptake of nutrition and eating of bacterium (Lee, 2008). More often *H. akashiwo* was found at the depths less than 10 meters in the
coastal zone of the United States, Canada, Chile, the Netherlands, Scotland, Ireland, Sweden, Norway, Japan, S. Korea, Hong Kong, Australia, and New Zealand (Rensel, 2007). A massive gathering of H. akashiwo cyst could form toxic red tides (Bowers et al., 2006; Rensel, 2007) and provoke the death of fish (Khan et al., 1997; Rensel, 2007).

Microalgae cells were cultured with Guillard’s f/2 medium (Guillard and Ryther, 1962). We used filtered seawater (pore diameter of the filter was 0.22 µm) with salinity 33 ± 1 ‰, pH 8.0 ± 0.2. The cultivation was carried out at a temperature of 20 ± 2 °C with an illumination intensity of 300 µmol photons/m²·s, with a light cycle of 12:12 hours.

2.3 Bioassays

Algae-bioassay was performed to test the toxicity of VEPs. Culturing of microalgae and toxicity test conditions were conducted in accordance with the guidance of OECD No.201 (OECD, 2011) with minor modifications as previously described (Pikula et al., 2019). The calculated 96-h and 7-day LC₅₀ (the concentrations at which a half of microalgae cells have died) for P. purpureum were 1.18 and 0.15 mg/l and for H. akashiwo 13.88 and 8.94 mg/l, respectively. For bioassays, we used algal cultures growing in the exponential growth phase with a density of 1-5 × 10⁶ cells/ml. For microalgae cultivation, we used 24-well plates with VEPs at concentrations of 1, 10, 100 mg/l. The wells with only f/2 medium were taken as a control group. All assays for each concentration of VEPs were conducted in four replications. The volume of microalgae aliquots in each replication was 2 ml.

Registration of cell death and biochemical changes of microalgae cells after exposure of VEPs was recorded by flow cytometer CytoFLEX (Beckman Coulter, USA) with the software package CytExpert v.2.0. The toxicity level assessment and determination of algal biochemical changes were evaluated using specific fluorescent dyes. A blue laser (488 nm) of the CytoFLEX flow cytometer was chosen as a source of excitation light since the light of this wavelength matches the absorption maximum of used dyes and their metabolites. The emission filters were selected according to the maximum emission of the dyes, provided by the manufacturer (Molecular Probes, USA). The chosen filters were used to record the fluorescence of each dye and its metabolites. Each sample was measured at a flow rate of 100 µl/min for 30 seconds. The toxicity endpoints of bioassay and test conditions are shown in Table 2.

<table>
<thead>
<tr>
<th>Toxicity endpoints</th>
<th>Test duration</th>
<th>Biomarker</th>
<th>CytoFLEX emission filter, nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitality</td>
<td>24 h, 96 h, 7 d</td>
<td>Propidium iodide (PI)</td>
<td>ECD, 610</td>
</tr>
<tr>
<td>Esterase activity</td>
<td>3 h, 24 h</td>
<td>Fluorescein diacetate (FDA)</td>
<td>FITC, 525</td>
</tr>
<tr>
<td>Membrane potential</td>
<td>6 h, 24 h</td>
<td>3,3'-dihexyloxacarbocyanine iodide (DIOC₆)</td>
<td>FITC, 525</td>
</tr>
<tr>
<td>Size</td>
<td>96 h, 7 d</td>
<td>Forward scattering</td>
<td>FSC (forward scattering of a blue laser 488 nm)</td>
</tr>
</tbody>
</table>

To determine the number of microalgae cells in each measurement, we selected a homogeneous population of events registered on the FSC/SSC dot cytogram (forward scattering to side scattering ratio). Subsequently, from that population were selected only those events that had an intense
fluorescence in the PC5.5 emission channel, which tallies the emission of chlorophyll \( a \). All the data of flow cytometric measurement analysis were interpreted as the mean fluorescence intensity (MFI).

Prior to the assessment of vitality, esterase activity, and membrane potential of each microalgae species, the series of preliminary measurements were carried out to determine the optimal concentration of fluorescent dyes and the optimal duration of staining.

2.3.1 Vitality

Cell viability was determined by staining with PI according to the standard bioassay protocol (Ostrander, 2005). The mechanism of PI action is the incorporation between DNA or RNA base pairs, whereupon the dye increases its fluorescence intensity by 20–30 times (Suzuki et al., 1997). Since PI is not able to penetrate intact membranes of living cells, the cells with dramatically increased fluorescent intensity in the ECD emission filter compared to control can be regarded as dead cells. The calculation of live microalgae cells was performed by identifying cells that have chlorophyll \( a \) autofluorescence and by the exclusion of dead cells from counting.

The optimal PI concentration and duration of staining for each microalgae species were determined using negative control (algae cells aged in solid-state climatstat for 10 minutes at 99 °C), positive control (intact cells), and a mixture of negative and positive control groups in a ratio of 1:1. The stock solution of the dye (1.5 mM) was prepared by diluting 1 mg of PI in 1 ml of distilled water. The working solution was obtained by diluting the stock solution with distilled water to 500 \( \mu \)M. To each group of cells, PI was added at concentrations ranging from 5 to 100 \( \mu \)M. The measurements were recorded for 30 minutes with 5-minute increments. We considered the dye concentration and duration of staining as optimal conditions for each microalgae species when negative control cells were stained at the level near 100%, positive control cells were almost not stained, and the mixture sample was stained approximately at 50%.

The optimal PI concentration for both tested microalgal species was determined as 20 \( \mu \)M with 10 min staining duration.

2.3.2 Esterase activity

Esterase activity of microalgae exposed to the VEPs was evaluated using non-fluorescent lipophilic dye FDA. Fluorescein diacetate can easily penetrate the microalgae cell wall and then decompose by interaction with nonspecific esterases inside the cells to form a brightly fluorescent constituent called fluorescein (Fontvieille et al., 1992). Thus, according to the intensity of fluorescein fluorescence inside the cells, the esterase activity of microalgae can be evaluated as a sensitive endpoint of algal sublethal toxicity (Yu et al., 2007; Hadjoudja et al., 2009; Wang et al., 2016).

After the addition of FDA to microalgae aliquots, MFI of microalgae cells rapidly increases due to the formation of fluorescein, then MFI reaches a maximum where it remains stable for several minutes and finally it starts to decrease. To identify the dye concentration and staining interval at which maximum fluorescence intensity stabilized, a stock solution of the dye (12 mM) was prepared by diluting 5 mg of FDA in 1 ml of acetone. The working solution was obtained by diluting the stock solution with distilled water to 1 mM. Fluorescein diacetate was added to each cell group at concentrations of 1 to 50 \( \mu \)M. The measurements were carried out for 60 minutes with 2-minute increments.
For *P. purpureum*, the optimal FDA concentration was 15 µM and the staining duration was 18-28 min. For *H. akashiwo*, the optimal FDA concentration was 5 µM and the staining duration was 24-34 min.

2.3.3 Membrane potential

The membrane potential of microalgae cells was assessed by a lipophilic, positively charged fluorescent dye DiOC₆, which is capable of binding to membranes (mitochondria and endoplasmic reticulum) and other hydrophobic negatively charged cell structures (Sabnis et al., 1997). In the case where the inner membranes of the cell became more electronegative compared to medium (hyperpolarization, i.e. an increase in the membrane potential), the dye will be absorbed. If the membrane potential decrease and the cell becomes less electronegative compared to medium (depolarization), the dye will be removed from the cell (Novo et al., 1999; Gregori et al., 2003).

To define the optimal concentration of DiOC₆ and the optimal duration of staining, a stock solution of the dye (10 mM) was prepared by dilution of 5.7 mg of DiOC₆ in 1 ml of acetone. The working solution was obtained by dilution of the stock solution with distilled water to 100 µM. 3,3′-dihexyloxacarbocyanine iodide was added to microalgae cells at concentrations of 0.1 to 5 µM. The measurements were carried out for 35 minutes with 5-minute increments.

For *P. purpureum*, the optimal concentration of DiOC₆ was 1.5 µM and the staining duration was 15-25 min. For *H. akashiwo*, the optimal concentration of DiOC₆ was 0.5 µM and the staining duration was 20-30 min.

2.3.4 Size

To determine the size of microalgae cells by flow cytometry, a size calibration kit (batch F13838, Molecular probes, USA) with the certified size distribution of 1, 2, 4, 6, 10, 15 µm was used for the FSC channel.

2.3.5 Morphological changes

Morphological changes of microalgae cells were captured by optical microscope Axio Observer A1 (Carl Zeiss, Germany).

2.4 Statistical analysis

Statistical analyses were performed using the software package GraphPad Prism 7.04 (GraphPad Software, USA). The one-way ANOVA test was used for analysis. A value of *p* ≤0.05 was considered statistically significant.

3. Results

3.1 Characterization of vehicle emitted particles

Images of VEP made by scanning electron microscope Carl Zeiss Ultra 55 (Carl Zeiss, Germany) are presented in Fig. 1. The metal content of VEP samples measured by XRF analyses is presented in Table 3. The results of laser granulometry analyses are presented in Table 4.
Fig. 1. Scanning electron microscopy images of vehicle emitted particles

Table 3
The metal content of VEP samples (mg/kg)

<table>
<thead>
<tr>
<th>Vehicle code</th>
<th>Cr</th>
<th>Mn</th>
<th>Co</th>
<th>Cu</th>
<th>Zn</th>
<th>As</th>
<th>Pb</th>
<th>Cd</th>
<th>Sn</th>
<th>Sb</th>
<th>Ba</th>
<th>Ni</th>
<th>Mo</th>
</tr>
</thead>
<tbody>
<tr>
<td>HusTE</td>
<td>374</td>
<td>12987</td>
<td>3560</td>
<td>2827</td>
<td>7209</td>
<td>228</td>
<td>113</td>
<td>48</td>
<td>491</td>
<td>834</td>
<td>300</td>
<td>22025</td>
<td>249</td>
</tr>
<tr>
<td>HonVT</td>
<td>425</td>
<td>1324</td>
<td>363</td>
<td>1371</td>
<td>4611</td>
<td>62</td>
<td>118</td>
<td>5</td>
<td>29</td>
<td>83</td>
<td>325</td>
<td>30</td>
<td>12</td>
</tr>
<tr>
<td>TMar2</td>
<td>21113</td>
<td>1446</td>
<td>1386</td>
<td>1187</td>
<td>25889</td>
<td>151</td>
<td>180</td>
<td>11</td>
<td>90</td>
<td>184</td>
<td>478</td>
<td>386</td>
<td>2389</td>
</tr>
<tr>
<td>THi</td>
<td>316</td>
<td>949</td>
<td>3369</td>
<td>760</td>
<td>511</td>
<td>107</td>
<td>283</td>
<td>28</td>
<td>265</td>
<td>559</td>
<td>98</td>
<td>589</td>
<td>2924</td>
</tr>
<tr>
<td>MiPaj</td>
<td>16739</td>
<td>4663</td>
<td>946</td>
<td>721</td>
<td>21707</td>
<td>39</td>
<td>796</td>
<td>7</td>
<td>65</td>
<td>96</td>
<td>139</td>
<td>255</td>
<td>1421</td>
</tr>
<tr>
<td>TLC80</td>
<td>1667</td>
<td>374</td>
<td>241</td>
<td>577</td>
<td>1135</td>
<td>60</td>
<td>50</td>
<td>2</td>
<td>18</td>
<td>33</td>
<td>120</td>
<td>204</td>
<td>15</td>
</tr>
<tr>
<td>KomPC</td>
<td>1070</td>
<td>1094</td>
<td>1210</td>
<td>1289</td>
<td>7260</td>
<td>64</td>
<td>47</td>
<td>12</td>
<td>71</td>
<td>176</td>
<td>211</td>
<td>1000</td>
<td>33</td>
</tr>
</tbody>
</table>

Table 4
Particle size distribution at the measured samples

<table>
<thead>
<tr>
<th>Diameter, μm</th>
<th>Percentage of the particles in the size range, %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HusTE</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.2 Results of algae-bioassay

For all samples of VEP, we calculated the concentrations that caused 50% inhibition (EC$_{50}$) of vitality, FDA fluorescence intensity, and DiOC$_6$ fluorescence intensity compared to control (Table 5). In the cases when VEP exposure stimulated the measured endpoint for microalgae cells, the data were presented as a percentage increase of that endpoints for the highest used VEP concentration (100 mg/l) compared to control.

Table 5
The calculated EC$_{50}$ value for vitality, esterase activity, and membrane potential of microalgae after VEP exposure, mg l$^{-1}$

<table>
<thead>
<tr>
<th>VEP</th>
<th>Vitality 24-h</th>
<th>96-h</th>
<th>7-d</th>
<th>3-h</th>
<th>24-h</th>
<th>Membrane potential 24-h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Porphyridium purpureum</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HusTE</td>
<td>219.7 (214.5-225)</td>
<td>n/a</td>
<td>n/a</td>
<td>217.8 (210.7-225.3)</td>
<td>stimulation (39-40%)</td>
<td>stimulation (44-58%)</td>
</tr>
<tr>
<td>HonVT</td>
<td>130.1 (127.5-132.7)</td>
<td>n/a</td>
<td>n/a</td>
<td>59.3 (58.8-59.9)</td>
<td>101.2 (100.4-102.1)</td>
<td>218.9 (210.3-228.1)</td>
</tr>
<tr>
<td>TMar2</td>
<td>72.7 (70.1-74.5)</td>
<td>n/a</td>
<td>n/a</td>
<td>246.4 (244.1-248.8)</td>
<td>n/a</td>
<td>155.7 (154.5-156.9)</td>
</tr>
<tr>
<td>THi</td>
<td>9.1 (8.9-9.2)</td>
<td>54.57</td>
<td>n/a</td>
<td>n/a</td>
<td>47.6 (46.7-47.4)</td>
<td>70.6 (73.1-74.2)</td>
</tr>
<tr>
<td>MiPaj</td>
<td>33.7 (33.3-34.2)</td>
<td>149.1</td>
<td>(141.3-157.4)</td>
<td>183 (176.3-190.2)</td>
<td>190.2 (188-192.4)</td>
<td>n/a</td>
</tr>
<tr>
<td>TLC80</td>
<td>31.3 (30.1-31.7)</td>
<td>46.1 (45.1-47.1)</td>
<td>119.3 (114.3-124.6)</td>
<td>50.9 (50.2-51.7)</td>
<td>47.8 (47-48.7)</td>
<td>57.5 (56.9-58)</td>
</tr>
<tr>
<td>KomPC</td>
<td>14.9 (14.5-15.2)</td>
<td>13.6 (13.3-13.9)</td>
<td>70.4 (67.4-73.6)</td>
<td>n/a</td>
<td>62 (61.3-62.8)</td>
<td>19 (18.7-19.3)</td>
</tr>
</tbody>
</table>

Heterosigma akashiwo

<table>
<thead>
<tr>
<th>VEP</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HusTE</td>
<td>n/a</td>
<td>stimulation (23-28%)</td>
<td>125.3 (114.9-132.9)</td>
<td>n/a</td>
<td>144.6 (142.1-147.2)</td>
<td>n/a</td>
</tr>
<tr>
<td>HonVT</td>
<td>n/a</td>
<td>n/a</td>
<td>stimulation (27-55%)</td>
<td>stimulation (6-10%)</td>
<td>stimulation (12-18%)</td>
<td>n/a</td>
</tr>
<tr>
<td>TMar2</td>
<td>n/a</td>
<td>n/a</td>
<td>155.5 (149.8-161.6)</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
</tbody>
</table>
Red microalga *P. purpureum* showed a significantly higher sensitivity to all samples, while in the dynamics from 24 hours to seven days of VEP exposure, the toxicity of all particle samples decreased. The most toxic particles for both microalgae species were the samples KomPC and TLC80, emitted by diesel-powered vehicles with higher displacement. At the same time, KomPC particles demonstrated the highest level of toxicity for *H. akashiwo* in the dynamics of all three measurements (24-h, 96-h, and seven days). High acute toxicity (24-h and 96-h) of KomPC sample and a lower level of chronic toxicity (seven days) was registered for *P. purpureum*.

The particles THi and MiPaj caused death and a significant reduction of growth rate only for *P. purpureum*. For *H. akashiwo*, THi particles showed a decrease in the total number of algae cells by 30–35% at a concentration of 100 mg/l for only 24 hours of exposure followed by a decline of this negative effect. The exposure of MiPaj particles to *H. akashiwo* did not cause a response to any of the observed toxicity endpoints. HusTE particles at a concentration of 100 mg/l had a positive effect on the growth rate of *H. akashiwo* at 96 hours of exposure, followed by a slight inhibition for seven days of exposure.

For *H. akashiwo*, we did not observe the inhibition of esterase activity. At 24 hours of exposure at a concentration of 100 mg/l of HusTE particles, inhibition of FDA fluorescence was 26-28% as compared to control. All the other VEP samples did not inhibit FDA fluorescence of *H. akashiwo*, while the HonVT and THi particles caused a slight increase in cell esterase activity.

For *P. purpureum*, TLC80 and HonVT particles were the most toxic at both three and 24 hours of exposure, according to the significant decrease of microalgae esterase activity. The particle sample KomPC caused considerable inhibition of FDA fluorescence for *P. purpureum* at 24 hours of exposure.

The highest decrease of membrane potential for both microalgae species was registered under the influence of KomPC particles. Also, significant inhibition of DiOC₆ fluorescence was recorded for both algae species under the exposure of THi and TLC80 particles.

The changes in the size of algae cells under the exposure of VEPs are presented in Fig. 2. The size distribution for red algae *P. purpureum* is displayed in the ranges of 4-6, 6-10, and 10-15 µm. In the control group of *P. purpureum* 55-60% of cells were in the size range of 6-10 µm and 31-35% of the cells were in the size range of 4-6 µm. The size distribution of *H. akashiwo* cells was in the ranges 6-10, 10-15, and more than 15 µm. In the control group for *H. akashiwo* 68-70% of cells had the size range of 10-15 µm, and 30-31% of cells were in the size range of 6-10 µm.
Fig. 2. The size distribution of microalgae cells after VEP exposure
* – VEP samples that caused the most significant changes in algae cell size distribution compared to control.

KomPC, TLC80, and THi particles caused the most significant size enlargement of both microalgae species. For *P. purpureum*, TLC80 particles at a concentration of 100 mg/l caused an almost complete disappearance of cells in 4-6 µm size range and increased the cells in size range of 6-10 µm to 91–93%. KomPC and THi particles provoke an increase in the size of *P. purpureum* cells to 10–15 µm (56–64% for KomPC and 8–14% for THi at particle concentrations of 100 mg/l) for 96 hours of exposure, following with a decrease in effect to 7-day measurement. Unlike *P. purpureum*, KomPC, TLC80, THi, and HonVT particles lead to *H. akashiwo* cell swelling up to the fraction of more than 15 µm on the seventh day of the exposition.

Morphological changes in algae cells under seven-day exposure of VEP, which caused the most pronounced effects are presented in Fig. 3.
4. Discussion

An initial objective of this research was to identify the toxic effects of particulate matter emitted from different types of vehicles using two marine microalgae species as test organisms of the bioassay. It should be noted that both microalgae species were highly sensitive to the particles emitted by diesel-powered vehicles (KomPC, TLC80, and THi). These samples had the highest impact on survival, esterase activity, and membrane potential of microalgae, and they caused the most significant increase in microalgae cell size compared to the other VEP samples. The samples of VEP produced by gasoline-powered vehicles (HusTE, HonVT, TMar2, and MiPaj) showed a low level of toxicity for all observed endpoints or absence of effects even at higher concentrations (Table 5). Our findings are in accord with the work of Wu et. al (2017) where authors demonstrated a higher toxic level of the particles emitted by diesel fuel combustion for human lung cells A549 compared to the particles emitted by gasoline combustion.

These results strongly correlate with the data of laser granulometry analyses (Table 4), which indicate that samples THi, TLC80, and KomPC have a significantly higher percentage of the particles in the size range less than 1 µm (54.0, 33.7, and 88.5%, respectively). In their recent work, Shen et al.
(2019) demonstrated that fine particulate matter in the size range smaller than 1.1 µm collected from the air of 10 urban, sub-urban, farmland, and background sites of China had significantly higher toxicity compared to bigger particles. Also, authors have shown that fine particles in that size range more intensively accumulate polycyclic aromatic hydrocarbons and represent high lifetime excess cancer risk for humans (Shen et al., 2019). The same results of sub-micron particles’ superior toxicity were demonstrated in an aquatic area with zebrafish embryotoxicity test (Mesquita et al., 2014), for human lung cells A549 (Druga et al., 2014), and for mice lung and kidney (Wardoyo et al., 2018). Therefore, the presence of high number of particles of less than 1 µm in VEP samples THi, TLC80, and KomPC should be considered as the most probable reason for their high toxic influence on microalgae (especially on vitality, membrane potential, and cell-size change, Table 5). We also observed an increase in the level of particle toxicity with an increase in engine displacement (Table 1 and Table 5).

In this study, the visual observation of microalgae cells by an optical microscope showed that samples KomPC, THi, and TLC80 intensively agglomerated with microalgae cells (Fig. 3). Apparently, that agglomeration increases the role of physical effects as the most probable mechanisms of toxicity (Ma et al., 2014). Meanwhile, these samples can easily cohere in an aquatic environment resulting in enlargement of individual particles and cause subsequent decreasing of toxicity as it was shown in chronic toxicity tests for all particle samples (Table 5).

The role of oxidative stress, caused by metal residuals in the particles, also might be an important aspect of aquatic toxicity (Pikula et al. 2019a). Oxidative stress induced by particulate matter from diesel-powered vehicles in bacteria, algae, daphnids, and fishes was demonstrated in the following research works (Li et al., 2009; Correa et al., 2017). Moreover, a number of studies displayed the influence of metals on the growth rate of microalgae and their direct and indirect toxic effects (Joonas et al., 2017; Miazek et al., 2017). Correa et al. (2016) showed the phytogenotoxic effect of particulate emissions from heavy-duty diesel-powered vehicles associated with the presence of metals and organic constituents.

Regarding the content of metals indicated in the samples KomPC, TLC80, THi (Table 3), we cannot highlight the predominance of any specific elements compared to the other samples. Moreover, the samples TMar2 and MiPaj, which demonstrated lower toxic influence on the algae, significantly exceed the other samples regarding chromium and zinc content, and the sample HusTE surpasses all other samples regarding magnesium and nickel content. Therefore, within the scope of this research, it is impossible to evaluate the direct influence of metal content in the VEP to their aquatic toxicity. However, the impact of metal combinations and their synergetic effect might be the highly promising subject of further investigations.

Earlier, we defined a high sensitivity of red algae P. purpureum as test-object of bioassays using common reference toxicant potassium dichromate (Pikula et al., 2019b). It matches the fact that P. purpureum demonstrated more pronounced responses to all particle samples than H. akashiwo. Also, it was shown that H. akashiwo can produce reactive oxygen species as a mechanism of defense (Twiner et al., 2001). At the same time, the ability of P. purpureum cells to produce hydrophobic biofilm (Oakley and Dodge, 1974) promotes the gradual decline of VEP toxicity to the algae cells over time, as indicated in Table 5.

Cell membrane depolarization was registered by flow cytometry in the case when the internal environment of the cell becomes less electronegative relative to the external environment, in
comparison with the control, which indicates a violation of the normal functioning of the cell membranes (Franklin et al., 2001). Cell wall deformation and damage of its integrity are very dangerous for cells. Cell membrane dysfunction can be expressed in terms of changes in its elasticity, the disappearance of lipid microdomains, changes in cation permeability, and various changes in other characteristics. In turn, the above-mentioned effects lead to various diseases or make algal cells easily susceptible to pathogens attacks (Maresova et al., 2009). KomPC sample demonstrated the most pronounced depolarization of the cell membranes for both microalgae species. This effect was produced by the highest concentration of sub-micron particles in the sample (Table 4) and by the intensive agglomeration of the particles with microalgae cells (Fig. 3).

Inhibition of the esterase activity was observed only for *P. purpureum*. In this case, the most pronounced effect was observed for the samples TLC80 and KomPC, that indicates a deficit of enzyme activity since esterases that decompose FDA to fluorescein are important elements in the metabolism of cell membrane phospholipids and they are often used as an indicator of sublethal toxicity (Li et al., 2011; Melegari et al., 2013).

Based on the results presented in this study, all samples of VEPs can be arranged in descending order of hazard level (EC50 concentrations in mg/l for more sensitive species to each pollutant are given in the parentheses; in the cases where inhibition was not indicated we used the symbol n/a).

**Vitality**: KomPC (48,8) > THi (54,6) > TLC80 (119,3) > HusTE (125,3) > TMar2 (155,5) > MiPaj (n/a) > HonVT (stimulation);

**Esterase activity**: TLC80 (47,8) > KomPC (62) > HonVT (101,2) > HusTE (144,6) > MiPaj (190,2) > THi, TMar2 (n/a);

**Membrane potential**: KomPC (25,3) > THi (68,1) > TMar2 (105,9) > TLC80 (125,2) > MiPaj (165,5) > HonVT (169,7) > HusTE (n/a).

Therefore, the environmental risk of VEP exposure for marine phytoplankton depends on used fuel (in descending order): diesel fuel, Russian standard AI-92 gasoline (lower octane number), Russian standard AI-95 gasoline (higher octane number). Furthermore, the toxicity of VEP rises with the increasing vehicle engine displacement, considering all other conditions as constant.

5. Conclusions

Our results demonstrated that the greatest risk to studied microalgae species was represented by particulate matter emitted from vehicles running on diesel fuel. Moreover, VEP samples, collected from the diesel-powered vehicle have the highest number of particles in the size range of less than 1 µm, and particles of these samples intensively agglomerate with microalgae cells. These facts and a lack of detectable dependence of toxicity on metal content in the samples confirm that physical damage is the most pronounced toxic effect of VEP. There was also determined the increase of exhaust gas particles toxicity with an increase in vehicle engine displacement. Despite the difference in microalgae sensitivity and their probable opportunity for adaptation to the toxic influence, the above-mentioned conclusions can be applied to both algae species.

We believe that presented results are important for planning further detailed strategies for VEP risk assessment and for stable maintaining of environmental and human safety.

Acknowledgements
This work was supported by the Russian Science Foundation (No. 15-14-20032-P).

The authors would like to thank the staff team of FEFU CCU (Center of Collective Use) Interdepartmental Center for Analytical Control of the Environment for their dedicated involvement in this study. Authors are also thankful to Dr. Muhammad Amjad Nawaz (Education and Scientific Center of Nanotechnology, Far Eastern Federal University, Vladivostok, Russian Federation) for his help in copyediting and English language editing of this manuscript.

References


**Highlights:**

- Vehicles running on diesel fuel emit particles with higher aquatic toxicity
- Vehicle emitted particles toxicity rises with an increase of engine displacement
- Vehicle emitted particles agglomerate with microalgae producing physical damage
- Aggregation of particles from diesel-powered vehicle can reduce chronic toxicity