ENZYME ACTIVITIES AND SHIFTS IN MICROBIAL POPULATIONS ASSOCIATED WITH ACTIVATED SLUDGE TREATMENT OF TEXTILE EFFLUENTS

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ABSTRACT
The enzyme studies on an activated sludge (AS) were aimed at providing information about adaptive response of microbial community during four significant alterations in textile wastewater treatment plant behavior associated with: shock loading with azo compounds; recovery after the xenobiotic shock; building up additional aerobic reactor; breakdown of facility exploitation for 5 months. The activity of several enzymes associated with target biodegradation processes was assessed. Induction of catechol 1,2–dioxygenase (C12DO), catechol 2,3–dioxygenase (C23DO), protocatechate 3,4–dioxygenase (P34DO) occurred under aerobic and notably under anoxic/microaerobic conditions, depending on the control point. Key microbial groups in the AS consortium were determined by cultivation-based methods. The densities of bacteria from g. Pseudomonas (Ps) and Acinetobacter (Acin) were increased during the toxic shock. Aerobic heterotrophs (AH) and g. Acinetobacter decreased after pause of facility exploitation. The numbers of denitrifying bacteria (Dn) in denitrifying reactor were significantly decreased after each of the critical events.

Keywords: activated sludge, enzyme activities, microbial community, textile wastewater treatment

Introduction
The effluents from textile industry are extremely variable in composition, a high content of organic compounds with complex structure which often contain aromatic xenobiotics resistant to biodegradation, makes textile wastewater difficult for treatment by conventional methods (7, 8). The final effluent from textile wastewater treatment plants always exhibits a certain degree of color intensity and often is out of compliance with the current legislative constraints. Serious concern over the pollution potential of textile dyes is prompted by their possible toxicity and carcinogenicity. Although not all dyes are highly toxic compounds, many of them are made from known carcinogens, such as benzidine and other aromatics (6), all of which might be reformed as a result of microbial metabolism. It has been shown that azo- and nitro-compounds are reduced in the sediments (12) and in the intestinal environment (2), resulting in regeneration of the parent toxic amines. In order to meet the public demand of color-free effluent as well as strict regulatory requirements, environmentally acceptable, affordable and flexible approaches for removing azo-dyes and their toxic by-products seems to be necessary.

The ability of biological systems to metabolize azo-dyes has been subject of intensive research in recent years (7, 9). Under anaerobic and anoxic conditions various bacterial strains have been reported to readily decolorize azo-dyes. Although the anaerobic reduction of azo-compounds is relatively easy to achieve, complete mineralization of the molecule is difficult. The reductive cleavage of the azo-bond is carried out by microbial cells and results in production of colorless aromatic amines, which can be further metabolized under aerobic conditions. Therefore, to overcome the problem of the relative recalcitrance of azo-dye breakdown products under anoxic conditions, it is repeatedly suggested to combine the anaerobic reduction with subsequent aerobic treatment of textile wastewater (9).

The microbial community in a biological wastewater treatment system largely defines the system’s treatment efficiency. In many cases the accumulation of azo-dyes and their toxic by-products in the bioreactors can be detrimental to the microbial population and may lead to unstable performance in the real technical applications.

Conventional activated sludge process is operated and controlled by monitoring influent and effluent characteristics (COD, BOD, nitrogen in different forms, phosphorus, etc.) and working parameters (dissolved oxygen, suspended solids, pH, recirculation flow rate, etc.). In general, process control is carried out by measuring parameters which are not directly representative of in situ activity of pollutant-degrading microorganisms.

In recent years, the enzymology of biological remediation and practical applications of enzyme technologies in wastewater treatment has been receiving increased attention (13). The study of enzyme activities can provide suggestive information regarding the processes that are taking place in AS system and may help the improvement of wastewater treatment strategy. A crucial step in biodegradation of toxic aromatic compounds is ring cleavage. The expression of specific enzymes associated with aromatic ring cleavage is indicative for the detoxification potential of the biosystem.
In this research the deviations of microbial and enzymatic characteristics of AS system were investigated under four different exploitation scenarios of textile wastewater treatment (WWT) plant. Results of the biological analyses were evaluated on the basis of plant behavior and performance. The aims of the present study are:

1. To analyze the distribution patterns of representative functional groups of microorganisms and enzyme activities associated with target biodegradation processes under different redox conditions during the wastewater treatment;
2. To investigate how significant alteration in WWT plant behavior can influence the distribution of bacterial groups and the induction of different specific enzymes;

Materials and Methods

Plant description

The wastewater treatment plant (WWTP) in a local textile factory “Giorgetti Bulgaria” AD – Elin Pelin was investigated in the course of this study. The facility receives exclusively industrial wastes originating from the pre-mentioned Industrial Estate. The treatment process includes primary filtration, homogenization (accumulating basin), secondary biological treatment with denitrifying step (denitrifying reactor) and subsequent aeration (aerobic reactors), secondary sedimentation (clarifier) and ozonation. The plant is continuous flow activated sludge system with sludge recycle.

Sample collection strategy

The following sampling scheme was applied: mixed samples of the studied wastewater and activated sludge were collected every time from the same locations in accumulating basin (AB), denitrifying reactor (DN), aerobic reactor 1 and 2 (AerR 1 and AerR 2), clarifier (Clrf) and effluent stream (Eff); biomass for microbiological analyses and enzyme assays was collected from DN, AerR 1 and AerR 2.

Bioassays were performed in four control points (CP) related to significant changes in exploitation scenarios. Two of the control points were addressed to significant risk factors like industrial accident resulting in shock loading of biosystem with xenobiotics (cP2), clarifier (Clrf) and effluent stream (Eff); biomass for microbiological analyses and enzyme assays was collected from DN, AerR 1 and AerR 2.

Microbiological analyses

Mixed samples of wastewater and activated sludge were sonicated for 3x10 sec (20 kHz, 12 μm, maximal output power 180 W) in order to obtain a homogenous microbial suspension. To count the total number of aerobic heterotrophic bacteria (AH) a nutrient agar medium was used. Facultative heterotrophic anaerobes (AnH) were routinely grown on standard nutrient agar medium and incubated anaerobically at 25°C for 14 days. The plates were placed in anaerobic jars (Merck) containing sachets Anaerocult A (Merck), which provided oxygen-free environment. Bacteria from genera 

Pseudomonas

(PS), Acinetobacter (Acin) and denitrifying microorganisms were also enumerated by the agar plate count method with appropriate media: King’s Medium B with the following composition (g per liter): peptone (20), glycercol (10), K2HPO4 (1.5), MgSO4.7H2O (1.5), for pseudomonads (Ps); minimal salt acetate (MSA) medium with the following composition (g per liter): CH3COONa (0.78), D-glucose (0.21), yeast extract (0.7), NH4Cl (0.5), Na2SO4 (0.09), MgSO4.7H2O (0.05), CaCl2.2H2O (0.015), NaHPO4.12H2O (0.3), KH2PO4 (0.045), EDTA-Na2H2O (0.00103), FeSO4.7H2O (0.0007), 0.2M Tris-HCl buffer (pH 7) (50ml), N,N’-dicyclohexylcarbodiimide (DCCD) (200 μM), for bacteria from g. Acinetobacter (Acin); bromothymol blue (BTB) medium with the following composition (g per liter): l-asparagine (1), KNO3 (2), KH2PO4 (2), FeCl3.6H2O (0.05), CaCl2.2H2O (0.2), MgSO4.7H2O (2), 1 ml of BTB liter-1 (1.6% in ethanol), for denitrifying bacteria.

Enzyme assays

All enzyme assays were done at 25°C. The biomass was resuspended in 0.033 mM phosphate buffer pH 7.5 (0.15 g wet weight per ml). The cell extracts were prepared by sonic disruption (20 kHz, 12 μm, maximal output power 180 W) at 4°C until 90% of the cells were disrupted. The unbroken cells and cell debris was removed by centrifugation at 13,000 x g for 30 min at 4°C and the supernatant was used as an intracellular crude enzyme source. One unit of enzyme activity was defined as the amount of enzyme required to reduce 1 μM of substrate per min per mg of protein. All enzyme activities were normalized back to total protein concentrations to obtain specific enzyme activities.

Catechol 1,2-dioxygenase (C12DO) was measured by determining the formation of cis,cis-muconate at 260 nm (ε cis,cis-muconate = 16 900 M–1 cm–1) as described previously by Willets & Cain (1972) (14). Reaction mixtures contained a total volume of 3 ml, 100 mM Na2EDTA, 2.2 mM catechol. The reaction was started by adding crude extract (4.5 – 7 mg of protein).

Catechol 2,3-dioxygenase (C23DO) was measured by determining product formation (2-hydroxyxmuconic semialdehyde) at 375 nm (ε 2-hydroxyxmuconic semialdehyde = 36 000 M–1 cm–1) as described previously by Farr & Cain (1968) (3). Reaction mixtures contained a total volume of 3 ml, 100 mM Na2EDTA, 2.2 mM catechol. The
reaction was started by adding crude extract (4.5 – 7 mg of protein).

Protocatechuate 3,4-dioxygenase (P34DO) activity was measured by determining substrate depletion (protocatechuate) at 290 nm (εprotocatechuate = 49 000 M⁻¹.cm⁻¹) as described previously by Fujisawa & Hayashi (1968) (4). Reaction mixtures contained a total volume of 3 ml, 100 mM K phosphate buffer (pH 7.5) and 0.8 mM catechol. The reaction was started by adding crude extract (4.5 – 7 mg of protein).

Unspecific azoreductase (AzoR) was measured by determining azo-dye-dependent NADH oxidation at 340 nm under anaerobic conditions. Reaction mixtures contained a total volume of 3 ml, 100 mM K phosphate buffer (pH 7.5), 1 mM NADH, and crude extract (4.5 – 7 mg of protein). After the unspecific reduction rate was determined, the reaction was started by adding 0.033mg.ml⁻¹ freshly prepared solution of azo dye Acid Black 1 (C.I. No. 20470). The values were corrected for endogenous NADH oxidation of the assay mixture.

The protein content of cell extract was determined by the method of Lowry, using bovine serum albumin as a standard (5).

Statistical analysis
All values are means of three independent observations and error bars indicate the standard deviation. Bacterial amounts in different types of bioreactors (denitrifying and aerobic reactors) and from different control points were compared using a one-way analysis of variance (ANOVA) and corresponding non-parametric test (Friedman test on ranks) on ln-transformed viable counts. Where significant differences were indicated (P<0.05), individual groups were then compared by post hoc test (Duncan’s multiple range test).

Other analyses
Mixed liquor suspended solids (SS) of activated sludge, chemical oxygen demand (COD) and N-NH₄ of wastewater were analyzed according to Standard Methods (1). The efficiency (E) of COD and N-NH₄ removal (in %) was calculated as follows: E (%) = [(Cᵢᶠˡᵘⁱˡᵉᵗ – Cₑᶠˡᵘⁱˡᵉᵗ) / Cᵢᶠˡᵘⁱˡᵉᵗ] x 100, where Cᵢᶠˡᵘⁱˡᵉᵗ is the concentration in the influent stream, Cₑᶠˡᵘⁱˡᵉᵗ is the concentration in the effluent stream.

Results and Discussion
Efficiency of COD and N-NH₄ removal at investigated control points
Three of the investigated exploitation scenarios were characterized by satisfactory plant efficiency, in terms of COD removal. The COD of influent wastewater at CP2, CP3 and CP4 was 388.16, 229.39, and 499.44 mg O₂.l⁻¹, respectively. The effluent COD decreased to less than 150 mg O₂.l⁻¹ (efficiency 52 – 92%). Isolated event of high COD concentration (286 mg O₂.l⁻¹) in the effluent and COD removal 51% was registered at CP1 during the shock loading of the AS with azo-compounds (Fig. 1). Concerning N-NH₄ removal, it was strongly influenced by the xenobiotic shock, although the removal efficiency was growing progressively from 2% (at CP1) to 96% (at CP3), it remained one of the critical stages of wastewater treatment in this particular facility (Fig. 1). The concentration in the effluent (25.19 – 68.54 mg.l⁻¹) at CP1, CP2 and CP4 exceeded significantly the norms according to the Bulgarian legislation (10 mg.l⁻¹).

Fig. 1. Efficiency of COD and N-NH₄ removal during different exploitation scenarios of textile wastewater treatment (WWT) plant (CP1 - shock loading of the biosystem with azo compounds; CP2 - one month after the xenobiotic shock; CP3 - after building up the additional aerobic reactor; CP4 – after breakdown (for 5 months) of WWT facility exploitation due to natural floods).

Effect of xenobiotic shock on the enzyme activity and microbial community structure in an activated sludge system
The distribution of studied enzyme activities among denitrifying and aerobic bioreactors is highly variable (Fig. 2). During the xenobiotic shock loading higher activity of C12DO (5-fold increase) and P34DO (2.7-fold increase) was observed in the denitrifying reactor compared to the aerobic reactor. The data showed that significant C12DO and P34DO expression levels can be realized under anoxic/microaerobic conditions and suggest that the activated sludge system has the potential to biodegrade aromatic products from azo-dye reduction using aerobic pathways under toxic shock conditions.

Conversely, there was very little effect on the activities of SDH and AzoR in anoxic/microaerobic (DN) and aerobic (AerR) environment. Stable activity of SDH under alternating redox conditions was expected, but our experimental results also showed that activated sludge possessed relatively low potential to carry out enzyme reduction of azo-dyes even under...
more favorable anoxic conditions. We found almost the same activity of AzoR in the DN and AerR (Fig. 2a).

This finding seems to indicate that stepwise raising concentrations of azo-dyes in activated sludge plant can be accompanied by an increase in azoreductase activity, but only to some critical concentration at which organisms reach their limits.

When wastewater treatment process was shifted to aerobic phase, biodegradation of monoaromatic compounds was initiated, and C12DO, C23DO and P34DO were induced to significant levels, respectively 5.5, 1.9 and 2.1-fold increase was found in the aerobic reactor.

The distribution of SDH activity had similar pattern to those observed at CP1 but the established activity has increased over 2-fold at CP2. Succinatdehydrogenase is a respiratory enzyme indicative of xenobiotic inclusion in the tricarbonic acids cycle. Apparently, high concentration of toxic substrate at CP1 exerts negative effect on metabolic activity of activated sludge, i.e. marked decrease in SDH activity, resulting in unsatisfactory COD removal system performance.

TABLE 1

Densities of bacterial population in denitrifying and aerobic reactor during shock loading with azo dyes - CP1 and one month later - CP2

<table>
<thead>
<tr>
<th>Groups of microorganisms</th>
<th>Shock loading with azo dyes - CP1</th>
<th>One month after xenobiotic shock - CP2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Denitrifying reactor</td>
<td>Aerobic reactor</td>
</tr>
<tr>
<td>Aerobic heterotrophs</td>
<td>18.000 ± 0.506</td>
<td>19.610 ± 0.135</td>
</tr>
<tr>
<td>Anaerobic heterotrophs</td>
<td>17.100 ± 0.310</td>
<td>17.860 ± 0.312</td>
</tr>
<tr>
<td>g. Pseudomonas</td>
<td>18.860 ± 0.282</td>
<td>20.260 ± 0.430</td>
</tr>
<tr>
<td>g. Acinetobacter</td>
<td>18.230 ± 0.315</td>
<td>19.650 ± 0.220</td>
</tr>
<tr>
<td>Denitrifiers</td>
<td>4.520 ± 0.201</td>
<td>10.720 ± 0.246</td>
</tr>
</tbody>
</table>

* Standard error of the mean
resulted in detectable increase of homogenic (free) cells from g. *Pseudomonas* and *Acinetobacter* in the wastewater. These results are consistent with our previous studies, indicating that both groups of microorganisms increased their densities during the processing of highly toxic wastewater (10). The amount of denitrifying bacteria were significantly ($P<0.05$) reduced in the denitrifying reactor at CP1 and increased one month later.

Apparently, during the shock loading with azo-compounds densities of denitrifiers decreased, and notably the most significant increase was observed in the densities of bacteria from g. *Pseudomonas* and *Acinetobacter*. Since pseudomonades and g. *Acinetobacter* can use nitrate as a final electron acceptor and some denitrifying bacteria can also use oxygen, we can conclude that under unfavorable conditions of high toxic loading denitrifying processes in the system are diminished and replaced by oxidative metabolism of toxic compounds. This finding seems to be supported by high activities of oxygenases observed in denitrifying reactor during the shock loading.

**Enzymatic and microbial characteristics of AS after construction of second aerobic reactor and after 5 months pause of WWTP facility exploitation**

The status of the biosystem was evaluated after building up the additional aerobic reactor (CP3) due to expanding the production capacity of the textile factory. The result of this reconstruction was expected to be reduction in xenobiotic loading of AS. The highest enzyme activity of C12DO was found under aerobic conditions in AerR1 and 2. The pattern of oxygenase activity at CP3 suggests the presence of factors inducing these specific enzymes. The activities of C12DO and C23DO were not detected in the denitrifying reactor (Fig. 3a).

The activities of oxygenase apparatus of AS and quite stable high levels of SDH activities in aerobic reactors suggest that the initiation of aromatic biodegradation at CP3 is carried out mainly during the aerobic phase of the wastewater treatment.

The data showed that azoreductase activity was repressed during aerobic but also during anoxic/microaerobic phase to levels comparable to those observed at CP1, showing that the first stage of azo-compound biodegradation was disrupted.

![Graphical representation of enzyme activities](image)

Some alterations were observed in CP4, the activity of the studied enzymes suffered modification depending on the long period (5 months) of passive condition of AS (Fig. 3b). During the anoxic phase, the activated sludge exhibited the highest unspecific azoreductase activity – Azor; catechol 1,2–dioxygenase – C12DO; catechol 2,3–dioxygenase - C23DO; protocatechate 3,4–dioxygenase – P34DO. Error bars represent the standard deviation between triplicates.

<table>
<thead>
<tr>
<th>Enzyme Activities</th>
<th>Reactor 1</th>
<th>Reactor 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDH</td>
<td>EAAerR1/EADN</td>
<td>EAAerR2/EADN</td>
</tr>
<tr>
<td>Azor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C12DO</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C23DO</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P34DO</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**TABLE 2**

Densities of bacterial population in denitrifying and aerobic reactors, after building up the additional aerobic reactor - CP3 and after pause for 5 months in the exploitation of WWTP facility – CP4

<table>
<thead>
<tr>
<th>Groups of microorganisms</th>
<th>Building up the additional aerobic reactor – CP3</th>
<th>Five months pause in the exploitation of WWTP – CP4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Denitrifying reactor</td>
<td>Aerobic reactor 1</td>
</tr>
<tr>
<td>Aerobic heterotrophs</td>
<td>19.140±0.443</td>
<td>18.440±0.139</td>
</tr>
<tr>
<td>Anaerobic heterotrophs</td>
<td>11.628±0.344</td>
<td>10.615±0.218</td>
</tr>
<tr>
<td>g. <em>Pseudomonas</em></td>
<td>10.921±0.395</td>
<td>9.192±0.0984</td>
</tr>
<tr>
<td>g. <em>Acinetobacter</em></td>
<td>18.185±0.112</td>
<td>16.803±0.409</td>
</tr>
<tr>
<td>Denitrifiers</td>
<td>9.668±0.229</td>
<td>8.366±0.0820</td>
</tr>
</tbody>
</table>

* Standard error of the mean
C23DO and P34DO expression levels were established under anoxic/microaerobic conditions.

In general, SDH activity showed decreased levels at CP4 compared to CP3 and the same typical stable distribution between denitrifying and aerobic reactors.

It is important to note that COD removal performance (92%) at CP4 was the best achieved by the system among the studied exploitation scenarios. Furthermore, the activity of the key enzymes involved in oxygen-dependent ring-cleavage of aromatic compounds in denitrifying reactor is comparable to those observed in aerobic reactors. This suggests that microaerobic conditions may be competitive with aerobic treatment strategies for wastewaters containing high concentrations of aromatic compounds.

In-situ response of AS microbial community after construction the additional aerobic reactor and after 5 months pause of WWT facility exploitation was assessed. The density of heterotrophic community was modified depending on the long period of passive condition of AS and ln counts of aerobic heterotrophs (Table 2) were decreased at CP4 compared with CP3. Another group of microorganisms with significantly reduced densities at CP4 is g. Acinetobacter. The numbers of anaerobic heterotrophs and bacteria from g. Pseudomonas were generally higher at CP4. The ln counts of denitrifying bacteria were markedly decreased in denitrifying reactor at CP4, conversely to the both aerobic reactors, where higher densities were observed at CP4 compared with CP3. These differences in counts were statistically significant (P<0.05) for all microbial groups.

Apparently, two different critical events affected the community structure of AS in two different ways. During the shock loading with azo-dyes shifts in the bacterial community were associated with detectable increase of bacteria from g. Pseudomonas and Acinetobacter (Fig. 4a). This adaptive strategy provides higher ability of AS consortium to resist toxic stress damage by raising the constitutive and inductive detoxification potential associated with microorganisms from these genera.

The long period of low heterotrophic activity showed effect related to decrease in the density of aerobic heterotrophs and g. Acinetobacter and higher numbers of anaerobic heterotrophs and bacteria from g. Pseudomonas (Fig. 4b). Both critical events were characterized with decreased counts of denitrifiers in denitrifying reactor. Under our experimental conditions, significant relationship between the densities of different microbial groups and the redox conditions in the reactors was not found.

**Conclusions**

The above-mentioned enzyme activities and functional microbial groups were chosen because their determination in AS system constitutes useful research tool for assessing the effect of significant alterations in WWT plant behavior on the functional diversity of microbial community.

All five studied enzymes have shown variations of their activities depending on the environmental conditions, on the presence of inducing factors and on the adaptive response of AS. The activity of SDH has stable distribution between denitrifying and aerobic reactors, but is markedly decreased under high toxic loading and after long period of low nutrient tension condition. Azoreductase activity is repressed during the xenobiotic shock but also after construction of second aerobic reactor, in both cases showing that the first stage of azo-compound biodegradation is disrupted. More favorable for enzyme reduction of azo-dyes are anoxic conditions, respectively in most of the investigated control points higher azoreductase activity is established in denitrifying reactor. Most significant variations are observed in the oxygenase activities. Induction of C12DO, C23DO and P34DO occurs under
aerobic and notably under anoxic/microaerobic conditions, depending on the control point and suggests that the activated sludge system has the potential to complete biodegradation of aromatic products from azo-dye reduction using oxidative pathways.

In general, the microbial characteristics of AS are more conservative than enzyme activities. There are no significant relationships between the densities of microbial groups and the redox conditions in the reactors. Nevertheless, our data suggested that during the shock loading with azo-dyes shifts in the bacterial community were associated with functional groups of denitrifiers and bacteria from g. Pseudomonas and Acinetobacter; 5-months period of low heterotrophic activity showed effect related to decrease in the density of aerobic heterotrophs and g. Acinetobacter. In terms of enzyme activity, enhanced the ability of investigated AS system to resist toxic stress damage was exhibited by induction of C12DO, C23DO and P34DO activities under microaerobic conditions. Additionally, the adaptive response occurred during the two critical events was of the same order of magnitude.

The objectives of our further studies are to use the knowledge of enzymatic and structural response of AS for evolution prognosis, thus improving wastewater treatment performance and ultimately to establish close collaboration between technology development and the studies of biosystem activity.

Acknowledgements
This work was financed by the National Scientific Fund of the Ministry of Education and Science (Republic of Bulgaria) - Project VU-B-4 – “Bioremediation Technologies for Detoxication of Water and Sediments, Polluted by Texile Industry” 2005-2008.

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