# Ecotoxicology TP Course

Concepts, Tests & Biomarkers

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# TP7 Effect and defense biomarkers


*Control*


*10 ug/L Glifosato*


*250 ug/L Glifosato*

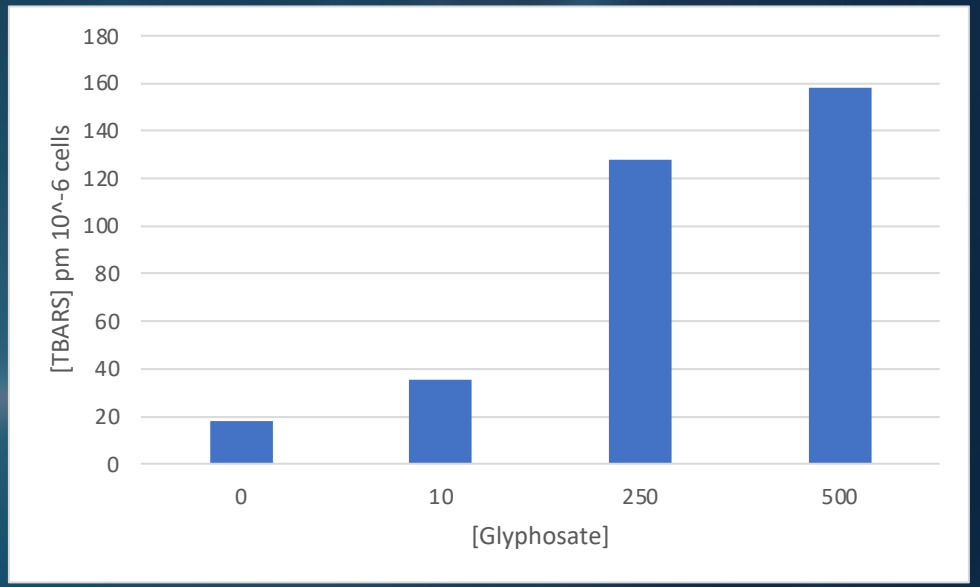

*500 ug/L Glifosato*

- Add 1.5 mL TCA 20% (w/v) to the pellet.
- Heat the sample for 20 min at 100 ºC.
- Take 1 mL of the TCA sample and add it to 1 mL TBA 0.5 %.
- Heat the sample for an additional 20 min at 100 ºC.
- Read the absorbance at 532 nm e 600 nm ($\varepsilon$ [MDA] = 155 $mM^{-1}$ $cm^{-1}$):

$$A_{532\,nm} - A_{600\,nm} = [MDA]mM \times \varepsilon MDA$$

# TBARS Results

| [Glyphosate] | [TBARS] pmol cell $10^{-6}$ |
|---|---|
| 0 | 18,1364 |
| 10 | 35,18561 |
| 250 | 127,7721 |
| 500 | 158,1783 |



# TBARS Results

| [Glyphosate] | [TBARS] pmol cell $10^{-6}$ | Enhancement (%) |
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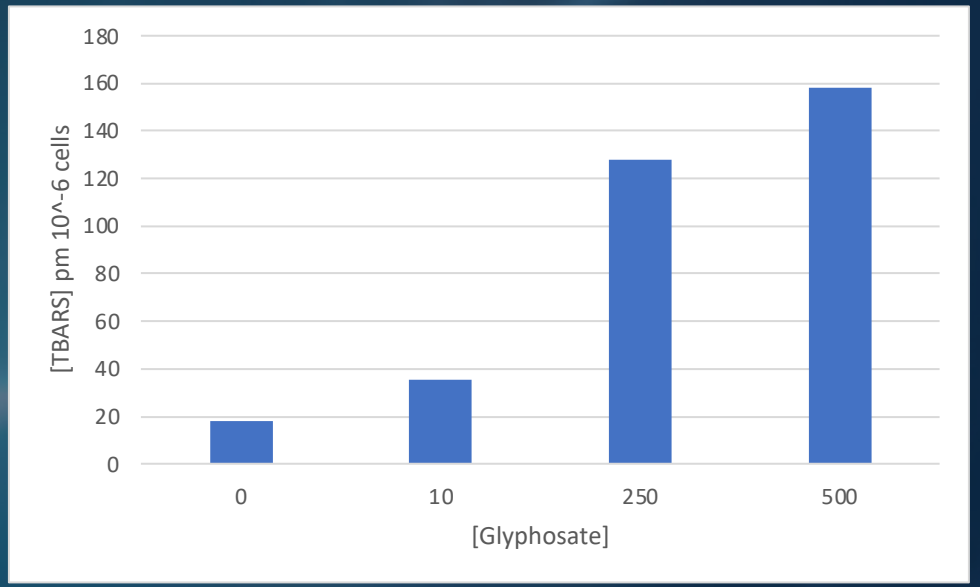

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| [Glyphosate] | [TBARS] pmol cell $10^{-6}$ | Enhancement (%) |
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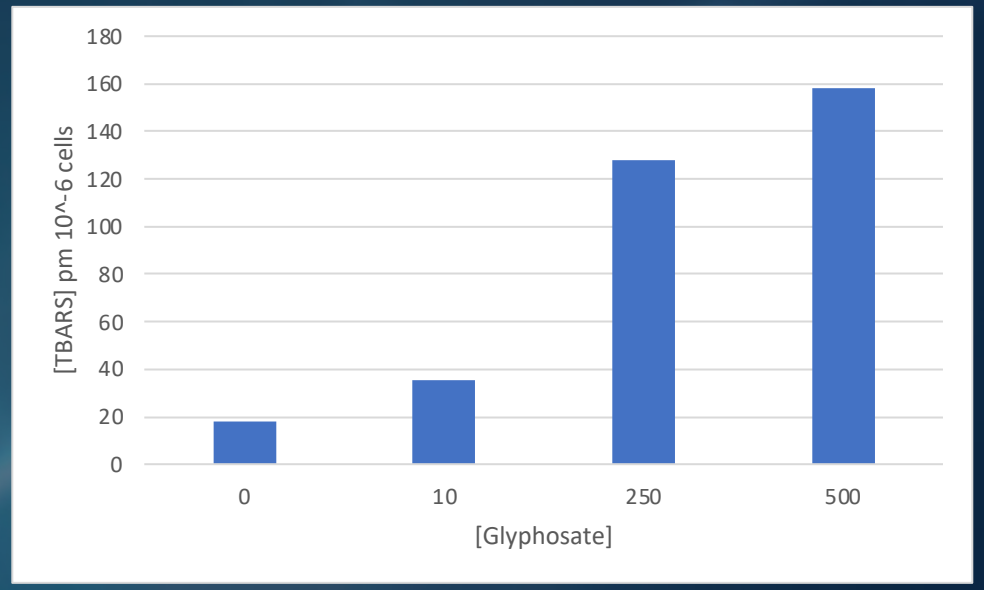
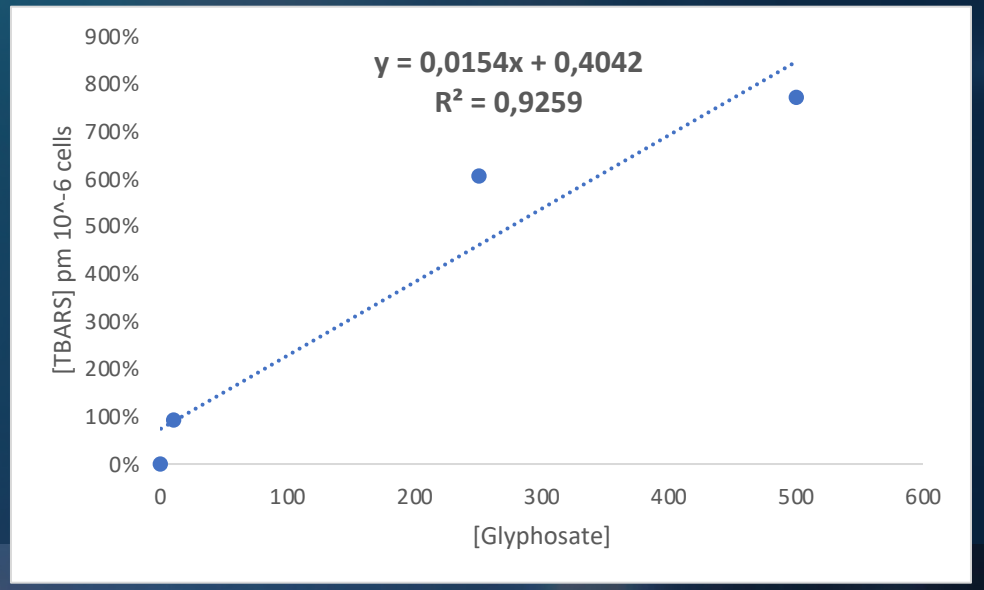
| 0 | 18,1364 | **0%** |
| 10 | 35,18561 | **94%** |
| 250 | 127,7721 | **605%** |
| 500 | 158,1783 | **772%** |

**$EC_{50}$ = ?**



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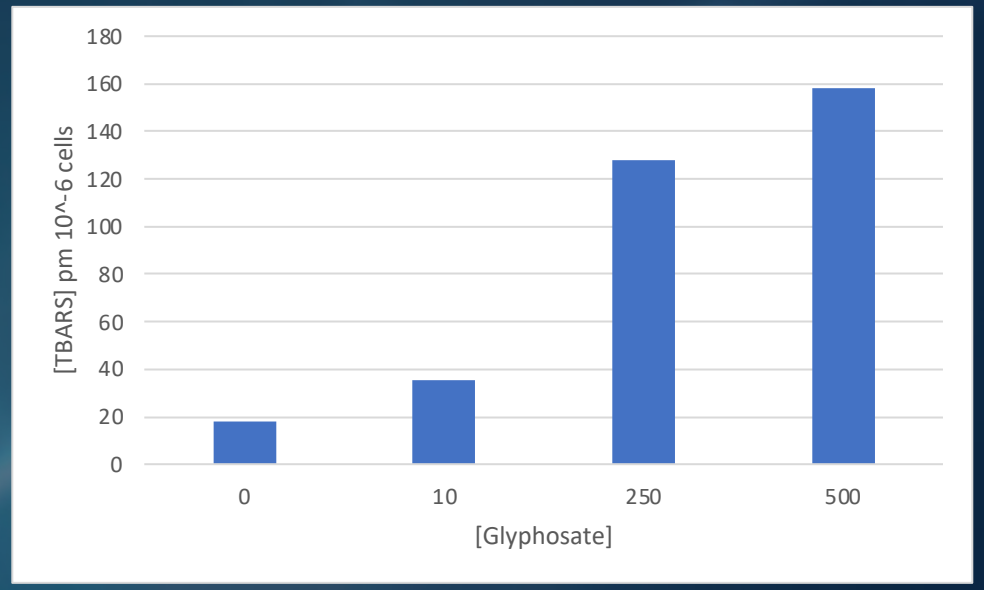
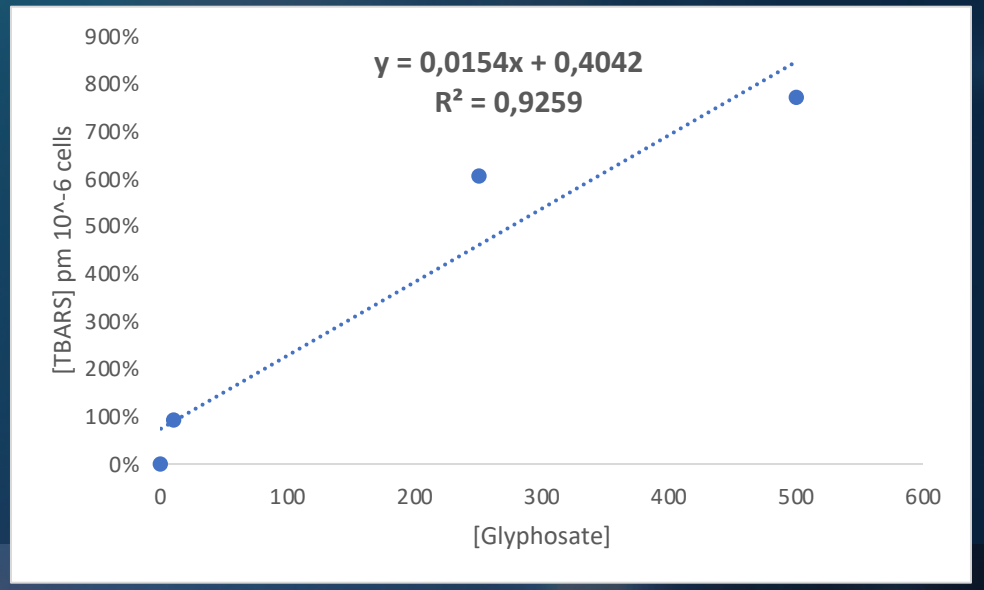
**$EC_{50}$ = 6.22 mg/L**



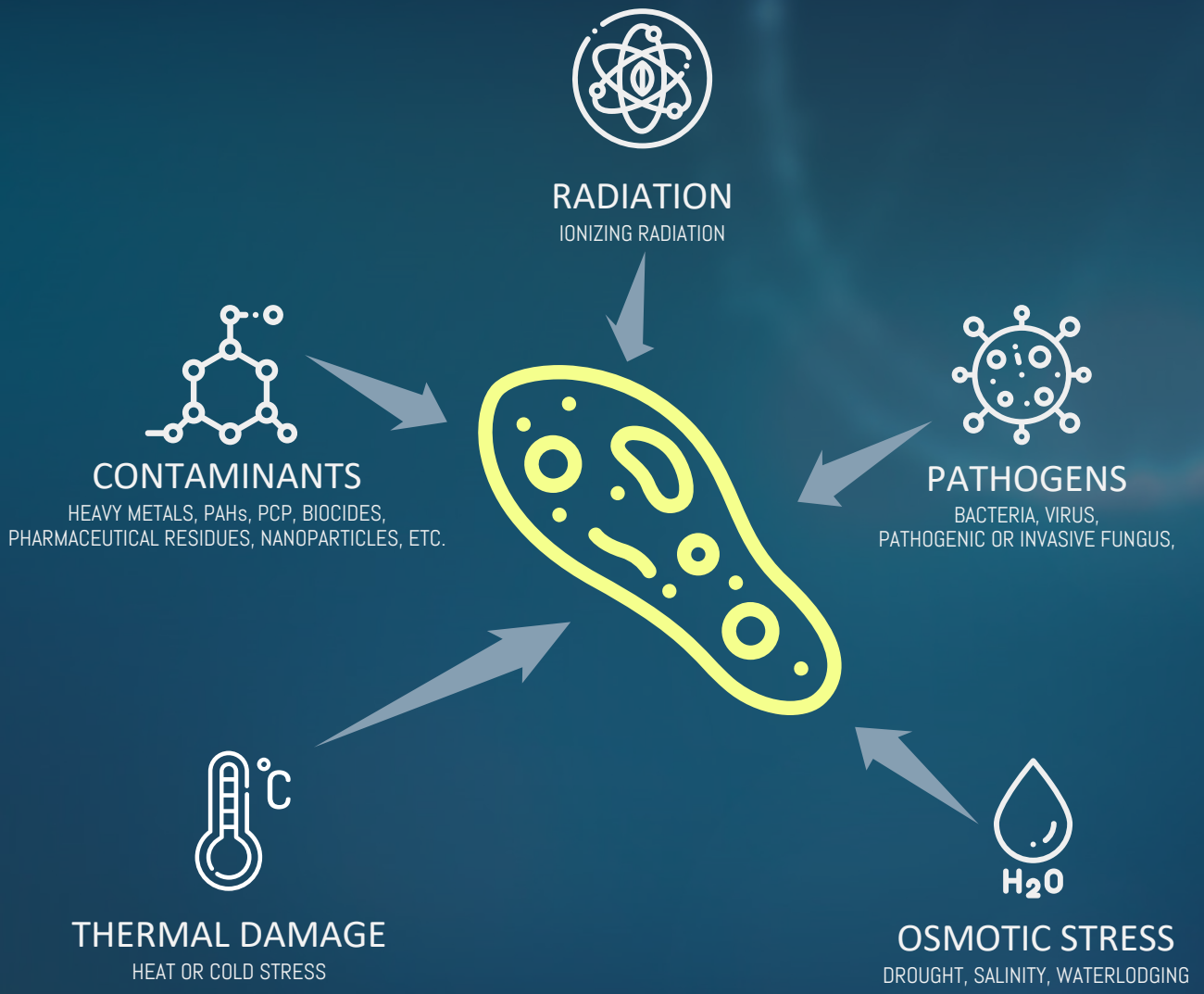



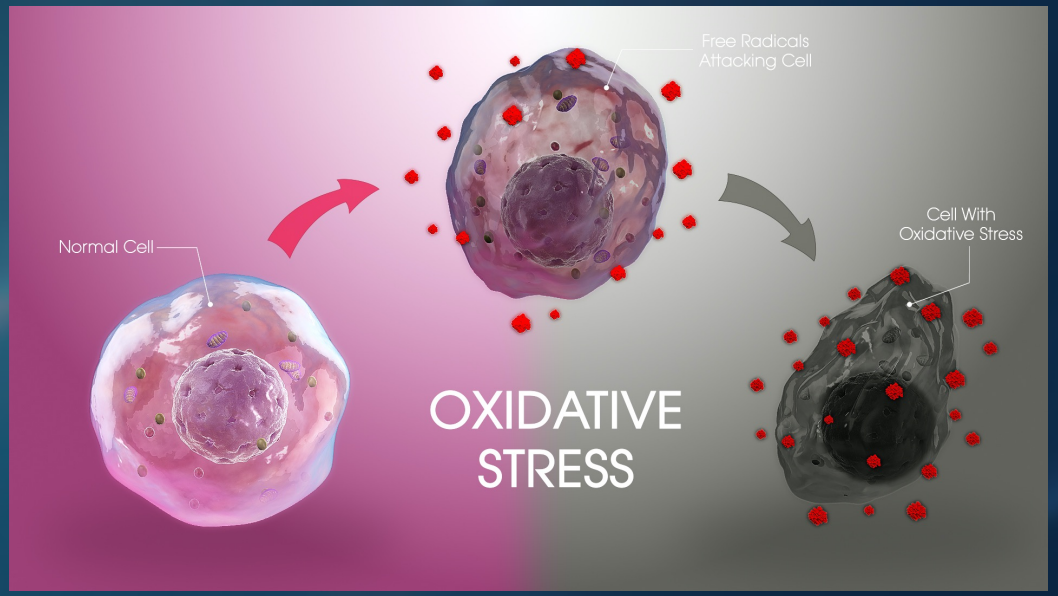
# OXIDATIVE STRESS

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.

# SOURCES OF OXIDATIVE STRESS



# PHASE I AND PHASE II ENZYMES

## PHASE I: BIOTRANSFORMATION

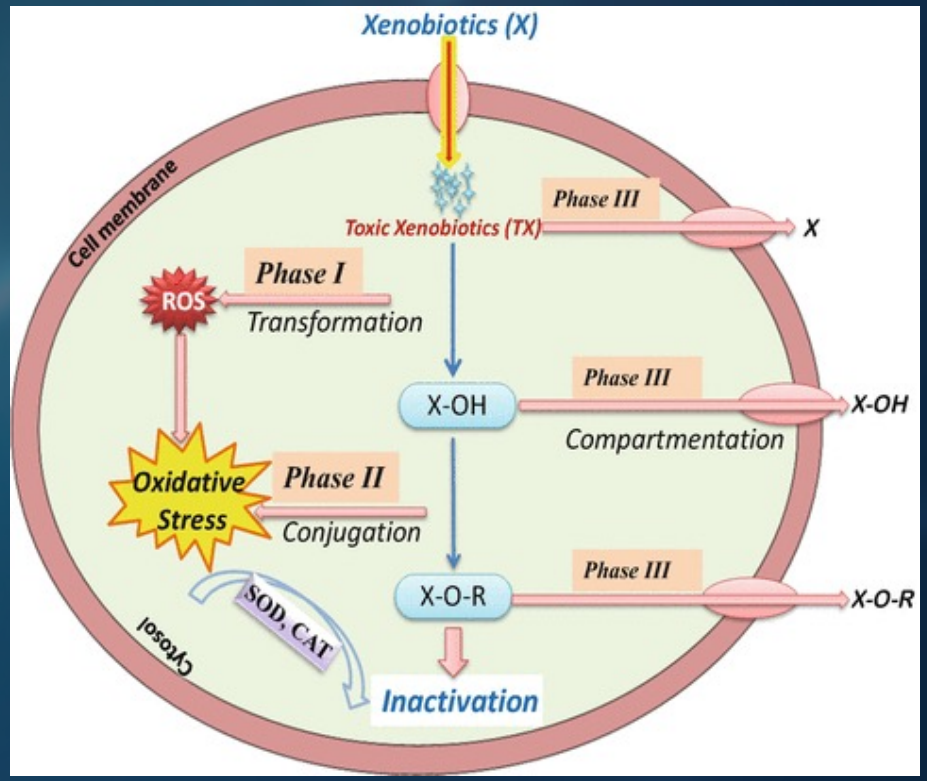
Phase I enzymes and mechanisms **transform xenobiotics into less harmful molecules**, but that may have a ROS-generating potential (for e.g.: CYPA1 enzymes).

## 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.

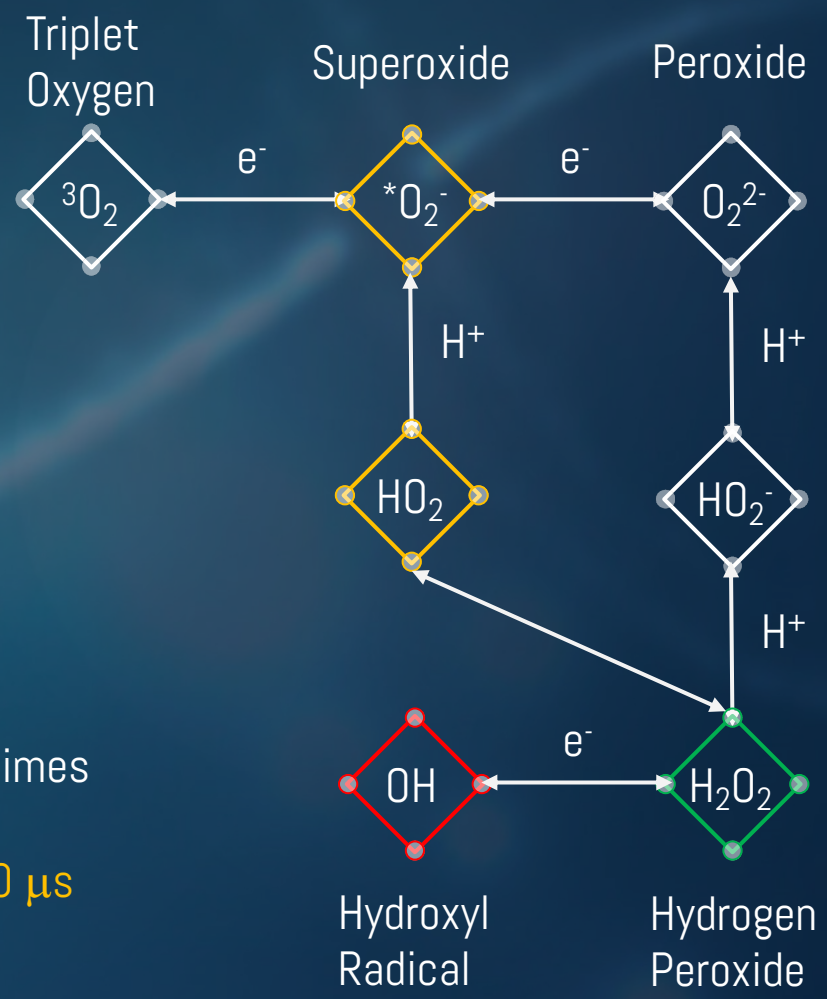
## PHASE III: EXCRETION

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.



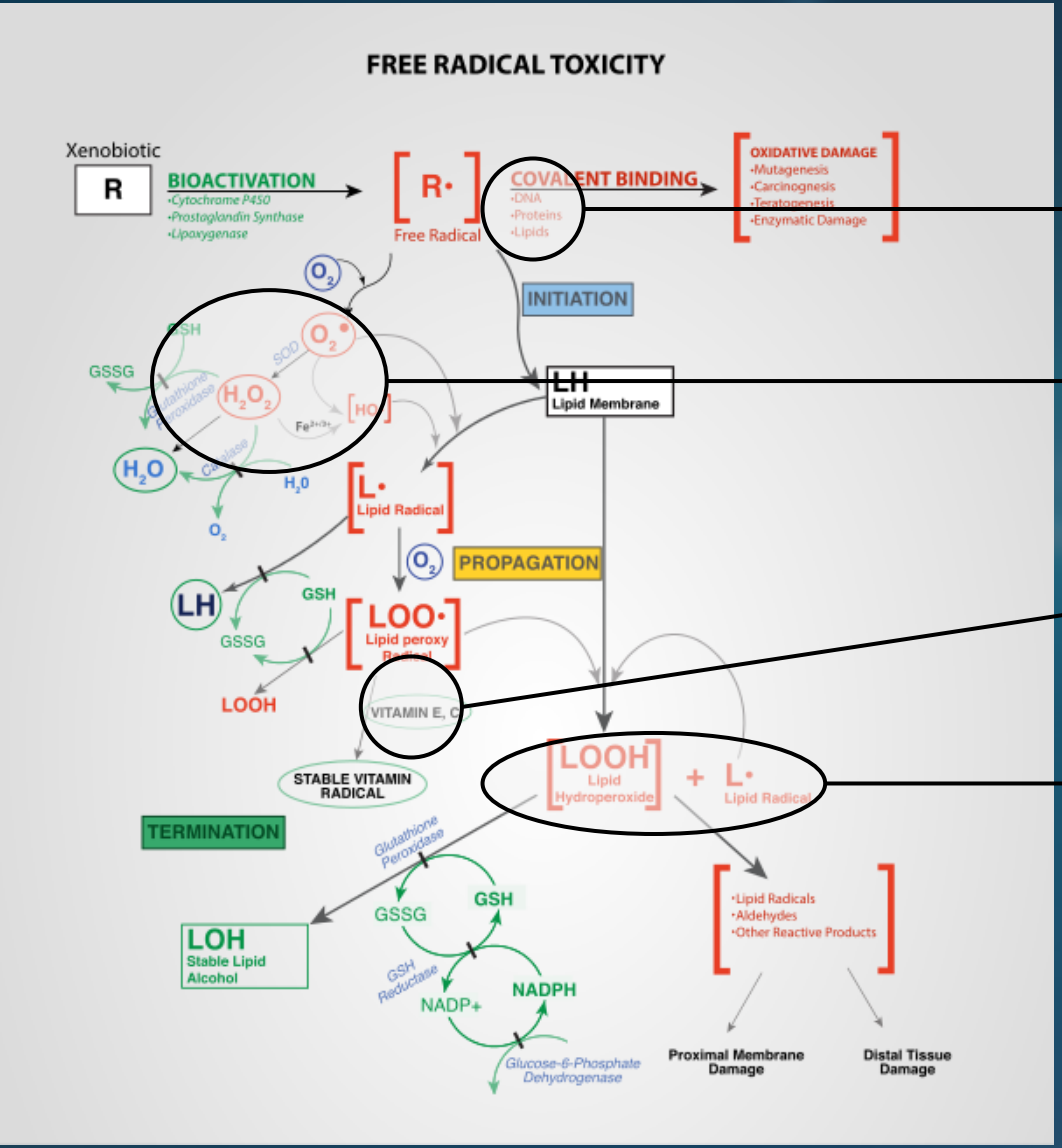
# ROS TYPES

| | |
|---|---|
| Oxygen | $O_2$ |
| Superoxide anion | $O_2^-$ |
| Peroxide anion | $O_2^{2-}$ |
| Hydrogen peroxide | $H_2O_2$ |
| Hydroxyl radical | OH |
| Hydroxyl anion | $OH^-$ |
| Singlet Oxygen | $^{-1}O_2$ |
| Hypochloric acid | HOCl |




ROS lifetimes
1 ns
100 – 10 µs
s – min

# ANTI-OXIDANT DEFENSE SYSTEM




## DNA AND PROTEIN DAMAGE

ROS covalent binding to DNA leads to double strand disruption.
ROS interaction with Proteins induces protein oxidation/carboxylation.

## ENZYMATIC DEFENSE SYSTEM

Superoxide dismutase isoforms and several peroxidases decompose ROS into harmless substances.

## NON-ENZYMATIC ANTIOXIDANT

Vitamins, phenolics, flavonoids, thiol molecules and other anti-oxidant are able to quench directly ROS molecules into stable harmless radicals.

## MEMBRANE DAMAGE

ROS interaction membrane fatty acids induces the formation of lipid hydroperoxides and lipid radicals inducing membrane disruption.

# 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.

# BIOMARKERS : ENZYME EXTRACTION


$N_2$ or Quartz sand grinding, Tissue Lyzer

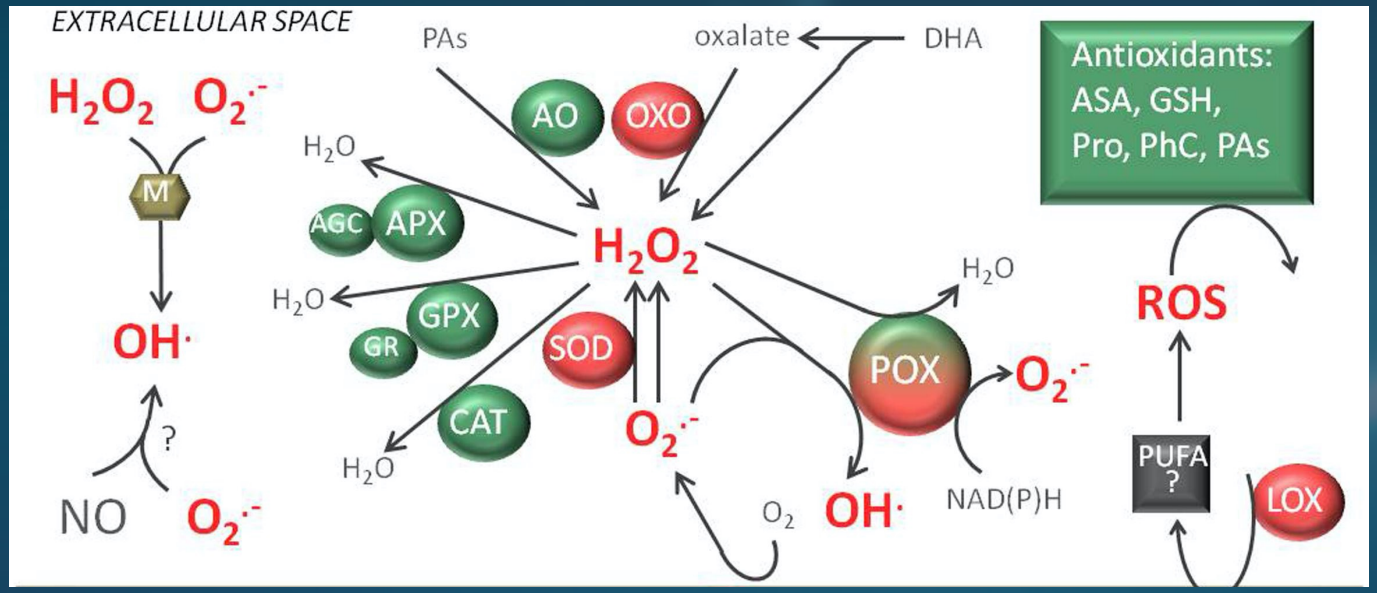
Ultra-sound in physiological buffer with protection cocktail

(PBS typically supplemented with DTT, PVP and PMSF)

Centrifugation and collection of the protein-containing supernatant

(Discard the pellet composed by cellular debris)

# BIOMARKERS : PHASE II ENZYMES




- All these enzymes are part of the normal functioning of the cells.
- Cells produce ROS as part of their normal cellular metabolism.
- In Ecotoxicology, the activity of these enzymes towards the basal cell functioning is evaluated under stress conditions as an measure of the cell stress.

## ASCORBATE PEROXIDASE (Apx, *ONLY PLANTS*)

Uses ascorbate molecules to quench the oxidative power from $H_2O_2$, converting hydrogen peroxide into $H_2O$.

## GLUTATHIONE PEROXIDASE (Gpx) AND REDUCTASE (GR)

Glutathione peroxidase used reduced glutathione molecules to converted $H_2O_2$ into $H_2O$. The oxidized form of glutathione (GSSH) is then reduced back by glutathione reductase at the expense of NADH.

## CATALASE (CAT)

Converts hydrogen peroxide into water.

## PEROXIDASE (POX)

A class of several peroxidasic enzymes that convert $H_2O_2$ into $H_2O$ or $OH^-$ at the expense of NAD(P)H.

## SUPEROXIDE DISMUTASE (SOD)

This enzymes converts the superoxide anion into hydrogen peroxide feeding the peroxidasic system..

# BIOMARKERS : SUPEROXIDE DISMUTASE ASSAY (PHASE II)

PRINCIPLES OF THE METHOD:

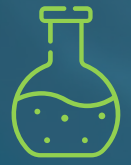
- Pyrogaloll (Pyr) has a absorption peak at 325 nm.
- Pyrogaloll has an auto-oxidation (degradation) rate.
- The method measures the inhibition of oxidized Pyrogaloll for 120 seconds at 325 nm by Superoxide Dismutase.

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

BLANK CELL

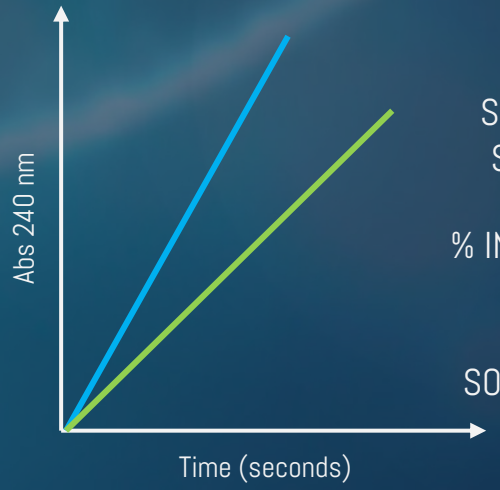
1. 100 µg protein (calculate the extract volume needed).
2. Remaining volume with PBS buffer.

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 (0.5 mL)
2. Sonicate
3. Centrifuge to precipitate cellular debris
4. 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 |