III. ENVIRONMENTAL BIOTECHNOLOGY

APPLICATION OF NANODIAMONDS IN WASTEWATER TREATMENT TECHNOLOGIES

Ivaylo Yotinov, Mihaela Belouhova, Irina Schneider, Yana Topalova

Abstract: Nanotechnology is a rapidly developing scientific area and nanoproducts continually increase their use. Thus, they become the subject of more research. Nanodiamonds (NDs) are a class of nanomaterials which attracted worldwide attention with high biocompatibility, non-toxicity and many opportunities for surface chemical interactions. Furthermore, the surface functional groups on NDs can be easily adapted so that they exhibit valuable chemical, physical and biological properties. This article discusses the main characteristics of the NDs, with their potential use in stimulation, indication and monitoring of the wastewater treatment and biodegradation processes.

The effect of diamond nanoparticles on the transformation of the azo-dye amaranth during the treatment process, modeled in sandy sequencing batch biofilter has been discussed as case study in this paper. In the described examples key parameters were evaluated: residual concentration of amaranth, enzyme activities, effectiveness of amaranth decolorization, chemical oxygen demand and total organic carbon. The data showed the positive role of the diamond nanoparticles as a modulator. These results discover perspectives for further research and applications of NDs in the stimulating of biodegradation processes in wastewater treatment technologies.

Key words: amaranth, biological wastewater treatment, enzyme activity, nanodiamonds, waters

INTRODUCTION

The intensive development of the nanotechnologies accelerated the studies and applications of nanodiamonds because of their mechanical, chemical, biological and optic properties [4]. The nanodiamond fluorescence is also of practical interest (in modification of the surface) and the high biocompatibility. All these characteristics contribute to the more effective application of nanodiamonds in different biomedical, ecological and other technologies in comparison to the other carbon nanoparticles. In the different biological applications the diamond nanoparticles are subjected to a number of modifications with a vast variety of active chemical groups, which in their turn allow the interaction with different biomolecules.

Other properties of the diamond nanoparticles that contribute to their wide and more increasing use are the strong adsorption characteristic [1] and the presence of hydrophilic surface groups (-OH, $-NH_2$, $-C(O)NH_2$), which are able to actively adsorb viruses and microorganisms. For example, the attachment of the biocompatible nanodiamonds to enzymes or different medicines has proven effectiveness against pathogenic bacteria [18] and cancer cells [9].

Regarding preservation of the environment nanodiamonds can be used in different aspects. For instance the synthesis of NDs can be achieved from explosive substances, as well as old ammunition. Such an activity can be rightfully considered to be an ecological and economically favorable method for recycling ammunitions [30]. Another basic application of NDs, discussed in the present article, is their use for remediation of the environment [12].

BASIC CHARACTERISTICS AND APPLICATION OPPORTUNITIES OF NANODIAMONDS IN PRACTICE

In their structure well-purified NDs are crystal structures. They have multiple walls with a diamond core, built from carbon in sp^3 hybrid state. It can be covered by graphite layers or amorphous carbon with free connections, which can serve to attach different functional groups. A summary of the available information about the properties of nanodiamonds is given in Table 1 [29].

An important distinctive trait of nanodiamonds is that in comparison to the other carbon nanoparticles (carbon nanotubes, graphen, etc.) they have a great number of different functional groups on their whole surface [14]. Moreover, the diamond nanoparticles are characterized with the unique property to have the chemical modification on their surface in full control, without disrupting the structure and the useful properties of the material. Different methods for the realization of these chemical modifications can be used such as heating in reactive gases or wet chemical methods. These methods are in the base of attaching different surface functional groups on NDs, which in their turn define the participation of the nanoparticles in different chemical interactions. Such modified NDs with different chemical surface can be applied in various biological directions. For example they can be

Table 1. Properties of NDs for biological application

Property	Characteristic	Application/Usage
Structural	Small size of the particles (~2-10 nm)	Unique interactions with biocompounds of the same size
	Different forms (particles, film, substrate, etc.)	Ability to adapt to different bioenvironments
	Large specific surface (300–400 m^{-2}.g)	High adsorption capacity for connecting with enzymes, proteins; for treatment/modification.
	High specific weight (3.5 $g.cm^{-2}$)	Dense structure for solid-phase carrier
Chemical	Chemically resistant to decomposition and corrosion, pH stability	Implants, covers, films, substrates for the cell growth
	High chemical purity	Biocompatible interface
	Free sp^2 carbon orbitals	Adsorption of hydrophobic biomolecules
	Multiple oxygen-containing groups on the surface	Hydrophilic, water-soluble suspensions for further connection with biounits
	Easy surface functionalizing (chemical, photochemical, enzymatic, plasmic)	Attachment of medicines and biomolecules
	Great number of free electrons on the surface	Capture and inactivation of the free radicals
Biological	High biocompatibility, low toxicity	Studies at cellular, tissue and organism level
	Easy connection of bioactive substances (DNA, proteins, etc.) while preserving the functionality	Orientation to therapies, hormones, inhibitors, antigens, medicines
	Solid-phase carrier	Multiple methods of delivery in the cell
Optical	Photoluminiscence: without photodegradation, resulting from nitrogen-vacant defects	Fluorescent probes and imaging as a tool for marking bioobjects
	High coefficient of light refraction, optical transparence	Possible UV sunscreen
	Unique signal in Raman spectroscopy	Non-destroying discovery of live cells
Mechanical	High strength and hardness	Possible cellular lysis; sterilization
Electrochemical	Electrochemical covering of metals	Improving the durability of medical instruments/implants, etc.
Thermal	Able to withstand high/low temperatures	Sterilization (i.w. autoclaving); storage of liquid nitrogen

used in a number of environmental biotechnologies, which will be discussed in the second part of the article [2, 23].

The variety of different chemical modification techniques, as it was mentioned above, can be summarized as processing in gas environment or chemical reactions in liquid environment (Fig.1).

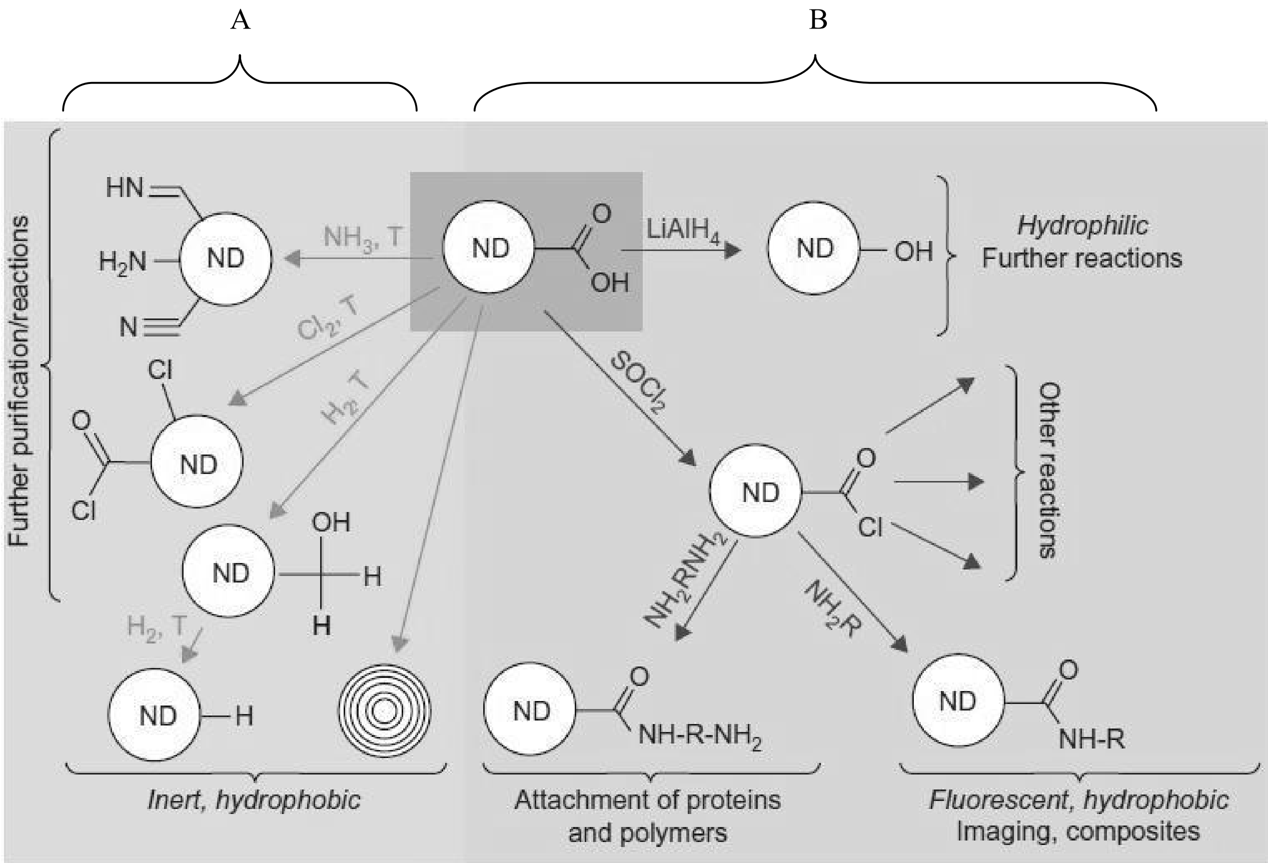

Fig.1. NDs with attached carboxylic groups (NDS-COOH). The surface of NDs-COOH can be modified by means of high-temperature gas processing (area A) or by means of temperature independent chemical reactions (area B) [14]

The processing of NDs in gas environment is realized at high temperatures (400-850^{o}C). The process of gas treatment is characterized with low selectivity. For instance when processing NDs in ammonium flow diamond nanoparticles are obtained with NH_2 groups and simultaneous reduction occurs of C=O to C-O-H, and the formation of C-H, C- - -N and C=N groups [15, 31, 32]. Chemical reactions in liquid environment, carried out on NDs, are realized in lighter conditions and a higher selectivity is ensured (Fig.1). The paths for carrying out modifications of NDs by means of chemical reactions in liquid medium are extremely varied and their number can be compared to the number of the possible chemical transformations of the different functional groups in organic chemistry. This is why they cannot be presented in a united scheme.

The application of the NDs modified by means of various methods in different biological directions is based on the fact that on their surface they carry carboxylic and hydroxylic groups. Moreover, the carboxylized NDs can be also viewed as prior materials for the different modification methods.(Fig. 1).

BIOCOMPATIBILITY OF THE NANODIAMONDS

Some of the important factors when discussing the biocompatibility of the NDs are concentration, exposure time and the specific cell responses. It is also proved that during the incubation of fluorescently marked NDs with three cellular structures of three different types – neuroblastomas from mice (N2A), human skin cells (HaCat) and alveolar macrophages from a rat (CRL-2192), no changes in the morphology of the cells are recorded in comparison to the control samples [25].

These and many other studies prove that in comparison to the other carbon nanomaterials (carbon nanotubes and fullerenes-C_{60}), NDs have better biocompatibility with cells [3, 6, 7, 11, 13, 16, 17, 19-22, 24, 26, 35]. The high biocompatibility of NDs provokes the interest to studies about their successful implementation in ecological biotechnologies in the field of wastewater treatment as nanostimulators in the biodetoxification processes.

FLUORESCENCE IN NANODIAMONDS

The presence of nitrogen-vacancy (NV) centers and a nitrogen atom to the very vacant spot in NDs leads to the presence of fluorescent properties. These nitrogen-vacancy centers can be created by irradiating NDs with high-energy particles. The fluorescent NV centers are studied for application in the biomedical images [5].

Fluorescent NDs can be obtained also by means of linking [8, 27] or adsorption [10] of different fluorescent dyes on NDs. The NDs linked with fluorescent dyes have the important property to be able to move through the cellular channels without decomposition of the surface-linked dyes or a change in the cellular viability for continuous periods of time [28]. Examples for such type of modified nanoparticles are the bright blue fluorescent NDs. They are obtained by means of the covalent connection of octadecylamine to the carboxylic groups of ND surface.

The fluorescent NDs have the benefits of the semi-conductor quantum points – small size, high photostability, bright multi-color fluorescence. Besides they are non-toxic, with rich chemical surface and biocompatibility, which shows they have potential for application in live cells or communities (activated sludge or biofilm). Moreover the possibility to attach modified fluorescent NDs to bacteria from the activated sludge would lead to the development of an effective method for ecological indication of the bacterial segment. An interesting application of this indication would be in the wastewater treatment processes and especially these processes which include an element of targeted and speedy neutralization of toxic pollutants. The available data about the applicability of NDs in the detoxification of xenobiotics as indicators or stimulators of the processes are scanty. In this relation we consider it appropriate to present two specific case-studies, illustrating the role of NDs in the technologized biodetoxification processes.

APPLICATION OF NDS IN THE WASTEWATER TREATMENT

In both examples a process of azo-biodegradation is modeled in a sandy sequencing batch biofilter. The cyclic charging of the biofilter is every 24 hours, with an increasing concentration of the azo-dye amaranth (from 10 to 47 $mg.l^{-1}$). Two model biofilters with quartz sand are used: a control one and a biofilter with added NDs at the 269^{th} hour of the process (Fig.2). The dynamics of key technological and enzymological indicators is followed in both biofilters. At the phase of biofilm destabilization and at the moment of decreasing the biodegradation rate (269^{th} hour) nanodiamonds are added to one of the biofilters. Their effect on the decolorization effectiveness has been followed (I phase of the amaranth biodegradation). NDs are added in concentration 3,12 $mg.g^{-1}$. A detailed description of these experiments is made in others our publications [2, 23]. In this article there are discussed other upgrading effects of the nanodiamonds on key parameters of the detoxification processes.

The biological system in the study is a biofilm, created by means of activated sludge from the Sofia Wastewater Treatment Plant "Kubratovo" as an inoculation material. The nanodiamonds applied as modulators of the processes are provided by Prof. Stavry Stavrev from the International Center for Nanomaterials, Nanotechnologies and Nanomedicine in the town of Smolyan, Bulgaria. The Laboratory of Environmental Biotechnology has been working with this center for over two years now [33].

Below we listed two specific experimental examples from sources of the study practice of the Laboratory for Biological Water Treatment to the Faculty of Biology of the Sofia University "St. Kliment Ohridski". In the first example there has been followed the effect of NDs on the technological parameters of the wastewater treatment process, and in the second – on the enzymes, catalyzing I and II phase of detoxification of the azo-dye amaranth. In both cases we speak about combined treatment of wastewater, containing easily degradable (trivial) and toxic pollutants (azo-dye amaranth), in the course of model processes in sequencing batch biofilters. A scheme of the technological setting is shown on Fig.2. The details about the processes are described in our article [23]. In Table 2 in a comparative plan there are shown the parameters of the wastewater treatment processes in the control biofilter and in the biofilters with adding NDs in the critical phase.

Case study 1: Effect of NDs on the technological parameters of the azo-biodegradation process - I phase (reduction of the azo-bond)

Data about the modulation effect of NDs on the wastewater treatment processes are obtained from experiments, carried out by Prof. Topalova's team [23]. In the experiments there have been studied the residual concentration and the removal effectiveness of the xenobiotic azo-dye amaranth in a biofilter, stimulated with NDs (Table 2). Besides, there has been followed the effect of NDs on the organic matter concentration and effectiveness of decreasing of COD and of total organic carbon (Table 2).

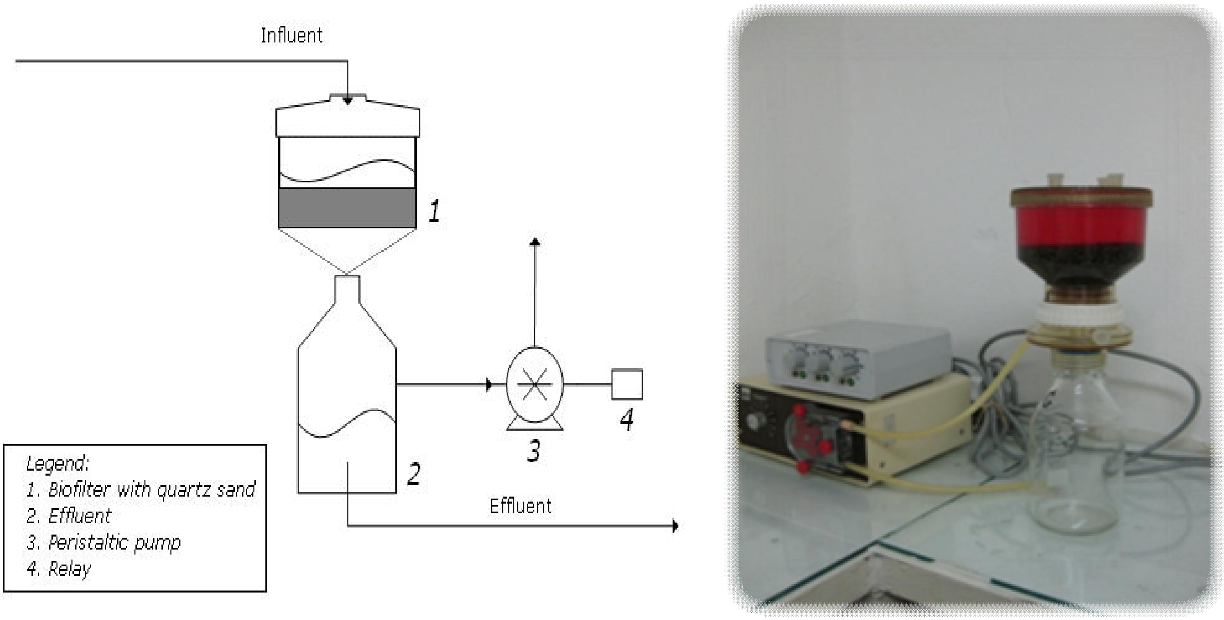

Fig.2. Biofilter with quartz sand for wastewater treatment in the textile industry [23]

Table 2. Technological parameters

Parameters	Control biofilter		Biofilter with NDs	
	Before adding NDs	After adding NDs	Before adding NDs	After adding NDs
Flow, $ml.h^{-1}$	23,13	26,56	22,31	23,28
Residual concentration of amaranth, $mg.l^{-1}$	1,90	4,71	1,61	2,20
Effectiveness of decolorization, %	89,78 %	89,68 %	91,80 %	95,21 %
Decolorization rate of amaranth, $mg.l^{-1}$	0,56	1,06	0,53	1,00
COD, $mgO_2.l^{-1}$	403,49	410,93	451,90	388,78
Effectiveness of COD decreasing, %	31,88 %	35,49 %	25,25 %	35,49 %
Total organic carbon, $mg.l^{-1}$	112,24	116,40	110,48	104,09
Total organic carbon removal effectiveness, %	52,55 %	53,32 %	53,28 %	57,44 %

The results for **the residual concentration of amaranth** in the biofilter, in which NDs are applied show that the values for this indicator are lower in comparison to those in the control biofilter (Fig.3). As a reason for this, the presence of NDs in the biofilter is considered. The average value of the residual concentration of amaranth in the phase before adding NDs in the control biofilter is 1,90 $mg.l^{-1}$, whereas for the phase after adding NDs is 4,71 $mg.l^{-1}$. The residual concentration of amaranth in the biofilter with added NDs during the phase before adding NDs is 1,61 $mg.l^{-1}$, and after adding NDs is 2,20 $mg.l^{-1}$.

The data for **effectiveness of amaranth decolorization** in both biofilters prove the stimulating role of NDs in the biotransformation processes of the xenobiotic (Fig.3). The two biofilters have similar effectiveness before adding NDs at the 269th hour. A decrease of the removal effectiveness is registered after the 269th hour in the control biofilter, whereas in the biofilter with NDs the effectiveness remains high. Keeping the high removal effectiveness during the most critical moments for the wastewater treatment process in the late phase confirm the NDs as a positive modulator for the amaranth biotransformation. An explanation of this could be the high activity, induced by the increase of the contact surface among the enzymes, the azo-dye and other modulators (protons, electrons, etc.) as their substrates [23].

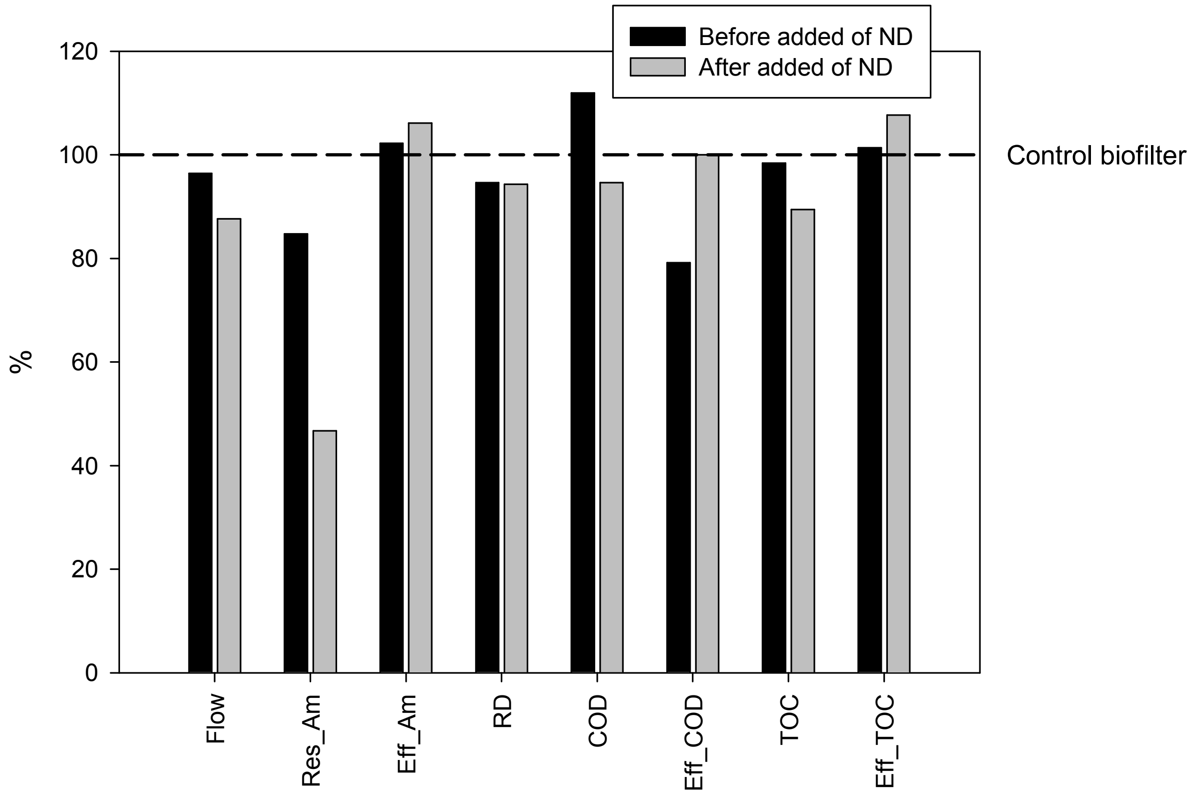

Fig.3. Effect of NDs on the technological parameters of the process in biofilter with added NDs: Flow, Residual amaranth (Res_Am); Effectiveness of amaranth decolourization (Eff_Am); Amaranth decolourization rate (RD); COD; effectiveness of COD decreasing (Eff_COD); TOC; TOC removal effectiveness (Eff_TOC). The values for control biofilter are 100%.

There has also been studied the effect of NDs on a key indicator in wastewater treatment – **chemical oxygen demand (COD)** (Fig.3). Higher COD values are registered before adding the nanoparticles in the biofilter with NDs in comparison to the control biofilter. After adding NDs there is a significant increase of the effectiveness of COD decreasing with 20,8%, compared to the phase before adding NDs in the same biofilter. The residual COD values decrease in the final phase of the wastewater treatment process.

In terms of the **total organic carbon (TOC)** the same tendency is found. After adding NDs there is an increase of the TOC removal effectiveness with 7,73% in comparison to the control biofilter (Fig.3). After adding the nano-modulator there is a decrease of the residual concentration of the total organic carbon in the effluent with 10,58% in comparison to the control biofilter [40].

Case study 2: Effect of NDs on key enzyme indicators, predicting the change of amaranth biodegradation

The effect of NDs is studied on four key enzymological indicators in the two biofilters – azo-reductase (Azo-R) (Fig.4), succinate-dehydrogenase (SDH), catechol-1,2-dioxygenase (C12DO) and catechol-2,3-dioxygenase (C23DO) (Fig.5). The enzymological indicators are analyzed in four critical control points (CCP), which are key for the wastewater treatment process: CCP 1 – 0 hour (beginning of the experiment); CCP 2 – 191st hour (early phase of functioning of the biofilters); CCP 3 – 455th hour (late phase of functioning of the biofilters); CCP 4 – 623rd hour (end of the experiment).

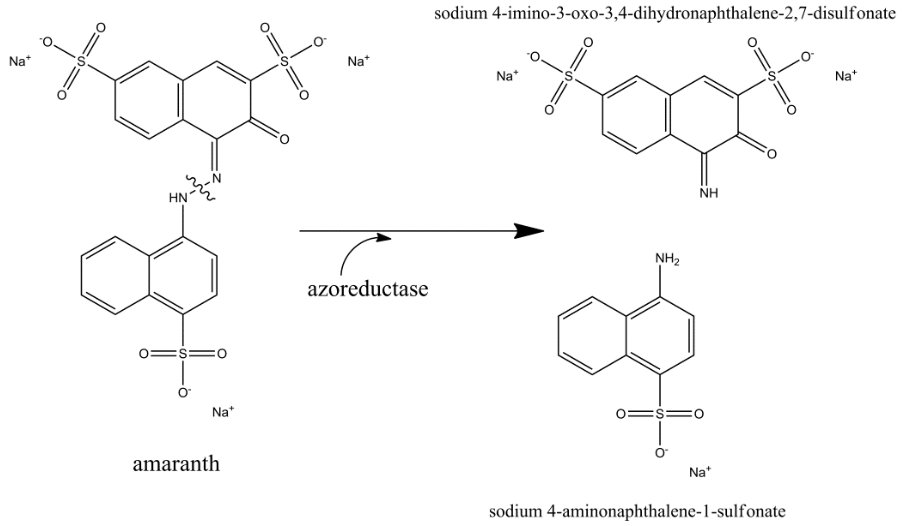

Fig.4. Catalytic function of the azo-reductase to the xenobiotic amaranth

Figures 4 and 5 show the mechanism of action of the used enzyme indicators. Figure 6 shows the sequence of including the studied indicators in the process of detoxification of the azo-dye amaranth. It is clearly seen that the enzymes we chose catalyze the key and most difficult reactions in the whole pathway of the amaranth detoxification.

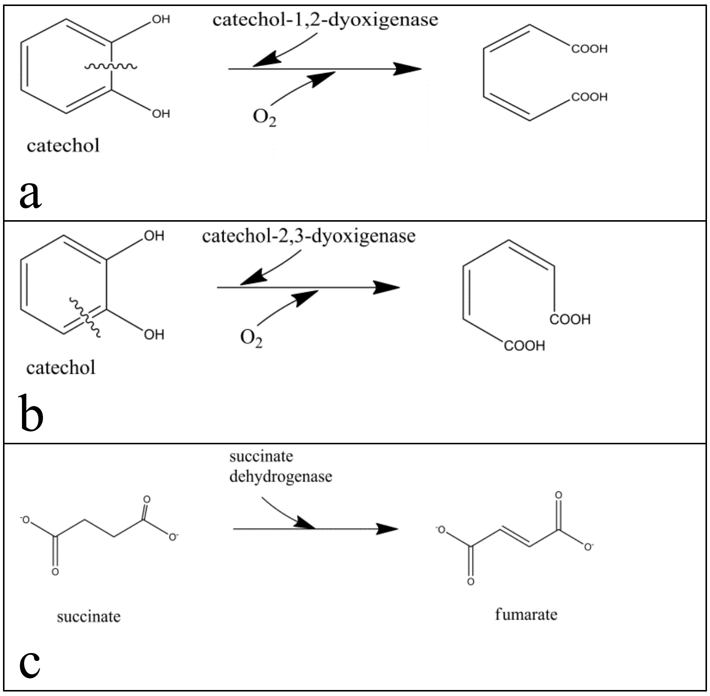

Fig.5. Catalytic function of the studied enzymes: a) catechol-1,2-dioxygenase b) catechol-2,3- dioxygenase; c) succinate-dehydrogenase

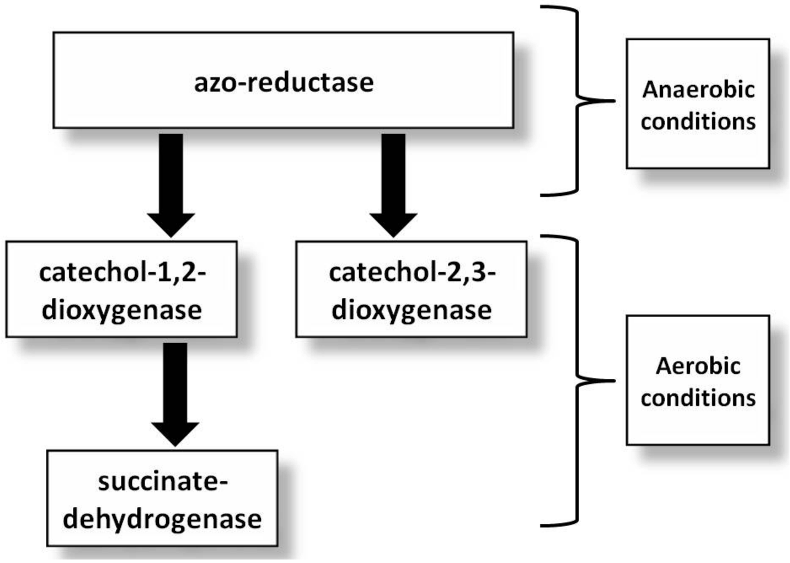

Fig.6. Sequence in the activity of enzymes in the course of the azo-detoxification process

Moreover one should bear in mind that the azo-reductase, catechol-1,2-dioxygenase and catechol-2,3-dioxygenase are mostly inductive enzymes with a very low residual constitutive background [34], and the succinate-dehydrogenase is an enzyme from the central catabolic pathways with constitutive nature. These characteristics of the enzyme indicators place them in a key regulating role in the whole detoxification pathway of the amaranth and give the chance to predict the detoxification rate depending on the toxicant concentration [34]. In the discussed experiment it is found out that after adding NDs in the biofilter the enzymes C12DO and C23DO are activated (Fig.7). The reason for the occurrence of this enzyme potential in the biofilm can be searched in the activating role of the NDs. In their absence the activities of these enzymes are under the critical registrations from the used methods. As to the succinate-dehydrogenase a decrease of the activity was found from CCP1 to CCP4, which is probably caused by the inhibitory effect of the xenobiotics (amaranth and the accumulated aromatic amines, metabolites from the amaranth reduction). For the azo-reductase in the biofilter with added NDs there is an effect of gradual increase of the enzyme activity from CCP1 to CCP4, while for the last point there is an increase of 17,45 % in comparison to the control one. This fact supports the higher effectiveness in terms of the eliminated xenobiotic, which is registered after adding NDs.

While the process is advancing under the influence of NDs, the Azo-R activity increases, and the SDH activity decreases (Fig.7). Most probably this is due to a number of reasons: 1) the SDH activity is inhibited by the produced aromatic amines; 2) Azo-R and SDH compete for protons, which progressively are depleted while the process is advancing; 3) Most probably these two factors work together and to their effect the different nature of Azo-R and SDH is added. Azo-R is above all an inductive enzyme and its activity increases with the increase of concentration of the amaranth, which is simultaneously substrate and enzyme inductor. The activity of SDH as a constitutive enzyme from the Krebs Cycle is strongly dependent on the accumulation of the biomass of the biofilm, which towards the end of the process is limited by the amaranth concentration. The dependency grows stronger - higher concentration of amaranth, higher activity of the detoxification inductive enzymes, inhibition and limitation of the constitutive enzymes from the citric acids cycle in the anoxic biofilter.

Most probably one of the mechanisms of impact of the nanodiamonds is that the contact surface is increased and the very processes on these surfaces are stimulated. This leads to the stabilization of the system as a whole, which causes an increase in the amaranth removal effectiveness. The addition of NDs during the wastewater treatment process helps to unlock the basic and additional pathways for aromatic ring cleavage of the azo-dye [2].

CONCLUSION

The following more important conclusions can be made from the obtained results:

- The nanodiamonds, added at the 269th hour of the experiment, have a stimulating and stabilizing effect in terms of the technological parameters. Indirectly this is shown in keeping a biofilm with a high activity, increasing the effectiveness of the organic matter removal and of the amaranth removal during the process.
- Adding nanodiamonds at the 269th hour helps to unlock the basic and additional pathways for aromatic ring cleavage, which is proved by the increased activity of C12DO and C23DO.

Thus the presented scientific prerequisites and technological experimental results about the role and the potential of NDs as a modulator of the detoxification processes are important arguments for future studies in this area.

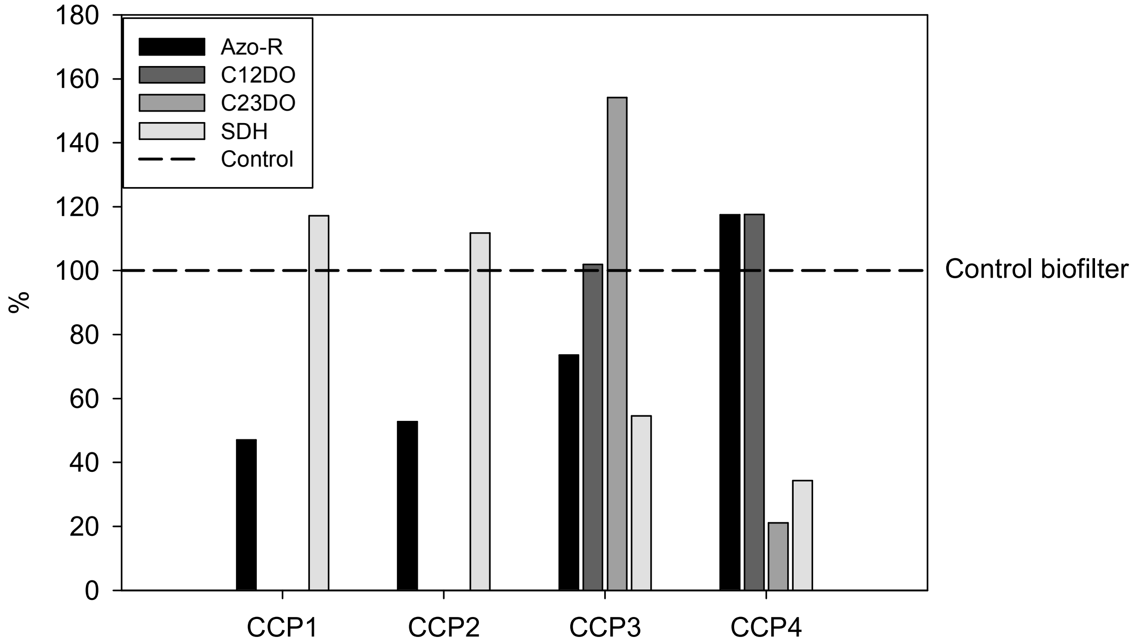

Fig.7. Effect of NDs on the enzymological indicators: Azo-R (azo-reductase); C12DO (catechol-1,2-dioxygenase); C23DO (catechol-2,3-dioxygenase); SDH (succinate-dehydrogenase) at four CCPs in biofilter with added NDs. The values for control biofilter are 100%.

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ПРИЛОЖЕНИЕ НА НАНОДИАМАНТИТЕ ВЪВ ВОДОПРЕЧИСТВАТЕЛНИТЕ ТЕХНОЛОГИИ

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Резюме: С интензивното развитие на нанотехнологиите и постоянно увеличаващото се използване на нанопродукти, те стават обект на все повече изследвания. Нанодиамантите (НД) са клас от наноматериали, които привличат вниманието по света с висока биосъвместимост, нетоксични свойства и много възможности за повърхностни химични взаимодействия. Освен това повърхностните функционални групи на нанодиамантите могат лесно да бъдат модифицирани, така че те придобиват желани химични, физични и биологични свойства. В настоящата статия се разглеждат основните характеристики на нанодиамантите, съвместно с потенциала им за приложение при стимулация, индикация и контрол на водопречиствателните и биодеградационни процеси.

В предложеното изследване е проучен ефектът на диамантените наночастици върху трансформацията на азо-багрилото амарант в хода на моделен пречиствателен процес в пясъчен биофилтър, функциониращ в sequencing batch режим. В хода на процеса са проследени ключови параметри: остатъчна концентрация на амарант, ензимни активности, ефективност на обезцветяване на амаранта, химично потребление на кислород, общ органичен въглерод. Данните показват положителната роля на диамантените наночастици като модулатор. Тези резултати разкриват перспективи за допълнителни изследвания и възможности за приложение на нанодиамантите при стимулиране на биодеградационните процеси в хода на водопречистването.

Ключови думи: амарант, биологично водопречистване, води, ензимна активност, нанодиаманти

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