Detoxification potential and rehabilitation of activated sludge after shock loading of Sofia’s wastewater treatment plant ‘Kubratovo’ with mazut

Yana Topalova, Yovana Todorova, Irina Schneider, Ivaylo Yotinov and Vesela Stefanova

ABSTRACT

The shock loading of wastewater treatment plants (WWTPs) with toxic pollutants remains a critical problem with crucial significance for the technologies. On 5 November 2014, 30 tons of mazut were emitted in Sofia’s WWTP ‘Kubratovo’, passing through equipment and damaging the functioning of the technological modules. The rehabilitation of activated sludge (AS) after shock loading as well as the development of detoxification activity were investigated. The hydrocarbon index of petroleum products, filamentous index (FI), sludge biotic index, sludge volume index (SVI), chemical oxygen demand (COD), biochemical oxygen demand (BOD5), oxygenases and succinate dehydrogenase activities were analyzed for a period of two weeks. The results show that independently from prolonged rehabilitation period, AS remained with filamentous bulking (SVI over 200 ml/g and FI over 1.107 μm/mg). At the same time, the detoxification potential of the AS was developed. Although the morphological and functional structure was still not fully recovered, the AS developed two adaptive mechanisms. First, activation of shorter, more effective ways for benzene ring cleavage, operated by catechol 2,3-dioxygenase; second, strong increase of succinate dehydrogenase activity, which is consistent with the activation of the degradation of trivial substrates for energy generating to overcome the intoxication and synthesis of oxygenases.

Key words | activated sludge, functional indicators, mazut, oxygenase activities, shock loading, Sofia’s wastewater treatment plant

INTRODUCTION

Pollution with oil and oil products as a result of spills is an increasing threat to the environment, human health, tourism and all living organisms. There are several known environmental disasters in the world related to oil, the largest being in California (Lakeview) – a spill of 1.2 million tons of oil and the Gulf of Mexico – 585,000 tons in 2010. The consequences of such catastrophes exert influence after decades, even centuries on the environment. It is estimated that the total spilled oil in the world is about 37 billion barrels, exceeding the amount of the consumed one (30 billion barrels) (Wang et al. 2011).

Mazut is a product from the dark heavy fractions of the liquid fuels, which are still widely used for heating. With respect to the hydrocarbons in the heavy oil fractions, the greatest amount is that of saturated and aromatic hydrocarbons (Okoh 2006; Abdel-Shafy & Mansour 2016). Some of the most dangerous components of the oil are aromatics (including benzene, xylene, toluene, ethylbenzene, phenol, naphthalene, other PAHs), as well as some intermediate metabolites formed during their biodegradation (Margot et al. 2015; Pintor et al. 2016). Benzene and benzoyprene have carcinogenic effects even at low concentrations (Sholz et al. 1999; Edokpolo et al. 2015).

The advent of petroleum products, in particular mazut in municipal wastewater treatment plants is detrimental to the structure and functions of the activated sludge (AS), deforms the purification process for a long time and is accompanied by risks of pollution of the water receivers with toxic pollutants (Amin et al. 2012). In a laboratory simulated wastewater treatment process of a 12-hour leak of toxic...
pollutants, in this case pentachlorophenol (PCP) in high concentration in a continuous system (biobasins and secondary settling tanks), the wastewater purification process is strongly deformed. Although the elimination of xenobiotic is about 60%, it is mainly due to the accumulation in AS and leakage of pollutants into the water receiver (Topalova 2009; Dimkov et al. 2017). The actual transformation is only about 10%. Upon impact with xenobiotics on the AS, important changes have been identified, such as deflocculation with a strong reduction of the skeleton, increase of the filamentous microorganisms and decrease in the representatives of micro- and metafauna (Kozuharov et al. 2003b; Sowinska et al. 2017). This is accompanied by further multiplication of homogeneous bacteria that quickly adapt to resistance and form a newly emerging component – a thick layer of homogeneous cells, which leads to impaired settling ability of the sludge (Topalova & Dimkov 2003).

A key role in the assessment and management of the rehabilitation and detoxification processes in the AS is played by a complex of indicators that can be applied for fast, effective and efficient diagnostics of the extent of damage to the AS and its potential to rehabilitation. The professional combination of appropriate methods for control of the rehabilitation and adaptation detoxification activity of sludge after shock loading with toxic xenobiotics is an important element in the management of such risk events. There is an increased use of the enzymological methods for diagnostics of the rate and mechanisms of biodegradation of toxic aromatic compounds (Topalova & Dimkov 2003; Topalova 2007; Gupta et al. 2015).

The purpose of this article is through a set of structural and functional indicators to assess the rehabilitation of the AS and the treatment process, as well as the development of detoxification sludge potential after shock loading of 30 tons of mazut in the WWTP ‘Kubratovo’ in Sofia (for purification of domestic wastewater of the capital Sofia in Bulgaria). The incident dates back to 5 November 2014 and in its scale is one of the most serious technological risk situations worldwide. At this critical accidental shock loading of mazut, the normal function of treatment facilities and biological system is damaged. The accent in experimental analyses is put on structural and enzymological parameters of AS as essential primary tools for realization of key biotic transformations. The successful recovery of biological system firstly needs improvement of its structure and development of wide enzyme profile and then the recovery of all metabolic functions can be registered. The biological stage in WWTP ‘Kubratovo’ is mainly based on the processes of nitrification and denitrification, as biobasins are divided into denitrification and nitrification zones. In the paper there is a purposeful selection of data on the examined indicators in the denitrification zone where wastewater initially enters and the sludge in it first meets mazut as a risk pollutant.

**MATERIALS AND METHODS**

After the accidental inflow of mazut in WWTP ‘Kubratovo’ the management of the plant made the necessary arrangements for action according to the damage risk, which included the addition of adsorbents in the aerated grit chambers and limiting as far as possible the entry of mazut in the biobasins. Sampling was conducted from the 16th to the 25th day after the occurrence of the risk situation for 10 days that we considered as rehabilitation period. The operator’s regular monitoring (data not presented here) showed that up to 7–8 days after the incident, the high concentrations of mazut (18 mg/L) were still registered at the entrance; afterward the 10th day the input concentrations were stabilized and retained at normal level (compared to these before the incident <0.2 mg/L). The presence of significant quantities of petroleum products visibly persisted for another week in the treatment facilities. This is the reason we choose this period for assessment of rehabilitation of AS – after the main passing of mazut through the treatment plant, normalization of the pollutant concentrations input and initial stabilization in facilities’ operation.

We traced the reaction of the sludge during the 10-day rehabilitation and recovery, and two phases of adaptation were differentiated. The first studied period is related to the early adaptation of AS to mazut (I AD). Questions to be answered here are the following: Is the xenobiotic deformed AS structure 16 days after the shock load? Is there deterioration in the conditions of biobasin based on the indicator groups of communities of micro- and metafauna? How has AS adapted to the degradation of the pollutant and what is its initial adaptive response in enzymological aspect? The next stage of adaptation (II AD) shows the late response of AS and how it improves its mechanisms for biodegradation of xenobiotic pollutant. The questions we should seek answer are: Is the structure of AS restored after the initial period of adaptation? Is the rehabilitation of the AS related to the restructuring of the communities and their activity, and in what direction?” To compare the studied indicators of sludge in both periods of adaptation we used AS from the same treatment plant but in normal
work mode (without registering xenobiotics in high concentrations, which can have a negative impact on the AS and the treatment process).

The studied parameters of the wastewater treatment processes and methods for their determination are presented in Table 1. The chemical indicators track the concentration of key pollutants. The hydrocarbon index (HCI) is used to specify the concentration of mazut in water and in biomass of AS; COD and BOD$_5$ to determine the total concentration of organics and biodegradable organics. COD and BOD$_5$ are determined on a mixed sample (AS and water). The enzymological parameters (activity of phenol 2-monooxygenase, catechol 1,2-dioxygenase, catechol 2,3-dioxygenase, protocatechuate 3,4-dioxygenase and the total dioxygenase and succinate dehydrogenase) are tested to determine the detoxification potential of the AS and its alteration in the rehabilitation stages.

Table 1 | Investigated parameters and methods for analysis

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Method</th>
<th>Description of method</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCl, mg/L</td>
<td>Internal laboratory method of Sofiyska voda JSC</td>
<td>Determination by gas chromatography.</td>
</tr>
<tr>
<td>Chemical oxygen demand (COD), mgO$_2$/L</td>
<td>APHA/AWWA/WEF (2012)</td>
<td>Method with K$_2$Cr$_2$O$_7$ with device WTW CR-2010.</td>
</tr>
<tr>
<td>Biochemical oxygen demand (BOD$_5$), mgO$_2$/L</td>
<td>BDS EN (1899)-1:2004</td>
<td>Determination of the BOD$_5$ by dilution and seeding with suppression of nitrification with addition of N-allythiourea.</td>
</tr>
<tr>
<td>SVI, ml/g</td>
<td>BDS EN 14702-1:2006 (2006)</td>
<td>Gravimetric method for the determination of the settleability of AS.</td>
</tr>
<tr>
<td>Filamentous index (FI).10$^7$, µm/mg</td>
<td>Sezgin et al. (1978)</td>
<td>Microscopic examination of the total length of filaments in AS to biomass quantity measured as mixed liquor suspended solids.</td>
</tr>
<tr>
<td>SBI</td>
<td>Madoni (1994)</td>
<td>Microscopic examination of qualitative and quantitative characteristics of microfauna in AS.</td>
</tr>
<tr>
<td>Biodiversity of micro- and metafauna</td>
<td>Foissner &amp; Berger (1996)</td>
<td>Microscopic examination of number of taxa and quantity of different groups of organisms from micro- and metafauna in AS.</td>
</tr>
<tr>
<td>Phenol 2-monooxygenase activity (P2MO), µMol/min-mgPr (EC.1.14.13.7)</td>
<td>Neujahr &amp; Gaal (1975)</td>
<td>Spectrophotometric method by measuring the decrease of NADPH$_2$ absorption at 340 nm.</td>
</tr>
<tr>
<td>Catechol 1,2-dioxygenase activity (C12DO), µMol/min-mgPr (EC 1.13.11.1)</td>
<td>Willets &amp; Cain (1972)</td>
<td>Spectrophotometric method by measuring the accumulation of the ring-fission product cis-cis muconic acid at 260 nm.</td>
</tr>
<tr>
<td>Catechol 2,3-dioxygenase activity (C23DO), µMol/min-mgPr (EC 1.13.11.2)</td>
<td>Farr &amp; Cain (1968)</td>
<td>Spectrophotometric method by measuring the accumulation of the ring-fission product 2-hydroxymuconic semi-aldehyde at 375 nm.</td>
</tr>
<tr>
<td>Protocatechuate 3,4-dioxygenase activity (P34DO), µMol/min-mgPr (EC 1.13.11.3)</td>
<td>Fujisawa &amp; Hayaishi (1968)</td>
<td>Spectrophotometric method by measuring the decrease of the protocatechuate at 290 nm.</td>
</tr>
<tr>
<td>Total dioxygenase activity (TDO), µMol/min-mgPr (EC 1.13.11.3)</td>
<td>Topalova (2009)</td>
<td>TDO is a mathematical sum of C12DO, C23DO and P34DO.</td>
</tr>
<tr>
<td>Succinate dehydrogenase activity (SDH), µMol/min-mgPr (EC 1.3.5.1)</td>
<td>Veeger et al. (1969)</td>
<td>Spectrophotometric method by measuring the accumulation of the product fumarate at 455 nm.</td>
</tr>
</tbody>
</table>

SBI determination was based on counting the total quantity individuals in unit volume, total number taxa, total number of small flagellates and determination of dominated groups of protozoan organisms in AS. The biodiversity of micro- and metafauna was analyzed by determining the quantity of protozoan organisms (naked and testate amoebae, flagellates, free-swimming ciliates, creeping ciliates, stalked ciliates, suctorian) and metazoan organisms (rotifers and nematodes). Mixed liquor suspended solids for calculation of sludge volume index (SVI) and filamentous index (FI) was determined by a standardized method (APHA 2012). The biomass for the measurement of enzyme activities was harvested according to the method described by Feist & Hegeman (1993). Enzyme activities were presented as µMol/min-mg protein and protein concentration in the cell extracts was specified by the micro-biuret method (Kochetov 1980).
RESULTS AND DISCUSSION

Part 1: Dynamics of key pollutants and basic technological indicators after shock entering of mazut in WWTP ‘Kubratovo’

This part of the article studies the dynamics of indicators 16 days after the accidental inflow of mazut. The 10-day period is divided into two phases. The first adaptation phase is carried out between the 16th and the 20th day of entry of the xenobiotic, and the second adaptation phase takes place between the 21st and the 25th day.

The concentration of key toxic pollutant is assessed by HCl that gives information about the concentration of oil products, including mazut (Figure 1). The data for the values of index in water phase and in sludge was presented for simultaneous assessment of processes of accumulation/deaccumulation. In the first adaptation phase, it was found that the hydrocarbon concentration in water decreases about four times, after which at the end of the phase an increase by about seven times was registered. In the second examined period we again registered a decrease of HCl, but the reduction is abrupt, followed by a sharp rise in values. At the end of the second adaptation phase, there is a reduction of the concentration of xenobiotic in water phase with values close to those from the 19th day after the shock loading (the end of the first adaptation phase). The opposite dynamics of this index was registered in the AS. The obtained results show that the key pollutant does not remove gradually but we register processes of adsorption and absorption in the biomass, followed by desorption. An important role in these processes is played by the complexes of microfauna and metafauna of AS. They absorb much of the pollutant and die while promptly releasing it in portions and thus contribute to the adaptation of other organisms in the AS, mainly microorganisms. Thus, segments of micro- and metafauna play the role of ‘distributor’ of toxic pollutants and ‘protector’ for the microbial segment of the AS. Similar results were obtained in the study of detoxification processes in shock load with model xenobiotics such as chlorophenols and nitrophenols (Topalova 2009). Topalova (2009) found that the removal of xenobiotics from water is mainly due to the accumulation of AS that is adapted not to biodegradation of xenobiotics but to development of resistance (Topalova 2009). The latter is associated with structural changes in the AS such as: increase of the amount of bacteria that are able to degrade xenobiotics (genus Pseudomonas and genus Acinetobacter), as well as reducing the amount of the more sensitive representatives of micro- and metafauna, and increase of more resistant groups of organisms (Kozuharov et al. 2003a, 2003b; Topalova et al. 2005; Topalova 2009). After the xenobiotic enters the biobasin, part of it is accumulated in and onto flocs; possibly manifesting the effect of biomagnification and thus the negative effect is strengthened. The pictures of the structure of AS (Figure 2) clearly show residual mazut drops on the flocs. It was also found that the flocs size decreases in the transition to the second adaptation stage, which is typical of conditions with the presence of toxic pollutants (Topalova 2009; Schneider et al. 2011). Overall, the presented data show accumulation of mazut by the AS and development of resistance, but not the actual...
process of biodegradation. The reason for this is the hydrophobicity of the molecule and the high molecular mass, which lead to poor bioavailability of mazut for microorganisms on the one hand, and its relatively high concentration, which is obviously above the critical one for the studied sludge, on the other.

The concentration of mazut in water decreases in the first phase (Figure 1(a)) while COD increases at the same time (Figure 1(b)). The data show that as a result of accumulation of xenobiotic in the flocs of AS there is registered an inhibition of the biological system to degrade even the trivial organic pollutants in wastewater. As it can be seen from Figure 1(b) the processes of degradation of the biodegradable organic matter, measured as BOD₅ are also inhibited. At the end of the first studied period (20th day) the sharp increase in HCl as a result of its release in the aqueous phase is accompanied by a sharp reduction of COD and BOD₅. Thus the removal of the inhibitory factor from AS promotes partial biodegradation of ordinary contaminants and the concentration of COD and BOD₅ decreases. At the beginning of the second adaptation phase, after the sharp decrease of the HCl (21st day), we registered a sharp increase of COD and BOD₅. Here again we consider the inhibitory effect of the pollutant on the metabolization of the biodegradable organic matter due to the absorption of oil drops in the AS. This effect of absorption and release of oil droplets from the AS is known as the ‘sponge effect’. After 21 days, the values of HCl fluctuate in a wide range, while COD and BOD₅ permanently increase and retain relatively stable values. The results show inhibition of AS. It was found that the reported concentrations of mazut in the biobasins, albeit relatively low, are above the critical ones for this AS and they have an inhibitory effect on the overall metabolic potential of AS. Thus, the biological system develops resistance to the toxic pollutant, as the long-term inhibitory mechanism is connected to the partial inhibition of the central metabolism and probably to blocking the synthesis of energy in the form of adenosine triphosphate (ATP).

In the denitrification zone throughout the studied period there are no sharp fluctuations in the value of the SVI, which varies around 200 mL/g (Figure 3(a)). The obtained results show that in both periods AS has a deformed structure, and the deformation is related to the bulking of the AS. As it can be seen from the data on FI which is greater than $1.10^7 \mu\text{m}/\text{mg}$ the bulking is a filamentous one (Sezgin et al. 1978). In the first adaptation phase SVI increased until the 18th day as a result of accumulation of the xenobiotic in biomass. The next peak is recorded on the 21st day, which again is accompanied by a reduction in the HCl and the absorption processes in the flocs (Figure 1(a)). By the end of the studied period we established small variations in the values of SVI, which indicate that the deformations in the structure of the AS remain. These results are probably due to the fact that the key pollutant is accumulated by AS and biodegradation processes are severely hampered. The adaptation is aimed primarily at developing resistance. Unlike the SVI, in the FI we established a more sudden change. The deformation in the structure of AS is more pronounced during the first adaptation phase, as it can be seen by the high values of the FI. In the second adaptation phase, we registered improvement, although the deformation is retained.

The reason for the excessive development of filamentous microorganisms can be found in the change in the
ratio between floc-forming and filamentous microorganisms. With the accumulation of mazut in the biomass there is an increased toxic effect of the xenobiotic on the floc-forming microorganisms and increased amount of the more resistant microorganisms. Filamentous microorganisms are known to be more resistant to toxic pollutants due to their low half saturation constant and the ability to use alternative sources of energy to carry out their metabolic processes. For example, the genera *Thiothrix* and *Beggiatoa* have intracellular sulfur granules, which accumulate nutrients and sulfur, which are used as an additional source of energy and electron donor (Bitton 2005). As a result of biotic and abiotic processes, part of the mazut is degraded with release of sulfur compounds that are utilized by the filamentous microorganisms. *Sphaerotilus natans*, another filamentous microorganism, commonly found in AS, can contain between 11 and 20% of its dry weight intracellular inclusions, containing a supplementary source of carbon as poly-β-hydroxybutyrate. The bacterial cell wall may also be a factor in the dominance of filamentous organisms because it makes them more resistant to toxic pollutants. For example, genus *Nocardia* is resistant to xenobiotics because actinomycetes inherently have a thicker cell wall, which prevents the damaging effect of the toxic compounds (Bitton 2005). The low half saturation constant of the filamentous microorganisms (e.g. *Microtrix parvicella*) is another important factor that leads to competition for substrates with floc-forming organisms at low concentrations of biodegradable organics (Bitton 2005). In the early phase of the process, in the course of two days, we reported lower values of BOD$_5$/COD (below 0.5), which indicates a decrease in the percentage of biodegradable organics (Figure 3(b)). Precisely in this first phase, the dominance of filamentous microorganisms is more pronounced (Figure 3(a)).

Sludge biotic index (SBI) determines the quality of AS based on the quantitative and qualitative characteristics of microfauna, its value ranging between 0 and 10. The method for determining the index is based on two main principles. First, changes in the dominant groups of protozoa are in direct correlation with changes in operating parameters in the reactors. Second, biodiversity and abundance of taxa decreased with the effectiveness of the treatment process (Madoni 1994). In the period, SBI ranges between 8 and 10, indicating that the sludge has a high biodiversity and the purification process should be carried out at relatively normal conditions. To establish this, however, it is necessary to identify which groups of micro- and metafauna are dominant. The index shows that during the first stage of adaptation the quantitative and qualitative characteristics of microfauna is more stable and has higher range (SBI around 10) compared to the second period of study (SBI is 8). The index value drops on the 20th day, which is associated with a sharp rise in the HCI in water (Figure 1(a)); i.e. as a result from the accumulation of mazut in flocs until the 19th day the xenobiotic has strong negative effect on the microfauna and after some of them die the mazut is released in portions in the aqueous phase. In the second adaptation phase, there is a decrease in the biodiversity of protozoa and SBI is lower (Figure 3(b)).

On the 18th day after the shock entering of mazut, we registered a sharp decrease in the amount of attached ciliates (Figure 4), leading immediately to a sharp rise in BOD$_5$ (Figure 1(b)). For sludges with a reduced amount of ciliates it is typical that the waters have high turbidity and a high concentration of biodegradable organic matter, as
well as an increased amount of suspended bacteria in the water, since here the predatory press of ciliates on microorganisms is removed (Madoni 1994). In the transition between the first and the second adaptation phase (20–21 days after the entry of mazut) we recorded restructuring of communities, which is associated with permanent change in the dominant groups (Figure 4). So at the beginning of the second adaptation phase, the stalked ciliates are replaced by the creeping ones, which indicate deterioration in the quality of the AS (Madoni 1994; Pedrazzani et al. 2016).

Upon deformation of the structural level the presence of xenobiotics leads to disintegration of the floc structure of the AS (Figure 2), and in this case the more affected ones are the stalked ciliates, which are attached on the surface of the flocs (Figure 4). This deformation is detected in the presence of azodyes and heavy metals, chlorophenols, etc. (Kozuharov et al. 2003a, 2003b). The increase of the creeping ciliates is probably related to the increase of the free area of the flocs (higher number of flocs with a smaller size), which provides more food substrate in the form of easily detachable particles of the flocs. The change in the amount of free-swimming ciliates is also important. It was found that when switching to second adaptation phase, their number slightly increases. It was established that free-swimming and stalked ciliates are in a competitive relationship since the two groups feed with suspended in the aqueous phase bacterial cells (Madoni 1994). After reducing the amount of stalked ciliates competition grows weak and the free-swimming ciliates increase their share. Another favorable factor for their development is reducing the size of flocs (Figure 2(b)) in the second adaptation phase, which leads to increased homogeneous cells in the water. In this regard we also found an increase in the amount of flagellates, which is an indicator of the presence of poorly degradable compounds in the biobasin. Jenkins et al. (2004) report that the stalked ciliates, rotifers and nematodes indicate low ratio of food substrates to microorganisms and low organic load. In the second phase these groups decreased and rotifers even disappear, which indicates a high organic load and disturbed treatment process. Flagellates, amoebas and free-swimming ciliates in this phase increase, which indicates a higher organic load and presence of toxic substances (Foissner 2016). The deflocculation of the sludge and the increase of the microorganisms dispersed in water increase the nutritional substrates for flagellates and free-swimming ciliates.

**Part 2: Comparison of the data for the first and second adaptation phase with the data from the control situation (before the entry of mazut in WWTP)**

The results show that the parameters indicating rehabilitation of the AS during the first phase of adaptation are elevated in comparison to the control situation. This applies to HCl, BOD₅, COD, SVI, FI, SBI (Table 2).

Figure 5 below shows the restructuring of complex of micro- and metafauna with the increase of the adaptation time, i.e. comparing the change in the structure of the complex between the first and the second phase of adaptation. It was found that in both phases, despite the regrouping of the dominant groups, the highest percentage share (over 70% of all studied groups of protozoan and metazoan) is had by the
ciliates. When analyzing the results, the following indicative fact becomes clear. In the second adaptation phase the stalked ciliates decrease by 30%, and creeping and free-swimming ciliates increase by about 13% and 5%, respectively. This result indicates that the AS recovers slowly, as a structure and in its trivial functions as well. Madoni (1994) states that the higher number of creeping ciliates (over 2.10^5 ind./L) is associated with SVI less than 200 ml/g, while the significant reduction in their number increases the SVI (over 400 ml/g). In both phases SVI is about 200 ml/g (Table 2), which corresponds well with the high levels of this group of protozoa (Figure 4) reaching up to more than 1.10^6 ind./L. The higher number in the second adaptation phase leads to slightly lower values of SVI.

Another important indicator is the free-swimming ciliates that survive better than other groups of microfauna in the presence of toxic pollutants; often in such conditions the amount of flagellates increases as well (Madoni 1994). During the second adaptation phase the share of free-swimming ciliates increases by 5%, and the one of flagellates by 7%. The share of testate amoebae increases probably due to their high resistance to xenobiotics as it has been found by Topalova et al. (2003) and Kozuharov et al. (2003a, 2003b) in the presence of latex or PCP in wastewater. In the second adaptation phase rotifers disappear, which is a sure indication that mazut and its metabolites have a strong negative impact on the complex of the metafauna. There is a confirmation of the findings in previous studies that contaminants with aromatic structure damage the filtration apparatus of Rotifera (Kozuharov et al. 2003a, 2003b; Topalova et al. 2003).

The results show that despite the elapsed period of 16 days, AS is still in a phase of damage due to the experienced intoxication shock. The detoxification potential, indicated by the activity of the oxygenases and succinate dehydrogenase in this first phase of adaptation shows a reduction of the oxygenase activity as P2MO, C12DO, P34DO and TDO. These enzyme activities also promote inhibition of the detoxification potential of AS compared to that of a normally functioning sludge, which relates to ortho-mechanism for cleavage of the benzene ring. At the same time in this first adaptation phase the meta-mechanism for cleavage of

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### Table 2

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Reference values for control situation</th>
<th>I AD</th>
<th>II AD</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCl, mg/L</td>
<td>below 0.04</td>
<td>0.18</td>
<td>0.16</td>
</tr>
<tr>
<td>BOD₅, mgO₂/L</td>
<td>1.150.00</td>
<td>2.164.21</td>
<td>3.044.71</td>
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<td>COD, mgO₂/L</td>
<td>2.915.43</td>
<td>3.181.00</td>
<td>3.648.20</td>
</tr>
<tr>
<td>SVI, ml/g</td>
<td>between 70 and 100</td>
<td>201.64</td>
<td>198.85</td>
</tr>
<tr>
<td>FL.10^7, μm/mg</td>
<td>below 1.00</td>
<td>4.40</td>
<td>1.92</td>
</tr>
<tr>
<td>SBI</td>
<td>over 9</td>
<td>9.60</td>
<td>8.40</td>
</tr>
<tr>
<td>P2MO, μMol/min-mgPr</td>
<td>0.0938</td>
<td>0.0522</td>
<td>0.0899</td>
</tr>
<tr>
<td>C12DO, μMol/min-mgPr</td>
<td>2.5554</td>
<td>1.7637</td>
<td>1.6318</td>
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<tr>
<td>C25DO, μMol/min-mgPr</td>
<td>0.2064</td>
<td>0.2291</td>
<td>0.3579</td>
</tr>
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<td>P54DO, μMol/min-mgPr</td>
<td>0.5401</td>
<td>0.4822</td>
<td>0.2184</td>
</tr>
<tr>
<td>TDO, μMol/min-mgPr</td>
<td>3.3020</td>
<td>2.4751</td>
<td>2.2082</td>
</tr>
<tr>
<td>SDH, μMol/min-mgPr</td>
<td>0.7413</td>
<td>3.0455</td>
<td>4.1498</td>
</tr>
</tbody>
</table>

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**Figure 5**

Restructuring of different groups organisms from micro- and metafauna in AS during first (I AD) and second (II AD) adaptation phase.
the benzene ring is slightly activated, which pathway is shorter, more effective for detoxification, while the enzymes are driven by coordinated synthesis or are predominantly constitutive. This is already a manifestation of the first small adaptive response of AS to detoxification of the aryl-containing hydrocarbons.

In the second phase of adaptation, this tendency of hampered rehabilitation of AS remains, although we find some improvement, estimated by HCl, SVI, Fl, P2MO and SDH. The detoxification activity also continues to be inhibited in comparison to that of the normally functioning AS. And in this case, the increased activity of C23DO is preserved.

While in the first adaptation phase C23DO increases the activity with 11% compared to that of a normally functioning AS, in the second phase of adaptation the same enzyme is activated by more than 73.6% relative to the control (normally functioning AS). Therefore, it is clear that despite the prolonged intoxication of AS and its troubled rehabilitation with the increase of time after the extreme load with mazut, it activates a short and efficient way to degrade the aromatic ring (the meta-pathway for benzene ring cleavage through a key catabolite catechol). This adaptive response is amplified with the increase of the adaptation time. Another fact is also established: the ortho-mechanism for opening the benzene ring for detoxification continues to be inhibited in the second phase compared with the rate of this mechanism in the control and in the first adaptation phase. This makes it possible to offer the hypothesis that during the adaptation of AS to detoxification of the aryl-containing hydrocarbons in mazut, the ortho-mechanism is inhibited and the meta-mechanism of the benzene ring cleavage is activated, which indicates that the adverse conditions direct the adaptive response towards expression of shorter pathways having faster detoxification result. Our assumption will be confirmed later by a deeper analysis of the detoxification potential of the AS.

Part 3: Key enzyme activities directly involved in detoxification of toxic pollutants

The studied enzymological indicators for the detoxification of the aryl-containing contaminants in mazut are the key enzymes of the preparatory pathways of the cleavage of the benzene ring. Parallel to this we also studied the succinate dehydrogenase activity, which is an indicator of the functioning of the cycle of tricarboxylic acids (CTC) on the one hand, and the infusion of the metabolic products of detoxification in the central metabolic pathways, on the other (Figure 6). Tracking the effect of mazut on the activity of these enzyme activities indicates the change in the detoxification function of AS in the course of mazut pollution. Here it is necessary to point out that normally functioning AS has a well developed detoxification function (Das & Chandran 2011). The reason for this is that WWTPs regularly receive and purify contaminants with aromatic structures (phenols, chlorophenols, nitrophenols, detergents, organic solvents, hormones, etc.) which, however, are not in such high concentrations as in the case of accidental entry of oil.

The biotic transformation of aryl-containing xenobiotics is dominantly an aerobic process with crucial stage – incorporation of molecular oxygen by mono- or dioxygenase enzymes to catechols, protocatechuates, gentisates, followed by real ring cleavage (El-Naas 2012; Prdeep et al. 2015). The cleavage is catalyzed by specific metalloenzymes – dioxygenases, divided into intradiol dioxygenases which cleave the ring on ortho-position to the hydroxyl substituent and extradiol dioxygenases which cleave on meta-position (Das & Chandran 2011; Hernández-Lopez et al. 2015). Ortho-pathway is the oxidation of catechol to cis, cis-muconate in a reaction catalyzed by catechol 1,2-dioxygenase (C12DO), and meta-pathway is the oxidation of catechol to 2-hydroxymuconic semialdehyde catalyzed by catechol 2,3-dioxygenase (C23DO). The cleavage of the benzene ring of protocatechuate is carried out by protocatechuate 3,4-dioxygenase (P34DO), with the product β-carboxy-cis, cis-muconate (Topalova 2009; Yotinov et al. 2016). These processes can be realized at the conditions of denitrification at low oxygen concentration. In the denitrification zone, the rate of detoxification processes are lower. The enzyme mechanisms of this low rate detoxification potential present applied and scientific interest.

Figure 7 shows the change of the oxygenase enzyme activities and the activity of the succinate dehydrogenase in the first and the second phase of the AS adaptation to biodegradation of the hydrocarbons in the mazut. It is clear that mazut stimulates catechol 2,3-dioxygenase and the succinate dehydrogenase activities. Catechol 2,3-dioxygenase, which is involved in the first stage of biodegradation of the aromatic ring via meta-mechanism has increased by 73.4% in II AD in comparison to the control variant. Sludge triggers this mechanism with greater force. The increase in succinate dehydrogenase activity, evidence of the operation of the tricarboxylic acids cycle, is significant (by 310.82%) in I AD phase and 459.79% in II AD phase.

For the other three studied enzyme activities, catechol 1,2-dioxygenase, protocatechuate 3,4-dioxygenase and phenol 2-monooxygenase, we found a decline of 5% for
phenol 2-monooxygenase activity in II AD and up to 60% for protocatechuate 3,4-dioxygenase again in the same phase. Summary of the change in the oxygenase and the succinate dehydrogenase activities with exact numerical values is given in Table 3.

The restructuring of detoxification potential, assessed on the enzyme activities of the ortho- and meta-pathway of the benzene ring cleavage, is presented in Table 4. In it, the individual oxygenase activities are calculated as a part of the total dioxygenase activity of the AS. In the control and both phases of adaptation the largest share of the total dioxygenase activity is taken by catechol 1,2-dioxygenase and the ortho-mechanism of the benzene ring cleavage through a key catabolite catechol, which can be defined as the dominant pathway in the whole detoxification mechanism. In I AD and II AD phase it becomes apparent that the
The proportion of catechol 2,3-dioxygenase activity increases. Where as the percentage share of protocatechuate 3,4-dioxygenase decreases in I AD and increases in II AD phase.

The detoxification potential of AS changes in quantitative terms and in mechanisms as follows: C12DO and P34DO are inhibited, but retain relatively high activity and the leading role of the ortho-cleavage mechanism of benzene ring in key catabolites catechol and protocatechuate. This correlates with the fact that the AS retains mazut and its metabolic products, which are in concentrations exceeding the critical one for a given biological system. This was clearly illustrated above with the photographs and the indicators of AS that show its poor functioning (Table 2 and Figure 2). The decrease of the share of C12DO in the total dioxygenase complex is by 4 to 7%, and by 6% in P34DO.

C23DO is activated in both phases of the rehabilitation of AS, which shows that in a critical situation of shock contamination with toxic pollutant with aromatic nature, the microorganisms choose shorter and more rational ways of detoxification and dealing with the intoxication shock, such as meta-mechanism. The relative proportion of the activity of catechol-2,3-dioxygenase in this case is increased by 10% in I AD and by 5% in II AD phase.

Harmonization in operation and synergism of the two stages in xenobiotic detoxification: (1) peripheral metabolism with critical step of aromatic ring cleavage and (2) effective function of the cell central catabolic pathways are the key factors governing the overall mechanism of degradation of toxic pollutants (Topalova 2009). The TDO activity is an indicator for the cleavage rate and for the total detoxification activity; the SDH is an indicator for the entrance of non-toxic intermediate metabolites in central metabolic pathways. The ratio between these two indicators gives information which process is predominant – the real detoxification phase or the subsequent metabolism of the aliphatic products. The quantitative ratio of these two enzyme activities in the three experimental variants is presented in Figure 8. Despite the diverse biodegradation mechanisms that are triggered over time it is clearly seen that the total rate of the benzene ring cleavage (measured as sum dioxygenase activity) varies in very close limits among the three experimental variants. In the values reported for SDH, however, the situation is not the same. The difference is almost 4 μMol/min·mgPr between the highest value in II AD and the lowest in control.

The ratio between the total dioxygenase activity and the succinate dehydrogenase in the adaptation phases are 0.81 for I AD and 0.53 for II AD, while the value for normally functioning AS is quite high (6.49). In the control, the higher dioxygenase activity may be a result of the constitutive background that prevails in the defense mode of the system. In evolution terms it is embedded in a wider range of the protective function. In normally functioning AS we also register the inductive oxygenase activities available due to the regularly incoming aryl-containing pollutants in the wastewater to WWTP ‘Kubratovo’ (Topalova 2009).

### Table 3 | Change of oxygenase activities and succinate dehydrogenase activity at I AD and II AD

<table>
<thead>
<tr>
<th>Stimulation</th>
<th>C23DO</th>
<th>C23DO</th>
<th>P2MO</th>
<th>C23DO</th>
<th>SDH</th>
<th>SDH</th>
<th>SDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>control to I AD</td>
<td>+11%</td>
<td>+73%</td>
<td>+73%</td>
<td>+56%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control to II AD</td>
<td>+61%</td>
<td>+460%</td>
<td>+36%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Inhibition</th>
<th>P2MO</th>
<th>C12DO</th>
<th>P34DO</th>
<th>TDO</th>
</tr>
</thead>
<tbody>
<tr>
<td>control to I AD</td>
<td>-44%</td>
<td>-31%</td>
<td>-11%</td>
<td>-25%</td>
</tr>
<tr>
<td>IAD to II AD</td>
<td>-4%</td>
<td>-36%</td>
<td>-60%</td>
<td>-33%</td>
</tr>
</tbody>
</table>
In both adaptation phases, the ratio TDO/SDH has almost similar values and close to 1. In both experimental variants, the enzymatic reaction of SDH has a higher rate of completion. In comparison with the control, we observe the opposite. In both adaptation phases the ratio between the total dioxygenase activity and the succinate dehydrogenase activity is much lower. This is logical since besides toxic pollutants the AS degrades and metabolizes in CTC and trivial pollutants.

In unloaded AS just the opposite is reported. TDO prevails in the enzymatic apparatus. The reasons for this may be again the above mentioned, namely AS normally purify other aryl-containing contaminants that maintain high activities of the dioxygenase apparatus. In the first and second phase of intoxication/detoxication of mazut the ratio TDO/SDH is changed due to factors that act in different degrees and different direction, but having as a final result activation of the dioxygenase and dehydrogenase apparatus to varying degrees. The dehydrogenase apparatus is activated to a significantly higher degree in the process of adaptation because it has to provide energy to overcome the toxic shock, to degrade trivial and toxic pollutants and for the synthesis of the studied oxygenase enzymes. On the other hand, the decrease of TDO from I AD to II AD may be the result of the longer action of the xenobiotic substance into cells. There is a secondary intoxication of the biological system after the formation of metabolic products with higher toxicity after the first adaptation phase.

The ratio of TDO/SDH is very high in normally functioning AS, which confirms the fact that the bioenergy needs of AS are met and its oxygenase apparatus is functioning well. In the two adaptation phases, that ratio is below one. There is energy deficit and the energy to nourish the hydroxylation and the breakdown of the aromatic ring of the pollutant is not enough. This is also evident from the high SDH. The high SDH is needed to ensure the extremely high energetic needs of the AS to recover and to overcome the intoxication shock, for synthesis of oxygenase activities and other similar adaptive responses. The exceptional increase of SDH and the change of TDO/SDH is the other registered adaptive mechanism of AS to detoxification and rehabilitation.

Our results are a scientific base to propose that the addition of easily biodegradable substrates will enable the energy production to overcome the intoxication of the AS, as well as to supply the process with enough energy and carbon for synthesis and activation of the oxygenases. This will stimulate not only the detoxification on the enzyme level, but will accelerate overall adaptation and rehabilitation of AS and process. The amount and type of the biodegradable substrate have to be selected carefully according to the requirements of the heterotrophic denitrification process, as well as according to the concrete conditions in the biobasins.

CONCLUSION

The mazut load causes damage to the AS and its rehabilitation even after a prolonged adjustment period (25 days after the shock load) is difficult. AS continues to be a filamentous bulking and accumulation of oil drops in flocs was ascertained.

In shock load with mazut and in the phases of adaptation, P2MO, C12DO and P34DO inhibited, although the ortho-mechanism of the benzene ring cleavage remains a leading mechanism for degradation of aromatic hydrocarbons. During the rehabilitation of biodegradation activity in the activated sludge, adaptive reactions take place, which lead to increased C23DO and SDH. In the critical situation of loading AS with mazut the adaptive detoxification function is directed towards increasing the relative role and the rate of meta-mechanism of the benzene ring cleavage. This path is shorter, faster and more energy-efficient. Parallel to this, SDH is activated as an additional

**Table 4** | Percentage of activities of C12DO, C23DO and P34DO from TDO activity

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>I AD</th>
<th>II AD</th>
</tr>
</thead>
<tbody>
<tr>
<td>C12DO</td>
<td>78%</td>
<td>74%</td>
<td>71%</td>
</tr>
<tr>
<td>C23DO</td>
<td>6%</td>
<td>16%</td>
<td>9%</td>
</tr>
<tr>
<td>P34DO</td>
<td>16%</td>
<td>10%</td>
<td>20%</td>
</tr>
</tbody>
</table>
adaptive response. This confirms the accelerated functioning of CTC, in which trivial substrates are degraded at a higher rate and energy is generated, necessary to overcome the toxic effects of mazut and for synthesis of oxygenase enzymes with constitutive and inductive character.

Shock loading with mazut decreases the ratio TDO/SDH in both phases of adaptation. This indicates that despite the activation of SDH in the course of the development of adaptation potential, the detoxification function, represented as a sum of the ortho- and meta-mechanisms of the benzene ring cleavage has a lower rate of 3.6 to 4.3 times, respectively, for I AD and II AD, although the recovery period of AS is prolonged (more than 25 days). All this suggests that in treatment facilities there must be purposefully developed and ready systems for risk management, including the risk of shock loads with toxic pollutants.

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