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Biological activity of carbonic nano-structures. Comparison via enzymatic bioassay

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Abstract

Purpose: The aim of the work is to compare the biological activity of carbonic nano-structures of natural and artificial origination, namely, humic substances (HS) and fullerenols.

Materials and methods: The representative of the fullerenol group, $C_{60}O_y(OH)_x$ where y=2–4, x=22–24, was chosen. Enzyme-based luminescent bioassay was applied to evaluate toxicity and antioxidant properties of HS and fullerenol; chemiluminescent lumonol method was used to study a content of reactive oxygen species (ROS) in the solutions. Toxicity of the bioactive compounds was evaluated using effective concentrations EC_{50}; detoxification coefficients D_{OxT} were applied to study and compare antioxidant activity of the compounds. Antioxidant activity and ranges of active concentrations of the bioactive compounds were determined in model solutions of organic and inorganic oxidizers – 1,4-benzoquinone and potassium ferricianide.

Results and discussion: Values of EC_{50} revealed higher toxicity of HS than fullerenol (0.005 and 0.108 g L^{-1}, respectively); detoxifying concentrations of fullerenol were found to be lower. Antioxidant ability of HS was demonstrated to be time-dependent; the 50-min preliminary incubation in oxidizer solutions was suggested as optimal for the detoxification procedure. On the contrary, fullerenol' antioxidant effect demonstrated independency on time. Antioxidant effect of HS did not depend on amphiphilic characteristics of the media (values of D_{OxT} were 1.3 in the solutions of organic and inorganic oxidizers), while this of fullerenol was found to depend: it was maximal (D_{OxT} = 2.0) in solutions of organic oxidizer (1,4-benzoquinone).

Conclusions: Both HS and fullerenol demonstrated toxic and antioxidant effects; however quantitative characteristics of these effects were different. The difference in toxicities was explained with (1) flexibility of fragments of HS, (2) their higher ability to decrease ROS content. The difference in antioxidant activity was attributed to flexibility of HS macromolecules. The paper demonstrates a high potential of luminescent enzymatic bioassay to study biological activity of nano-structures of natural and artificial origination.

Keywords Antioxidant activity • Bioactive compounds • Fullerenol • Humic substances • Toxicity • Reactive oxygen species

Abbreviations

HS: humic substances;

F: fullerenol $C_{60}O_y(OH)_x$, where y=2–4, x=22–24;

NADH: nicotinamide adenine dinucleotide disodium salt reduced;

FMN: flavinmononucleotide;

OxT: oxidative toxicity

ROS: reactive oxygen species

1 Introduction

Biological activity of carbonic nano-objects is of great current interest for modern fields of medicine, biotechnology, pharmacology, and ecology. Biological activity presupposes that high-dose exposures inhibit physiological functions of organisms (toxic effect), but lower-dose exposures might activate physiological functions as a result of optimization of metabolic processes. Comparison of biological activity of natural or artificial 'nano-objects' is an important subject in biomedical investigations. Humic substances (HS) and fullerene derivatives can serve as representatives of carbonic structures of natural and artificial origination, respectively.

HS are natural bioactive compounds, which are able to attenuate environmental toxicity. HS are supposed to be irregular polymers of a complex structure (Orlov 1997; Perminova et al. 2001). Another concept is a supramolecular one; it assumes that HS 'consist of relatively small molecules linked by hydrogen, hydrophobic, or π-π bonds, as well as polyvalent cations' (Piccolo 2001; Richard et al. 2009; Trubetskoj et al. 2009; Lipczynska-Kochany 2018). Both conceptions assume that HS involve different functional groups, which can provide interactions with toxic compounds in water media, decreasing, by this, an aquatic toxicity. Mechanisms of these interactions include ion exchange, complexation, redox transformations, hydrophobic binding, coagulation, peptization, etc (Perelomov et al. 2018). Direct polar and non-polar interactions of HS with components of biological assay systems (cells, water-soluble biological compounds of low and high molecular weight) contribute to

biological effect of HS (Tarasova et al. 2011; Tarasova et al. 2012; Kudryasheva and Tarasova 2015; Tarasova et al. 2015). HS can take part in physical and chemical membrane irritation or stimulation through the enhanced surfactant-like interactions (Lipczynska-Kochany 2018). Because of the low molecular masses of their building blocks, HS are capable to pass bio-membranes, be metabolized intracellularly like xenobiotics, affect the enzymes, and stimulate oxidative stress defense.

Current paper compares the properties of HS and artificial bioactive compound, fullerenol (F), water-soluble polyhydroxylated derivative of fullerene-60, rigid nanosize carbonic particle. It is known that fullerenols are electron deficient structures; this property makes them as efficient catalyzers in biochemical reactions, and hence, perspective pharmaceutical agents. Additionally, fullerenols are amphiphilic structures: 'hydroxyl groups provide them with aqueous solubility, while the fragments of fullerene skeleton – with affinity to hydrophobic enzymatic fragments and lipid structures of cellular membranes' (Foley et al. 2002; Grebowski et al. 2013).

Luminescence bioassay systems are convenient tools to compare toxic and detoxifying properties of bioactive compounds; their main advantage is a high rate of registration of the biological response. This advantage provides possibility to carry out a lot of experiments under comparable conditions and, hence, an adequate statistical processing. This advantage is very important for biological analyses which are usually characterized by lower reproducibility than chemical assays. Luminous marine bacteria (Girotti et al. 2008) and their enzyme reactions (Kratasyuk and Esimbekova 2015) are among the most useful luminescence bioassays. They are considered as bioassays of different structural levels (cellular and enzymatic) and provide microbiological and biochemical analysis of the toxic effects, respectively.

The enzymatic luminescence bioassay provides monitoring of Oxidative Toxicity (*OxT*),

additionally to the conventional general toxicity. The *OxT* is attributed to redox characteristics of media (Kudryasheva and Tarasova, 2015). Using the bioluminescent enzymatic assay, the *OxT* of organic and inorganic oxidizer' solutions, quinones and polyvalent metals, have been previously studied (Kudryasheva et al. 2002; Vetrova et al. 2007; Tarasova et al. 2011); the importance of amphiphilic property of the organic oxidizers was shown in these studies. Bioluminescence assays were previously adapted for monitoring changes of *OxT* under exposure to bioactive compounds in (Tarasova et al. 2012; Kudryasheva and Tarasova 2015; Tarasova et al. 2015; Kudryasheva et al. 2017; Sachkova et al. 2017).

The aim of the work is to compare the biological activity of carbonic nano-structures of natural and artificial origination, namely, HS and fullerenol (F). Difference in rigidity of these structures is taken into consideration. The representative of the fullerenol group, fullerenol of lowest toxicity, $C_{60}O_y(OH)_x$ where y=2–4, x=22–24 (Eropkin et al. 2013; Kudryasheva et al. 2017; Sachkova et al. 2017) was chosen in this study. Section 3.1 presents toxicity evaluation of HS and F using enzyme-based luminescent bioassay; a role of reactive oxygen species in the toxic effects of HS and F is evaluated using chemiluminescent luminol method. Section 3.2 studies antioxidant effect of HS and F in model solutions of organic and inorganic oxidizers.

2 Materials and methods

2.1 Preparations of bioactive compounds

Humic Substances (HS) and fullerenol $C_{60}O_y(OH)_x$ where y=2–4, x=22–24 (F) were used as bioactive compounds.

The Gumat-80 preparation (Gumat, Russia) was produced by non-extracting treatment of coal with alkali (KOH, NaOH) (Levinsky 2000). Characteristics of the preparation are humic acids – 85 %, soluble potassium – 9 %, iron – 1 %, water – 5 %, and pH 8-9 in 1 % water solution.

The F was produced by fullerene-60 hydroxylation in nitric acid followed by the hydrolysis of the polynitrofullerenes (Goncharova et al. 2009; Isakova et al. 2011; Churilov et al. 2013). The F preparations were characterized with IR and photoelectron spectroscopies (Goncharova et al. 2009; Isakova et al. 2011; Juan et al. 2012). Fullerene-60 was preliminary synthesized by carbon helium high-frequency arc plasma at atmospheric pressure (Churilov et al. 2013). The fullerene content in carbon soot was about 12.6%. Fullerene mixture was extracted with toluene, and separated by liquid chromatography with turbostratic graphite (with interplanar distance 3.42 Å) as a stationary phase and toluene/hexane (4:6) mixture as a mobile phase.

2.2 Bioluminescence enzymatic assay and experimental data processing

Toxicity and antioxidant activity of HS and F were evaluated using bioluminescence enzymatic assay system, i.e. enzyme preparation, based on the coupled enzyme system NADH:FMN-oxidoreductase from *Vibrio fischeri* (0.15 a.u.) and luciferase from *Photobacterium leiognathi*, 0.5 mg ml^{-1} (Kuznetsov et al. 1996). The enzyme preparation was produced at the Institute of Biophysics SB RAS (Krasnoyarsk, Russia). Antioxidant activity of HS and F was assessed in water solutions of model oxidizers $K_3[Fe(CN)_6]$ (potassium ferricyanide) and 1,4-benzoquinone.

The chemicals used were: NADH from ICN, USA; FMN, and tetradecanal from SERVA, Germany; potassium ferricyanide of analytical grade from Khimreactiv, Russia; 1,4-benzoquinone from Aldrich, USA; sodium chloride (NaCl) from Khimreactiv, Russia.

To construct the enzyme system, 0.1 mg ml^{-1} enzyme preparation, $5 \cdot 10^{-4}$ M FMN, $4 \cdot 10^{-4}$ M NADH, and 0.002% tetradecanal solutions were used. The assay was performed in 0.05 M phosphate buffer (pH 6.8) at room temperature.

Measurements of bioluminescent intensity were carried out with bioluminometers BLM-3606 (Nauka Special Design Bureau, Russia) and TriStar LB 941 (Berthold Technologies, Germany). Time-course of the bioluminescent intensity was recorded.

Toxic effects of HS and F were evaluated by relative bioluminescent intensities, I_{HS}^{rel} or I_{F}^{rel}, respectively:

$$I_{HS}^{rel} = I_{HS} / I_{contr} \text{ or } \qquad I_{F}^{rel} = I_{F} / I_{contr} \qquad \text{(eq. 1)}$$

Here, I_{contr} and I_{HS} or I_F are maximal bioluminescent intensities in the absence and presence of HS or F, respectively.

Antioxidant activity of bioactive compounds (HS and F). Bioluminescence kinetics curves of enzymatic assay system are presented in Figure 1. The HS were chosen here as a representative of bioactive compounds. The evaluation was carried out in model solutions of oxidizers (1,4-benzoquinone or potassium ferricyanide). To compare toxic effects of the bioactive substances (HS and F), the effective concentrations of oxidizers, EC_{50}, (at $I_{ox}/I_{contr} = 0.5$) were determined. Here, I_{contr} and I_{Ox} are bioluminescence intensities in the absence and presence of oxidizers, respectively. Values of I_{contr} and I_{Ox} are shown in Figure 1. Changes of bioluminescence kinetics under addition of HS are shown in Figure 1, too.

Changes of Oxidative Toxicity (*OxT)* under exposure to HS or F were characterized with detoxification coefficients, D_{OxT}:

$$D_{OxT} = (T_{0.5})_{Ox} / (T_{0.5})_{Ox+HS}, \quad \text{or} \quad D_{OxT} = (T_{0.5})_{Ox} / (T_{0.5})_{Ox+F} \qquad \text{(eq. 2)}$$

Here, $(T_{0.5})_{Ox}$, $(T_{0.5})_{Ox+HS}$, or $(T_{0.5})_{Ox+F}$ are bioluminescence delay periods in the oxidizer

solutions in the absence and presence of HS or F, respectively, Figure 1. Values of D_{OxT} were determined at different HS or F concentrations.

Values of D_{OxT} >1 showed a decrease of *OxT* in oxidizer solutions under the exposure to HS or F, i.e. detoxification of the oxidizer solutions. Values of D_{OxT} <1 showed a toxic effect of HS or F.

Values of SD for D_{OxT} did not exceed 0.1. The data for the calculations of D_{OxT} were obtained in three parallel experiments with five samplings for all HS, F and control solutions.

The D_{OxT} values of HS were determined at different times of incubation with oxidizers: 0, 7, 15, 50, 130, and 160 min.

2.3 Luminol chemiluminescence assay

Luminol from Sigma-Aldrich, Russia, potassium hydroxide (KOH) from Khimreactiv, Russia, 3% hydrogen peroxide solution (H_2O_2) from Tula Pharmaceutical Factory, Russia, were used in chemiluminescence method.

The 3% hydrogen peroxide solution (H_2O_2) was applied to prepare the model peroxide solutions. Concentration of aqueous alkaline luminol was 10^{-4}M.

The chemiluminescence reaction was initiated by $K_3[Fe(CN)_6]$ solution through TriStar LB 941 bioluminometer injector system. Maximal chemiluminescence intensity was determined. Measurements of chemiluminescence intensity were performed in 25-40 replicates for all solutions; average and SD values were calculated, they did not exceed 0.1.

Dependence of chemiluminescence intensity on H_2O_2 concentration was determined; it was used in the following experiments to evaluate concentrations of peroxide compounds in the solutions of HS or F. The peroxide content was plotted vs. concentrations of HS or F. To compare effects of HS and F, their effective concentrations decreasing chemiluminescence intensity by 50%, EC_{50}, were determined.

3 Results

3.1 Toxicity of bioactive compounds

To compare the toxicity of HS and fullerenol (F), the dependencies of relative bioluminescence intensities I^{rel} on concentration of the compounds were determined. It is seen from Fig 2 that both HS and F demonstrate inhibition (toxic) effects ($I_{HS}^{rel} < 1$; $I_{F}^{rel} < 1$), however their inhibiting concentration intervals differ.

It should be noted that all experiments with "colored" solutions of HS and F excluded effect of 'optic filter' (Fedorova et al. 2007), and this effect did not skew the results the toxicological measurements.

To compare toxicity of HS and F in water solutions, values of EC_{50} were determined using enzymatic bioluminescent assay (Table 1).

Enzymatic assay revealed higher values of EC_{50} for F than HS, showing higher HS toxicity for enzymatic reactions. Probably nonrigid structure of HS, flexibility of the macromolecular' fragments and functional groups provided their intervention to the enzyme-substrates binding in water solutions. Additionally, it is known that bioluminescence enzymatic reaction includes a peroxide compound (peroxyhemiacetal) as an intermediate (Nemtseva and Kudryasheva 2007), hence, a decrease of peroxide content in HS solutions can be a reason of the inhibition of bioluminescence intensity of the enzymatic assay system. Following this suggestion, we used luminol chemiluminescent method to compare the peroxide contents (as representative of Reactive Oxygen Species, ROS) in the solutions of HS and F. Dependences of ROS content on HS or F concentrations were studied, a suppression of chemiluminescence signal was demonstrated, Fig.3. Concentrations of HS and F decreasing ROS content by 50%, $EC_{50,}$ were determined and presented in Table 1, second column.

The Table shows that HS decrease amount of ROS more effectively than F (values of EC_{50} for

the chemiluminescence assay are 0.008 and 0.235 g L^{-1}, respectively). This ability of HS is concerned with involvement of their redox chemical groups, decreasing the amount of peroxides in solutions, and hence, inhibiting the bioluminescence reaction.

Inhibition and activation of bioluminescence signal by ROS was reported previously for bacterial and enzymatic assays (Remmel et al. 2003; Alexandrova et al. 2011); hydrogen peroxide was applied by the authors as a representative of ROS.

Hence, the differences in toxic effects of HS and F on the enzymatic and chemiluminescence assay systems (Table 1, Figs 2-3) can be concerned with (1) flexibility of fragments and functional groups of HS, (2) their higher ability to decrease ROS content. These properties of HS might contribute to their antioxidant activity, as well. The following chapter 3.2 compares the antioxidant activity of HS and F.

3.2 Antioxidant activity of bioactive compounds in solutions of oxidizers

ROS content is directly concerned with Oxidative Toxicity (*OxT*) of the solutions. To monitor changes of *OxT* under exposure to bioactive compounds, HS and F, the bioluminescent enzymatic assay was applied. This assay is known to be specific to the group of oxidizers (Vetrova et al, 2007): in their presence, the bioluminescence delay period takes place (Figure 1); it depends on concentration and redox potential of oxidizers, and evaluates quantitatively the *OxT* of the solutions (Kudryasheva and Tarasova, 2015). Preliminary, we determined C_{50} in model solutions of organic and inorganic oxidizers (1,4-benzoquinone and potassium ferricyanide, respectively). Model oxidizers chosen are of high oxidative activity (standard redox potentials of 1,4-benzoquinone is 0.7 V, and this of ferricyanide is 0.36 V (Vanýsek 1983), but differ in polar/apolar characteristics). The changes of *OxT* in the solutions exposed to HS and F were studied; values of D_{OxT} were calculated and compared.

(a) Effect of oxidizers on bioluminescence assay system

Values of EC_{50} of the 1,4-benzoquinone and potassium ferricyanide were determined as 10^{-4} M and $2 \cdot 10^{-5}$M, respectively. These values are close to those determined earlier (Tarasova et al. 2011; Tarasova et al. 2012).

The EC_{50} values of the oxidizers were applied in the following experiments to evaluate the antioxidant activity of the bioactive compounds, HS and F.

(b) Change of toxicity of oxidizer solutions under exposure to bioactive compounds

Change of bioluminescence intensity of enzymatic assay in solutions of oxidizers was studied under variation of concentrations of HS or F. Low concentrations of HS or F which do not produce toxic effects (< 0.002 g L^{-1} for HS, or < 0.010 g L^{-1} for F Fig.2) have been chosen. Detoxification coefficients D_{OxT} have been calculated according to eq.2 at different concentrations of HS and F. Detoxifying (antioxidant) effects (D_{OxT} >1) of HS and F were found in solutions of organic (Fig. 4) and inorganic (Fig. 5) oxidizers. Detoxification coefficients of F presented in Fig. 4b and Fig.5b were determined previously (Sachkova et al. 2017).

The detoxifying effect of HS was found to depend on time of preliminary incubation with the oxidizers: only high incubation times (> 7 min) provided statistically significant antioxidant effect (D_{OxT} >1), however, shorter incubation times did not. Two times of incubation (0 and 50 min) were chosen for presentation in Figs 4a and 5a. The choice of 50 min incubation time was justified by saturation of detoxifying effect of HS at higher incubation times.

Such peculiarity of HS behavior is concerned with their nonrigid and nonregular structure, which determines the importance of time-dependent diffusion processes in water solutions.

The detoxifying effect of F did not depend on the incubation time.

Table 2 presents intervals of detoxifying concentrations of HS and F, as well as maximal

values of D_{OxT}. This Table and Figs 4, 5 show that the detoxifying concentrations of HS and F differ: the interval for F is moved to lower concentrations as compared to HS. In (Sachkova et al. 2017) a low-concentration effect of fullerenols was concerned with hormesis phenomenon (Calabrese 2015; 2014) that can be explained with structural effects of water media (Iavicoli et al. 2018; Zheng et al. 2017). Following this hypothesis, diffusion-dependent processes involving macromolecules of HS might prevent from aqua structuring, minimizing low-concentration effects of HS.

Antioxidant effect of HS is of 'soft' character: average values of D_{OxT} do not exceed 1.3 (Table 2). This effect is similar in solutions of both organic and inorganic oxidizers. The latter means that the difference in polar/apolar characteristics of oxidizers is not important for HS. This difference appeared to be important for F; value of D_{OxT} is much higher in solutions of organic amphiphilic oxidizer: 2.0 and 1.3 for 1,4-benzoquinone and ferricianide, respectively, Table 2, Figs 4,5. Probably, hydrophobic interactions of the organic oxidizer in complex enzymes+F+1,4-benzoquinone solutions contribute to the catalytic activity of the fullerenol. Rigid structure of hydrophobic fragments in fullerene carcass of F nanoparticle might be important for such interactions.

4 Conclusions

Current study compares toxic and antioxidant effects of humic substances, natural detoxifying compounds, with fullerenol – a representative of a group of fullerene C-60 derivatives, a carbon nanosize structure, which is perspective in modern medical, biological and chemical technologies. Differences in toxic effects and antioxidant activity were attributed to structure of these compounds. Non-rigidity of humic macromolecules determines their diffusion restrictions, which result in higher toxicity and time-dependence of their antioxidant ability. The same property is probably responsible for unification of their antioxidant ability to

oxidizers of different hydrophilic/hydrophobic characteristics. On the contrary, antioxidant effect of fullerenol was found to depend on amphyphilic properties of oxidizers. However, further study of mechanisms of toxic and antioxidant effects of the bioactive compounds is of special applied and fundamental prospectivity.

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Table 1 Effective concentrations of bioactive compounds, EC_{50}, in enzymatic and chemiluminescence assays

Bioactive compounds*	EC_{50}, g L^{-1}	
	Enzymatic bioluminescence assay	Chemiluminescence assay
HS	0.005	0.008
F	0.108	0.235

**Bioactive compounds: HS – humic substanses; F – fullerenol*

Table 2 Maximal values of D_{OxT} and a range of detoxifying concentrations (D_{OxT}>1) of bioactive compounds in model solutions of organic and inorganic oxidizers (1,4-benzoquinone and $K_3[Fe(CN)_6]$)

Bioactive compound*	Range of detoxifying concentrations, g L^{-1}; Maximal value of D_{OxT}	
	1,4-benzoquinone	$K_3[Fe(CN)_6]$
HS (50 min incubation)	$4 \cdot 10^{-5} – 5 \cdot 10^{-3}$; 1.3	$2 \cdot 10^{-6} – 5 \cdot 10^{-3}$; 1.3
F	$10^{-14} – 5 \cdot 10^{-5}$; 2.0	$10^{-14} – 2 \cdot 10^{-5}$; 1.3

**Bioactive compounds: HS – humic substanses; F – fullerenol*

Figure captions

Fig. 1 Bioluminescence kinetics in solution of a model oxidizer (Ox) and Humic Substances (HS)

Fig. 2 Relative bioluminescence intensity I^{rel} at different concentrations of bioactive compounds: humic substances (HS) and fullerenol (F). Enzymatic assay

Fig. 3 Concentration of ROS vs. concentrations of bioactive compounds: humic substances (HS) and fullerenol (F). Chemiluminescence assay

Fig.4 Detoxification coefficients D_{OxT} vs. concentration of HS (a) and F (b) in solution of 1,4-benzoquinone ($C_{50} = 10^{-4}$ M). Times of preliminary incubation of HS with 1,4 - benzoquinone are indicated in Fig. a

Fig. 5 Detoxification coefficients D_{OxT} vs. concentration of HS (a) and F (b) in solution of potassium ferricyanide ($2 \cdot 10^{-5}$ M). Times of preliminary incubation of HS with potassium ferricyanide are indicated in Fig. a

Fig.1. Bioluminescence kinetics in solution of a model oxidizer (Ox) and Humic Substances (HS)

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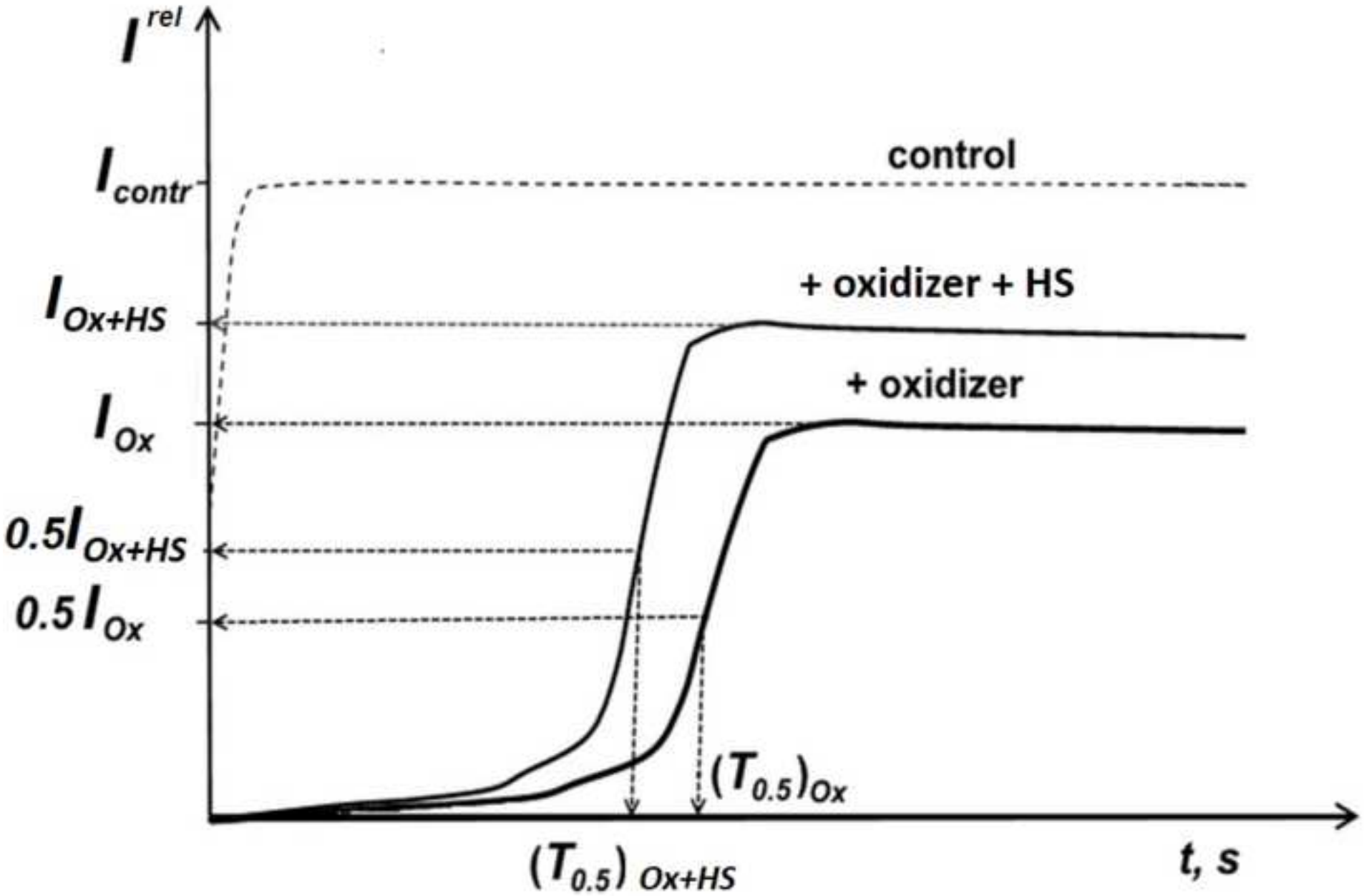

Fig.2 Relative bioluminescence intensity Irel at different concentrations of bioactive compounds: humic substances (HS) and fullerenol (F). Enzymatic assay

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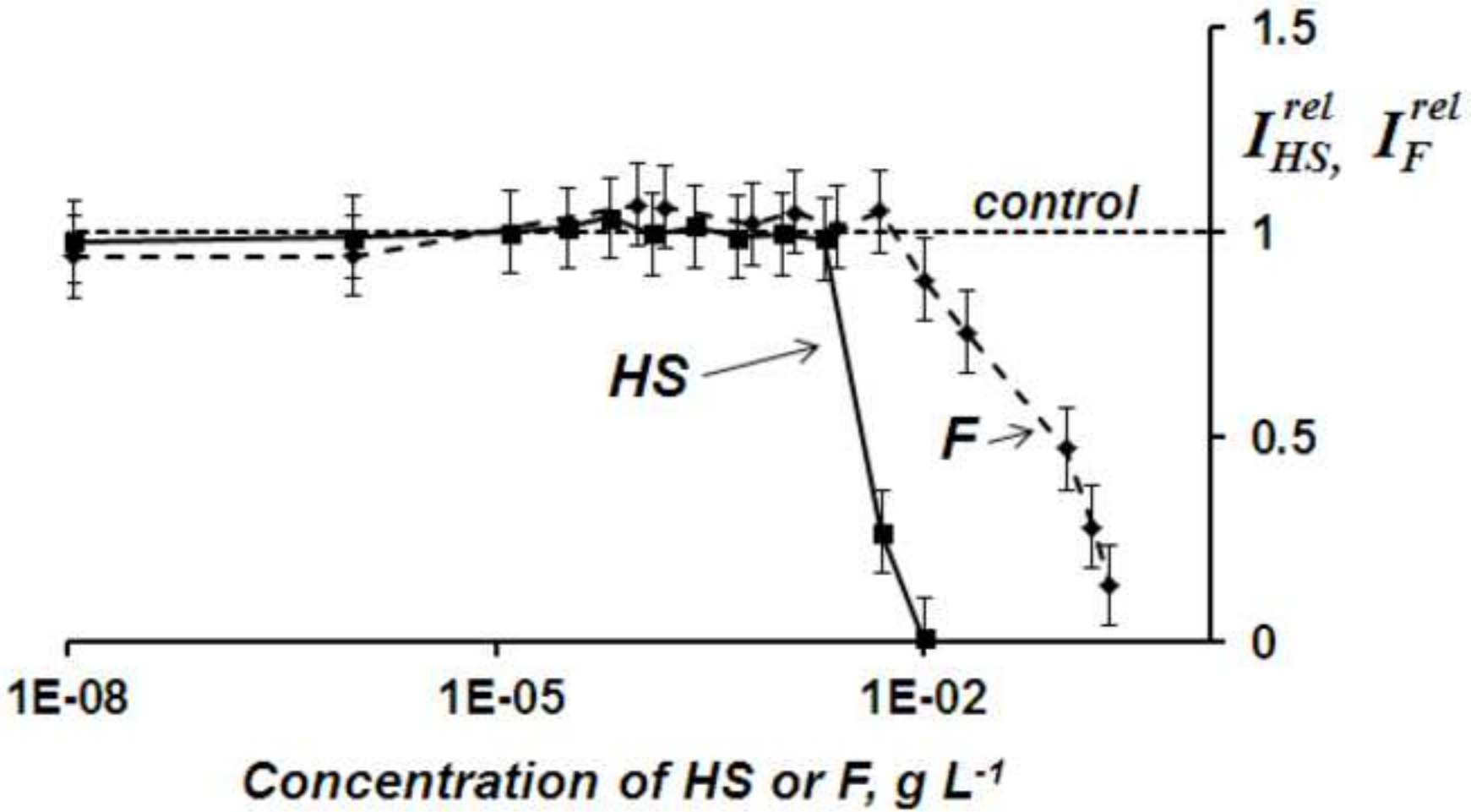

Fig. 3 Concentration of ROS vs. concentrations of bioactive compounds: humic substances (HS) and fullerenol (F). Chemiluminescence assay

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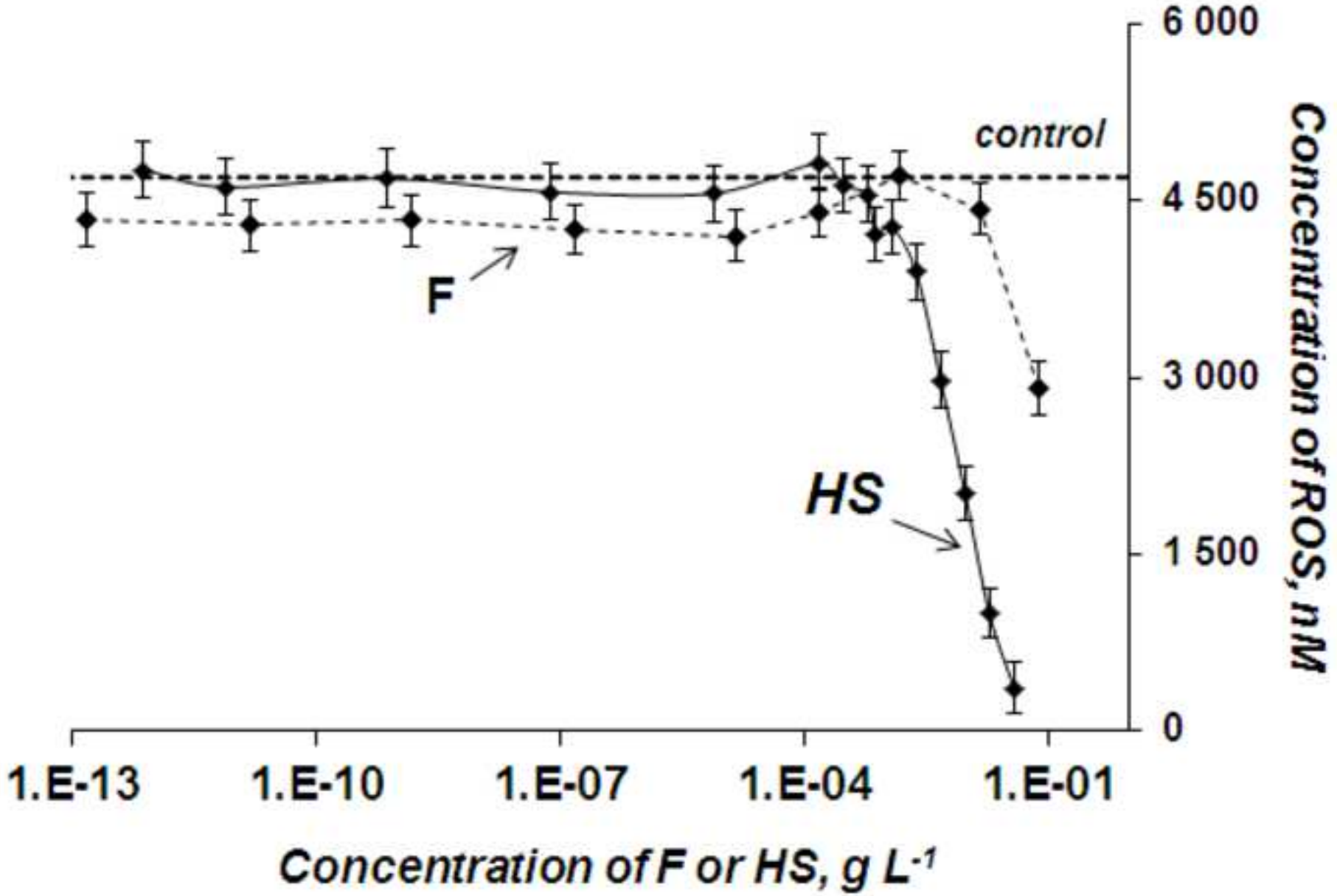

Fig.4 Detoxification coefficients DOxT vs. concentration of HS (a) and F (b) in solution of 1,4-benzoquinone (C50 = 10-4 M). Times of preliminary incubation of HS with 1,4 -

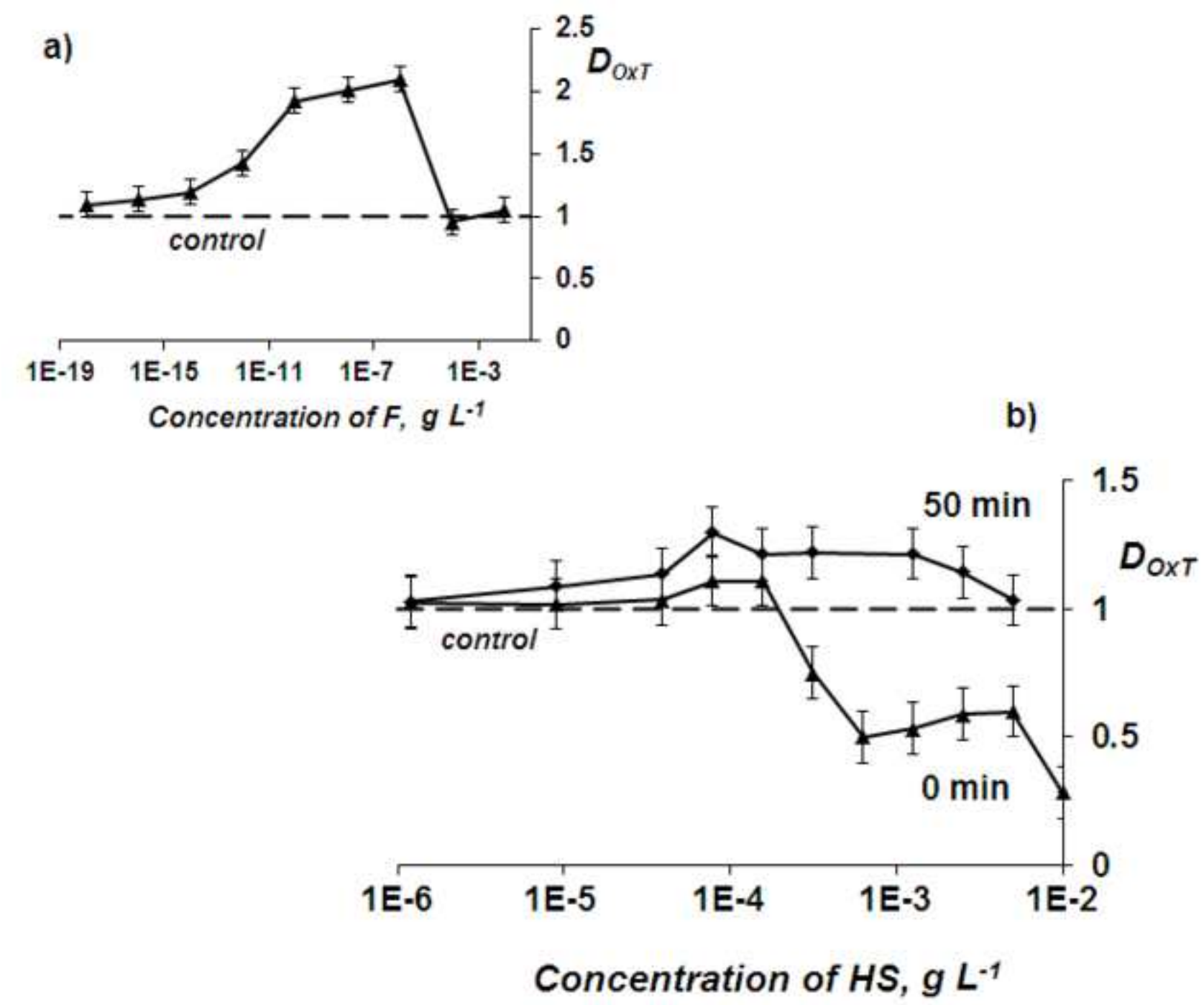

Fig. 5 Detoxification coefficients DOxT vs. concentration of HS (a) and F (b) in solution of potassium ferricyanide (2*10-5 M). Times of preliminary incubation of HS with