Ecotoxicology TP Course

Concepts, Tests & Biomarkers

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TP8 Effect and defense biomarkers

Control

10 ug/L Glifosato

250 ug/L Glifosato

500 ug/L Glifosato

PHASE I AND PHASE II ENZYMES

PHASE I: BIOTRANSFORMATION

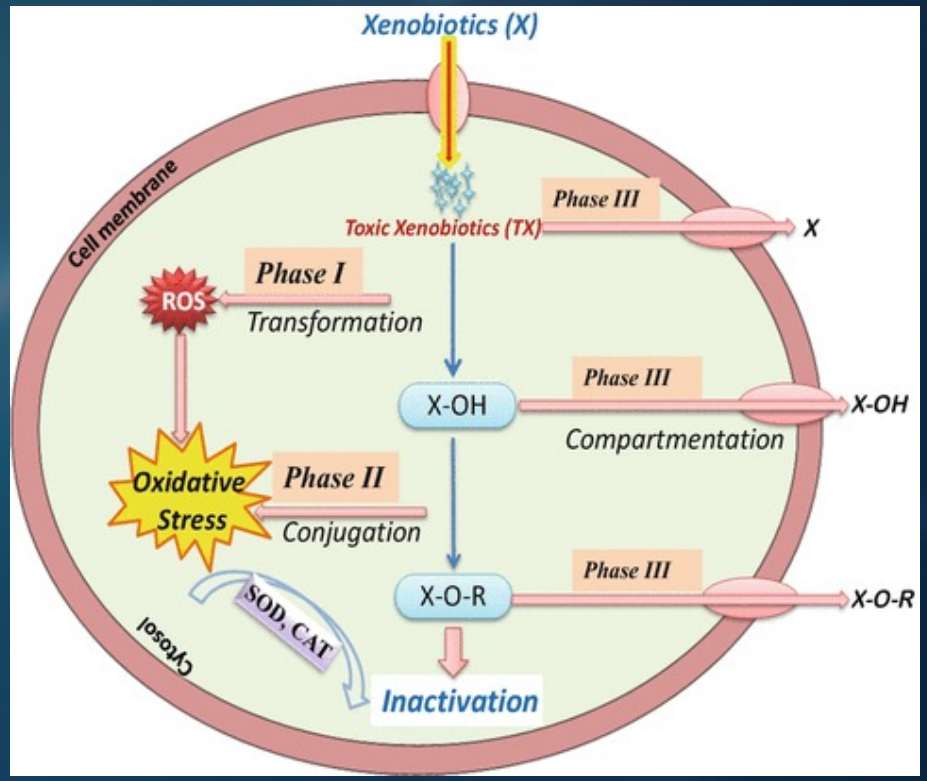
Phase I enzymes and mechanisms **transform xenobiotics into less harmful molecules**, but that may have a ROS-generating potential (for e.g.: CYPA1 enzymes).

PHASE II: CONJUGATION

Phase II mechanisms are composed by enzymatic and non-enzymatic **anti-oxidant mechanisms** that work in conjugation to quench the ROS generated directly by the xenobiotic or by the Phase I biotransformed xenobiotic.

PHASE III: EXCRETION

Phase III mechanisms are based in **membrane proteins that can excrete** directly or throughout vesicle compartments the transformed and/or inactivated xenobiotic to the extracellular environment.



BIOMARKERS : SUPEROXIDE DISMUTASE ASSAY (PHASE II)

PRINCIPLES OF THE METHOD:

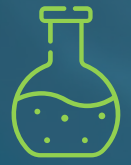
- Pyrogaloll (Pyr) has a absorption peak at 325 nm.
- Pyrogaloll has an auto-oxidation (degradation) rate.
- The method measures the inhibition of oxidized Pyrogaloll for 120 seconds at 325 nm by Superoxide Dismutase.

$$\text{Pyrogaloll} + 2\,O_2^{\cdot-} \xrightarrow{\text{SOD}} O_2 + H_2O_2$$

BLANK CELL

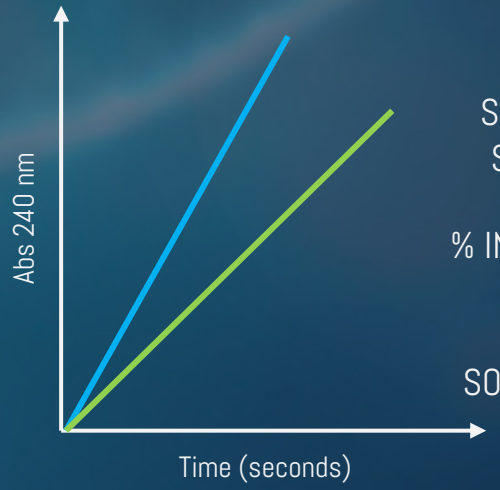
1. 100 µg protein (calculate the extract volume needed).
2. Remaining volume with PBS buffer.

To zero the spectrophotomer.



SOD ASSAY

1. 100 µg protein (calculate the extract volume needed).
2. Remaining volume with PBS buffer.
3. 0.3 mM Pyrogaloll



CALCULATIONS

$$\text{Slope}_{BLANK} = (\text{Abs}_{t=120} - \text{Abs}_0)_{BLANK} / (120 - 0)$$
$$\text{Slope}_{SOD} = (\text{Abs}_{t=120} - \text{Abs}_0)_{BLANK} / (120 - 0)$$

$$\%\ \text{INHIBITION PYROGALOLL} = \text{Slope}_{SOD} / \text{Slope}_{BLANK}$$

$$\text{SOD (U/mL)} = \%\ \text{INHIBITION PYROGALOLL} \times 50\%$$

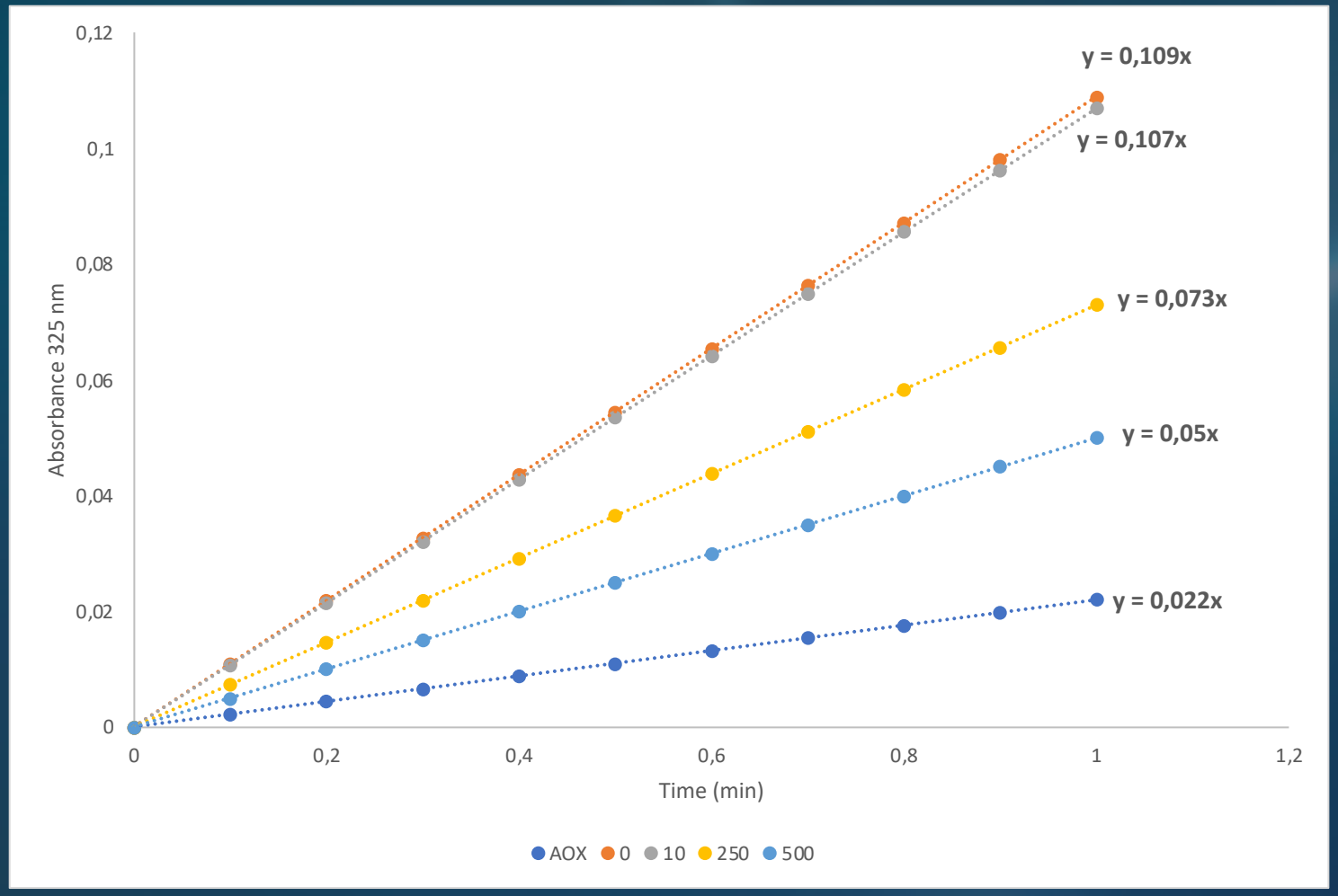
BIOMARKERS : SUPEROXIDE DISMUTASE ASSAY (PHASE II)

1. Extract the proteins with Extraction PBS Buffer (1 mL)
2. Centrifuge to precipitate cellular debris
3. Use the extract for spectrophotometric assay (60 sec at 325 nm)

	Blank	Reaction
Ultra-pure Water	360 µL	360 µL
Buffer	630 µL	550 µL
Extract	10 µL	10 µL
Pyrogalol	0 µL	80 µL

BIOMARKERS : SUPEROXIDE DISMUTASE ASSAY (PHASE II)

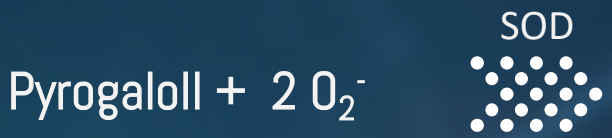
Pyrogaloll + $2\ O_2^-$ —SOD→ $O_2 + H_2O_2$

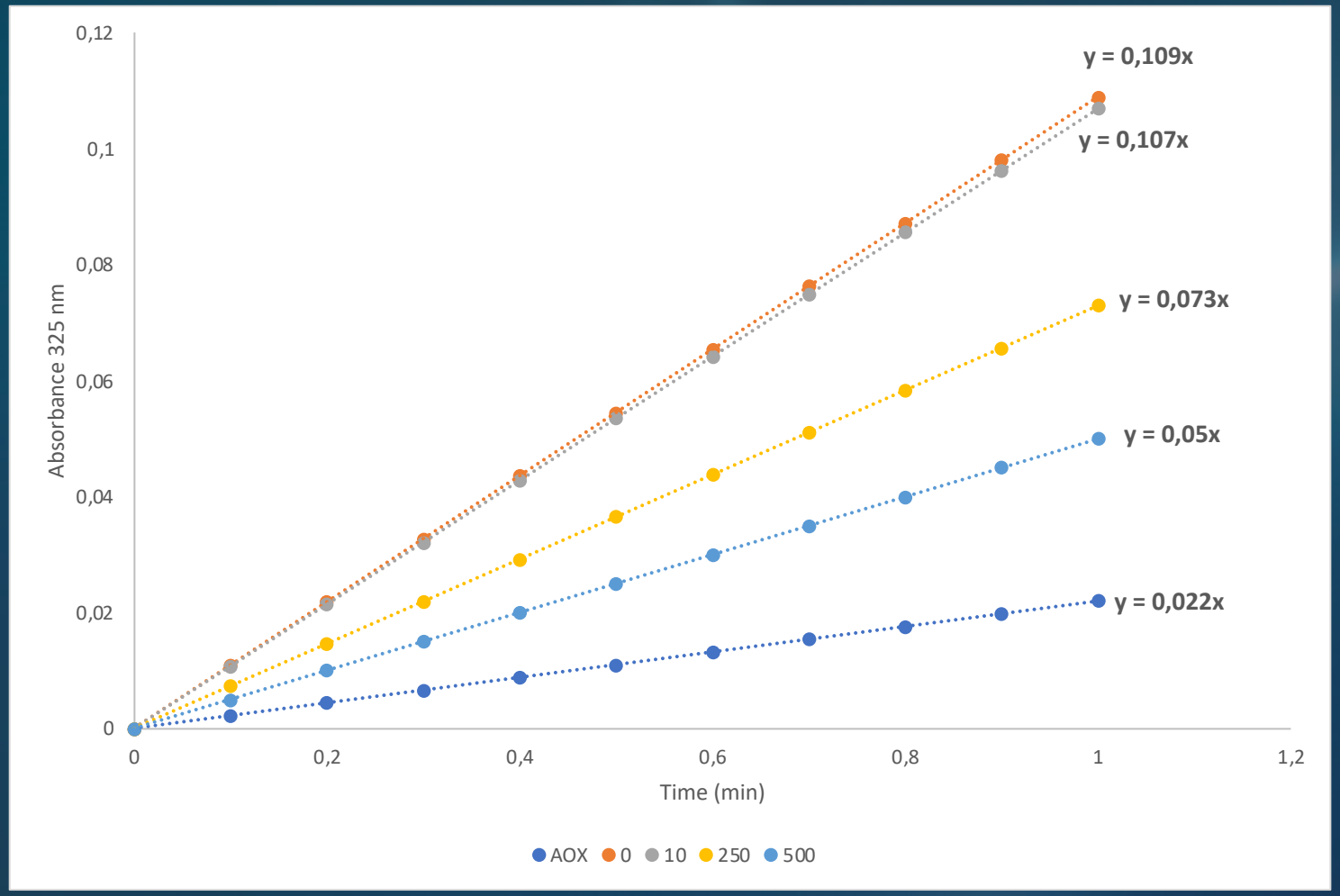


SOD Activity = Enzymatic Rate / (0.5 x AOX Rate)

[Glifosate]	Rate	Activity
0	0.109	
10	0.107	
250	0.073	
500	0.050	
AOX	0.022	

BIOMARKERS : SUPEROXIDE DISMUTASE ASSAY (PHASE II)





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[Glifosate]	Rate	Activity
0	0.109	9.91
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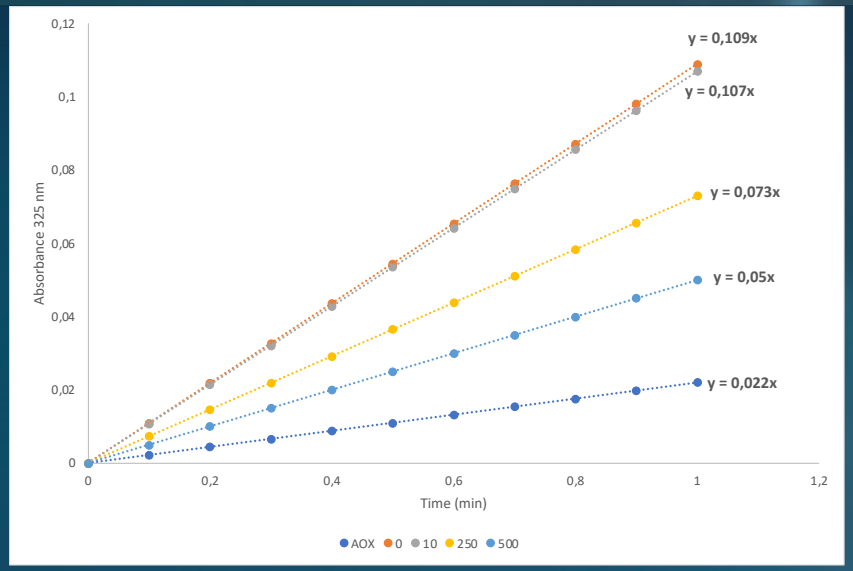

Pyrogaloll + $2\ O_2^-$ —SOD→ $O_2 + H_2O_2$

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Reaction volume = 10 uL

[Glifosate]	Rate	Activity	[Protein] ug/mL	SOD (U ug Prot)
0	0.109	9.91	208	
10	0.107	9.73	220	
250	0.073	6.64	270	
500	0.050	4.55	186	
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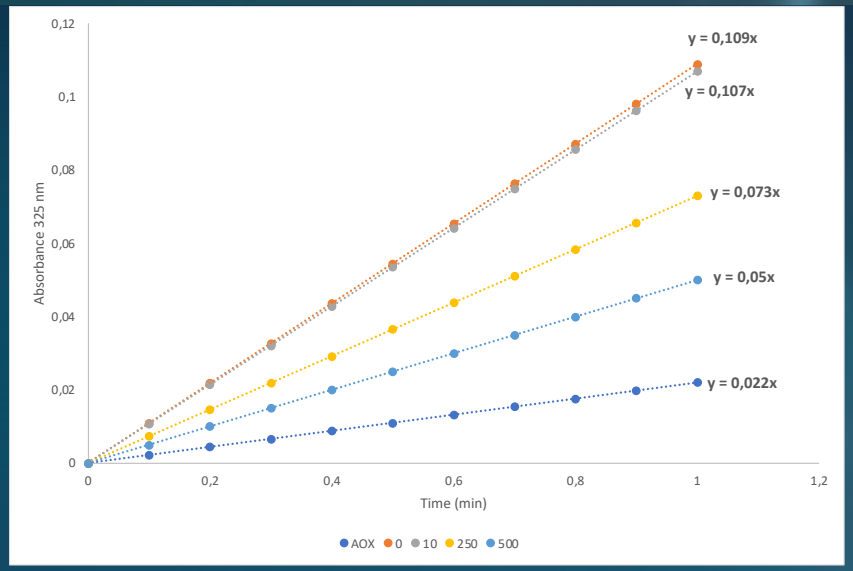

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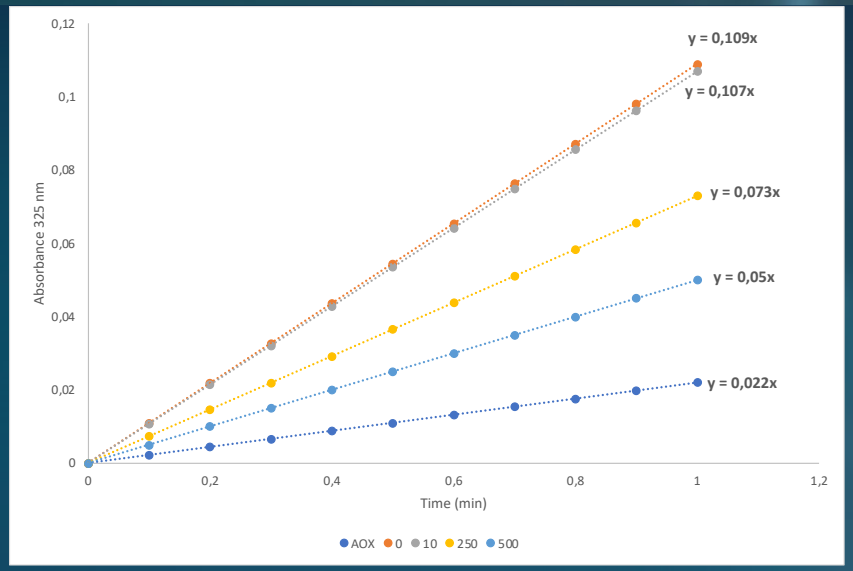

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10	0.107	9.73	220	4.42
250	0.073	6.64	270	3.90
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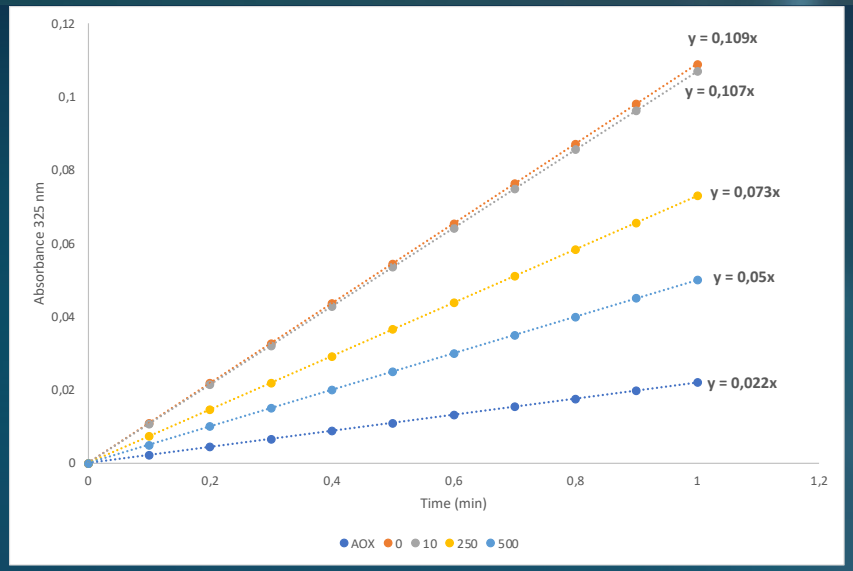

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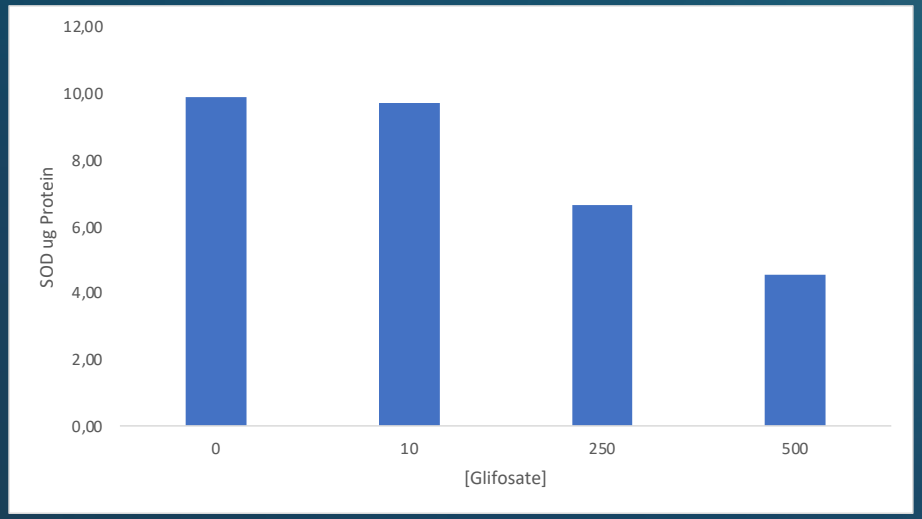
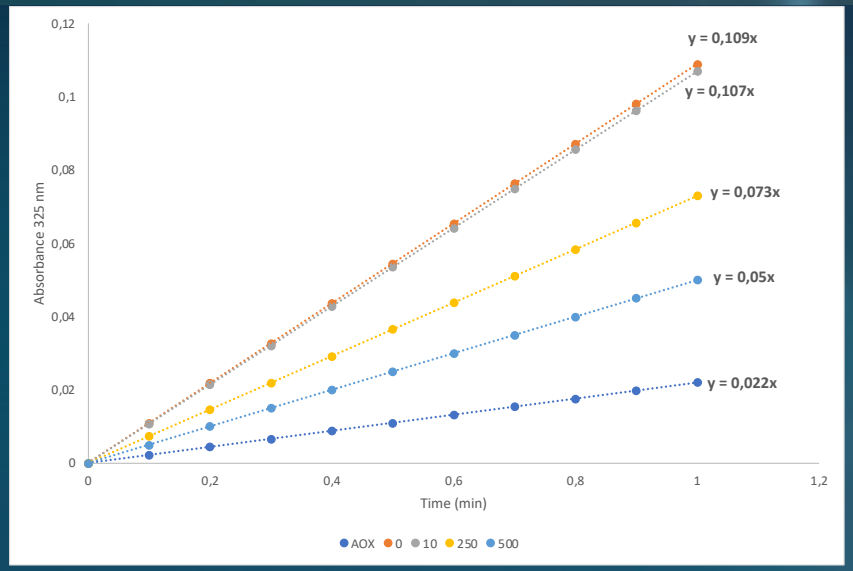


[Glifosate]	SOD (U ug Prot)	% Inhibition
0	4.76	
10	4.42	
250	3.90	
500	2.44	

BIOMARKERS : SUPEROXIDE DISMUTASE ASSAY (PHASE II)



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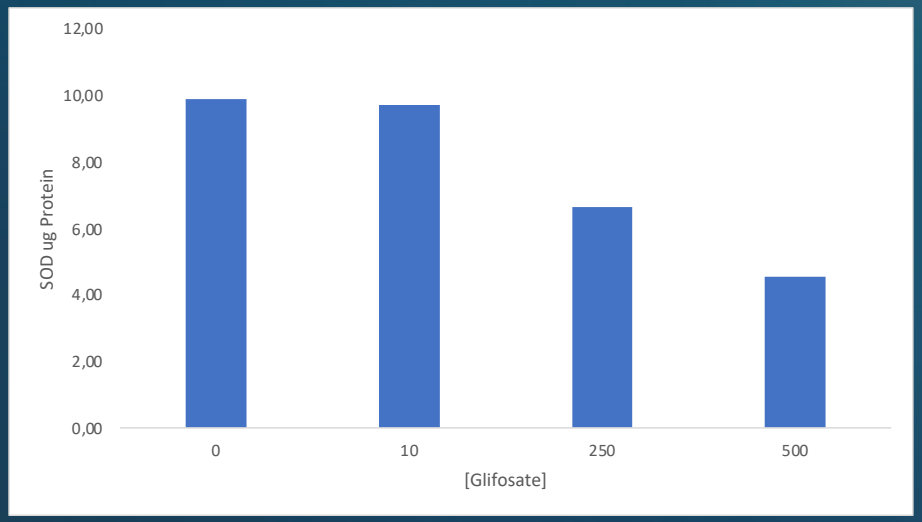
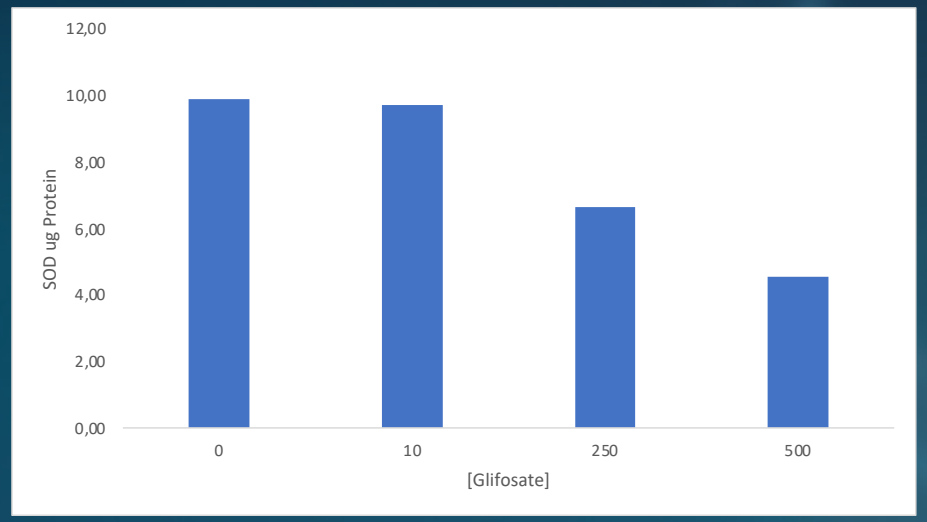
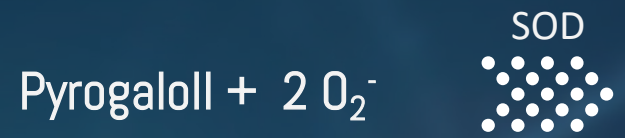
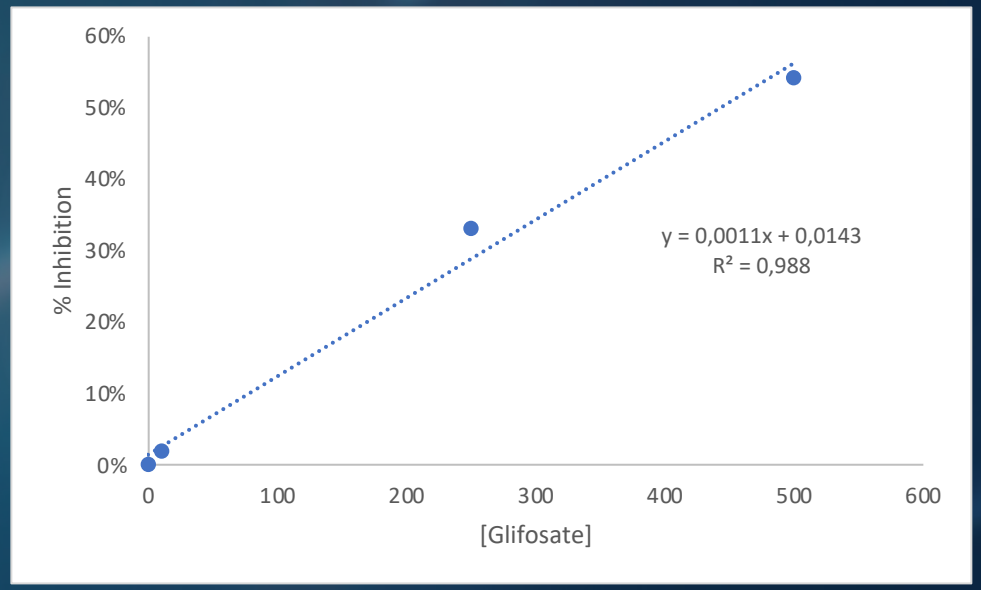


[Glifosate]	SOD (U ug Prot)	% Inhibition
0	4.76	0
10	4.42	2
250	3.90	33
500	2.44	54

BIOMARKERS : SUPEROXIDE DISMUTASE ASSAY (PHASE II)



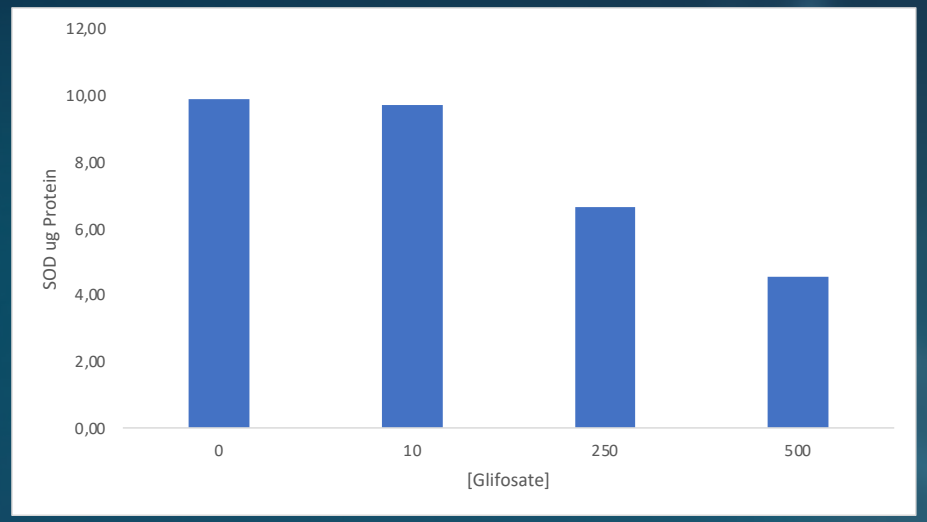


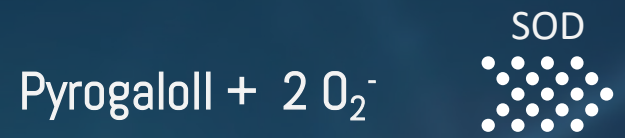


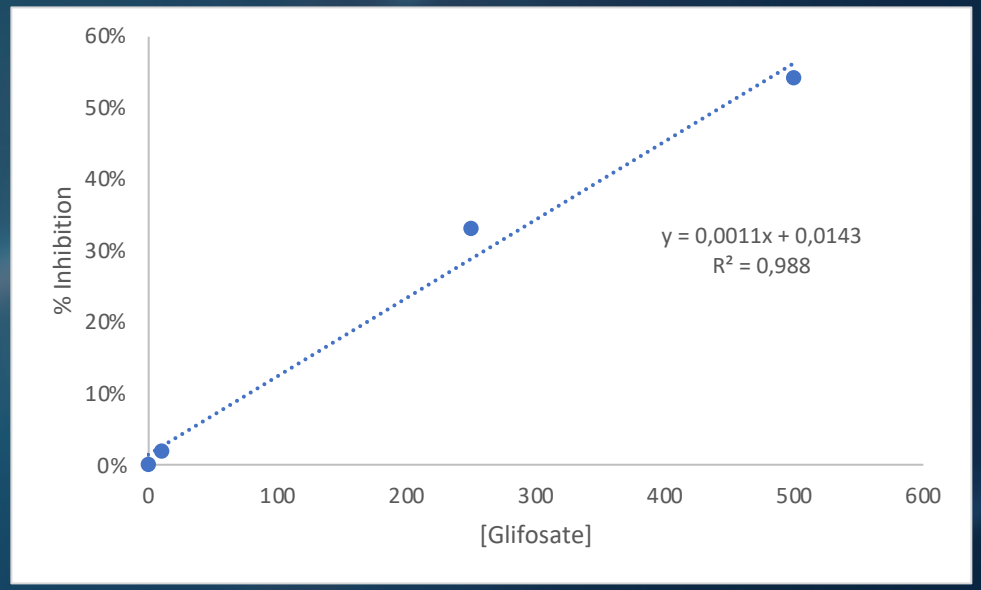
[Glifosate]	SOD (U ug Prot)	% Inhibition
0	4.76	0
10	4.42	2
250	3.90	33
500	2.44	54

IC_{50} = ?

BIOMARKERS : SUPEROXIDE DISMUTASE ASSAY (PHASE II)



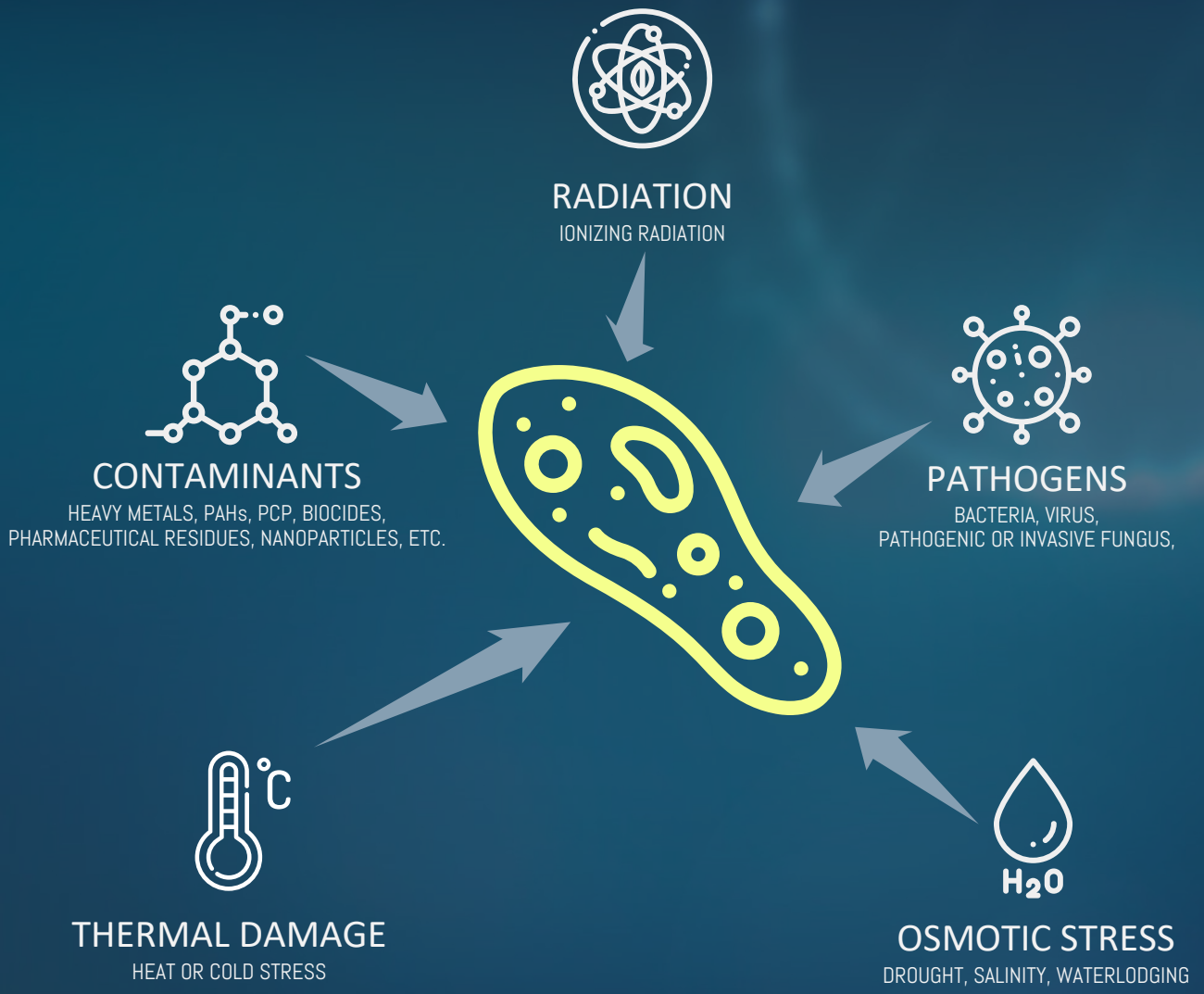


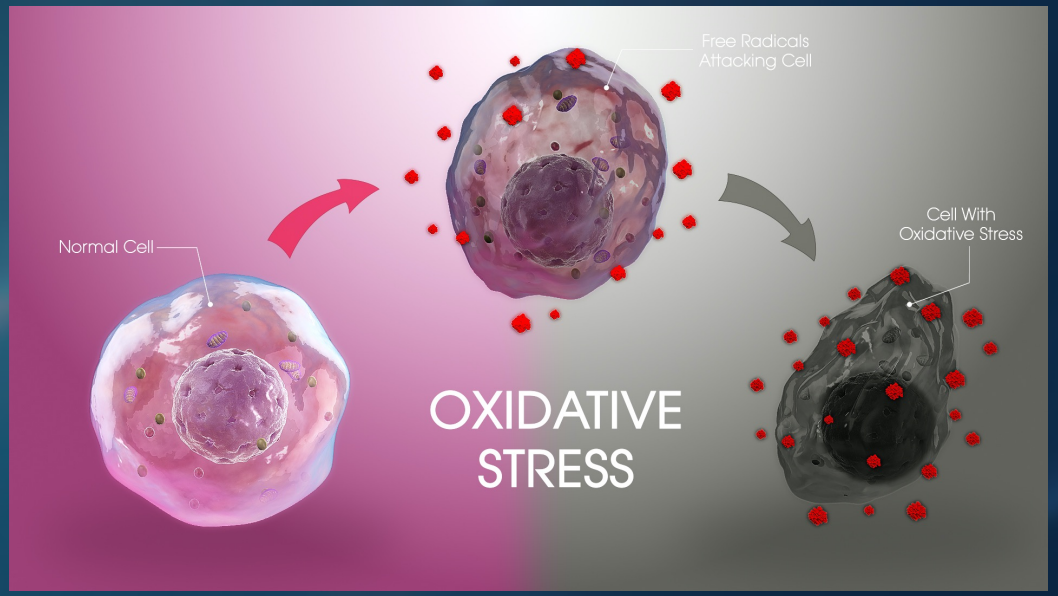


[Glifosate]	SOD (U ug Prot)	% Inhibition
0	4.76	0
10	4.42	2
250	3.90	33
500	2.44	54

IC_{50} = 44.15 ug/L

SOURCES OF OXIDATIVE STRESS





OXIDATIVE STRESS

OXIDATIVE STRESS:

- Oxidative stress reflects an **imbalance** between the systemic **manifestation of reactive oxygen species** and a **biological system's ability to readily detoxify** the reactive intermediates or to repair the resulting damage.
- Disturbances in the normal redox state of cells can cause toxic effects through the production of peroxides and free radicals that damage all components of the cell, including proteins, lipids, and DNA.
- Oxidative stress from oxidative metabolism causes base damage, as well as strand breaks in DNA. Base damage is mostly indirect and caused by reactive oxygen species (ROS) generated, e.g. O^{2-} (superoxide radical), OH (hydroxyl radical) and H_2O_2 (hydrogen peroxide).
- Further, some reactive oxidative species act as cellular messengers in redox signalling. Thus, oxidative stress can cause disruptions in normal mechanisms of cellular signalling.
- IMPORTANT: ROS are generated during the normal cell functioning thus the cells also developed anti-oxidant mechanisms to counteract the negative effects produced by ROS.
- In Ecotoxicology, the production or activity of these ROS counteractive measures as well as the ROS-biomolecules reaction products are used to evaluate the cell oxidative stress level.

BIOMARKERS : TYPES

BIOMARKER

A trait or molecular entity that can be measured experimentally and indicate the occurrence of a certain function (normal or pathological) of a certain organisms towards a specific stressor. Ideally these biomarkers should produce a dose related response towards the stressor applied.

ENZYMES

Typically oxidative stress triggered enzymes, which the activity is related to the stress level imposed.

DEGRADATION PRODUCTS

Molecules or compounds that result from the destruction of a cellular structure or metabolite in reaction to stress.

NON-ENZYMATIC ANTI-OXIDANT MOLECULES

Molecules or compounds that are produced in reaction to oxidative stress.

ANALYTICAL PROTOCOL

The DNA strand breaks are measured using the DNA alkaline precipitation assay as described by Olive, 1988. The method includes the precipitation of SDS associated nucleoproteins and genomic DNA followed by the determination of the DNA concentration that remains in the supernatant, which constitutes the damaged DNA. This damaged DNA is labelled with a fluorescent dye and the fluorescence is measured using 360:460 nm to excitation:emission wavelength filters.

SAMPLE PREPARATION

- Extract the sample with 0.5 mL Phosphate Buffer and ultrasounds.
- Centrifuge the sample (fast spin).
- Add 50 uL extract (or 50 uL PBS for the blanks) + 250 uL Reaction Mixture + KCl
- Heat the sample for 10 min at 60 ºC.
- Place on ice for 10 min.
- Centrifuge at 8000 x g for 5 min.

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SAMPLE ANALYSIS

- Add 50 uL of the reaction extract to the well.
- Add 200 uL of Hoesch dye
- Read the fluorescence with 360 nm Excitation and 460 nm Emission
- Compare with the DNA standard curve