# BIOMARKERS

Oxidative stress, ROS, biochemical reactions, enzymes, anti-oxidant, damage biomarkers

### BIOINDICATOR

Organisms that express specific symptoms or responses that indicate environmental changes. Produce **QUALITATIVE** information regarding these changes (better, worse than a previous or reference condition).

### BIOMONITOR

Organisms or populations which the distribution is studied over time and space and compared to a model where the deviations to the expected behaviour are evaluated. Produce **QUANTITATIVE** information regarding the environmental changes.

### 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.

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.

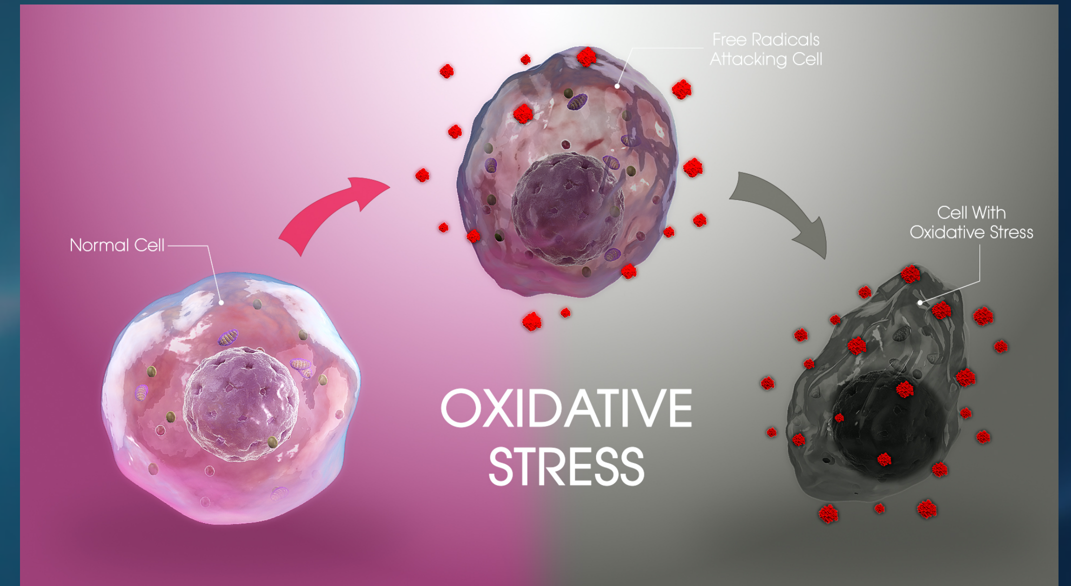
## RADIATION
IONIZING RADIATION

## CONTAMINANTS
HEAVY METALS, PAHs, PCP, BIOCIDES, PHARMACEUTICAL RESIDUES, NANOPARTICLES, ETC.

## PATHOGENS
BACTERIA, VIRUS, PATHOGENIC OR INVASIVE FUNGUS,

## THERMAL DAMAGE
HEAT OR COLD STRESS

## OSMOTIC STRESS
DROUGHT, SALINITY, WATERLODGING



## PHASE I: BIOTRANSFORMATION

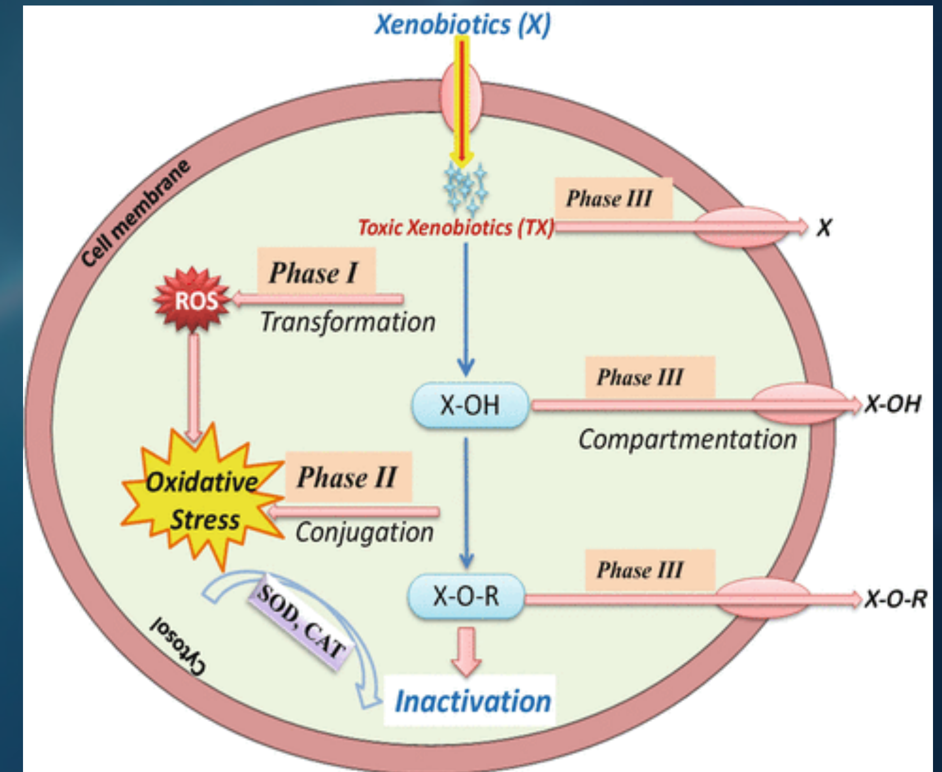
Phase I enzymes and mechanisms transform xenobiotics into less harmful molecules, but that may have a ROS-generating potential (for e.g.: CYPA1 enzymes).

## PHASE II: CONJUGATION

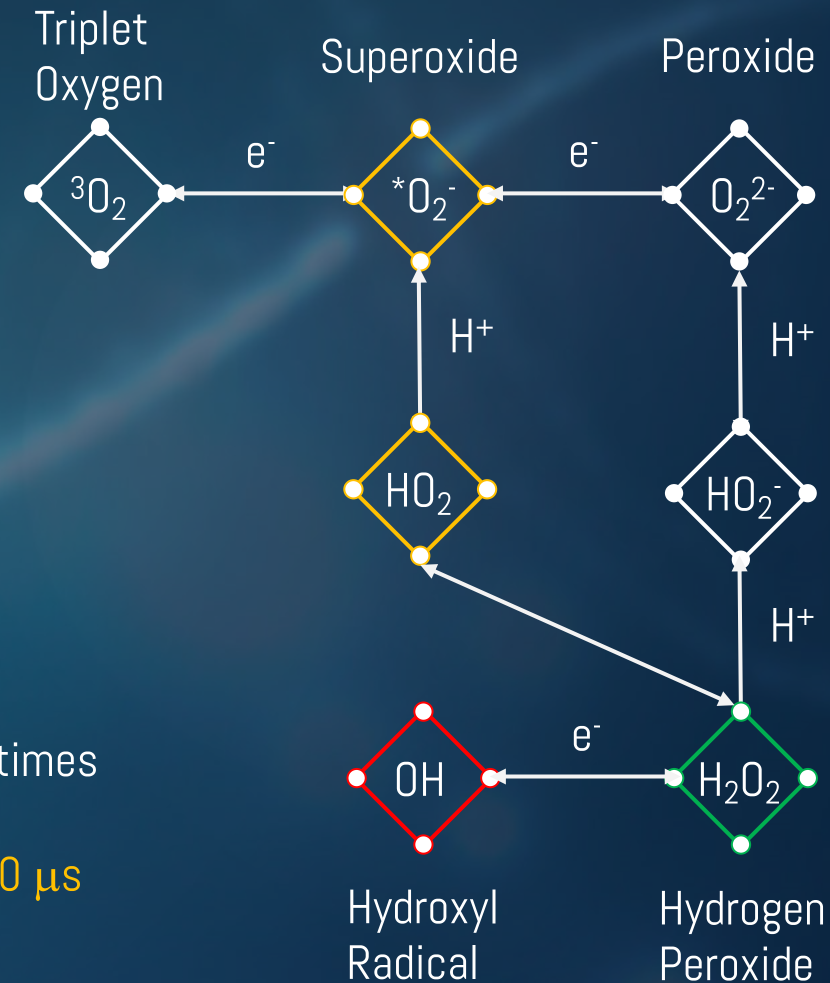
Phase II mechanisms are composed by enzymatic and non-enzymatic anti-oxidant mechanisms that work in conjugation to quench the ROS generated directly by the xenobiotic or by the Phase I biotransformed xenobiotic.

## PHASE III: EXCRETION

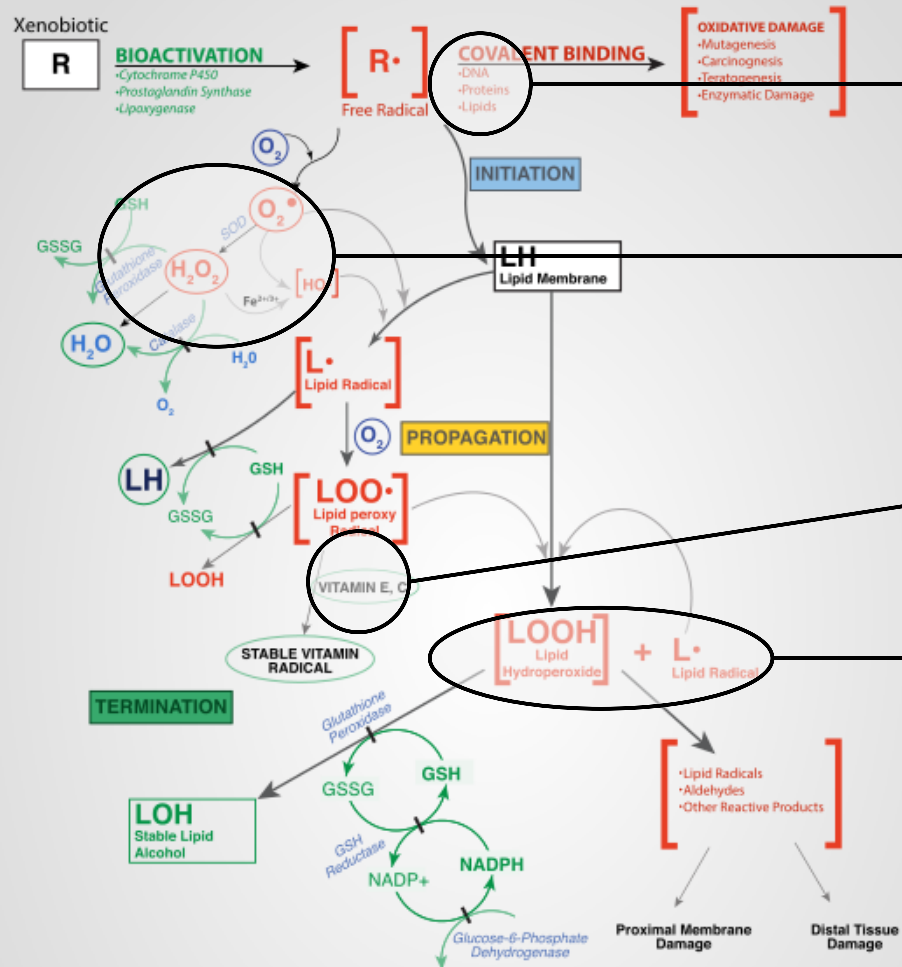
Phase III mechanisms are based in membrane proteins that can excrete directly or throughout vesicle compartments the transformed and/or inactivated xenobiotic to the extracellular environment.



| | |
|---|---|
| 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




## 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.

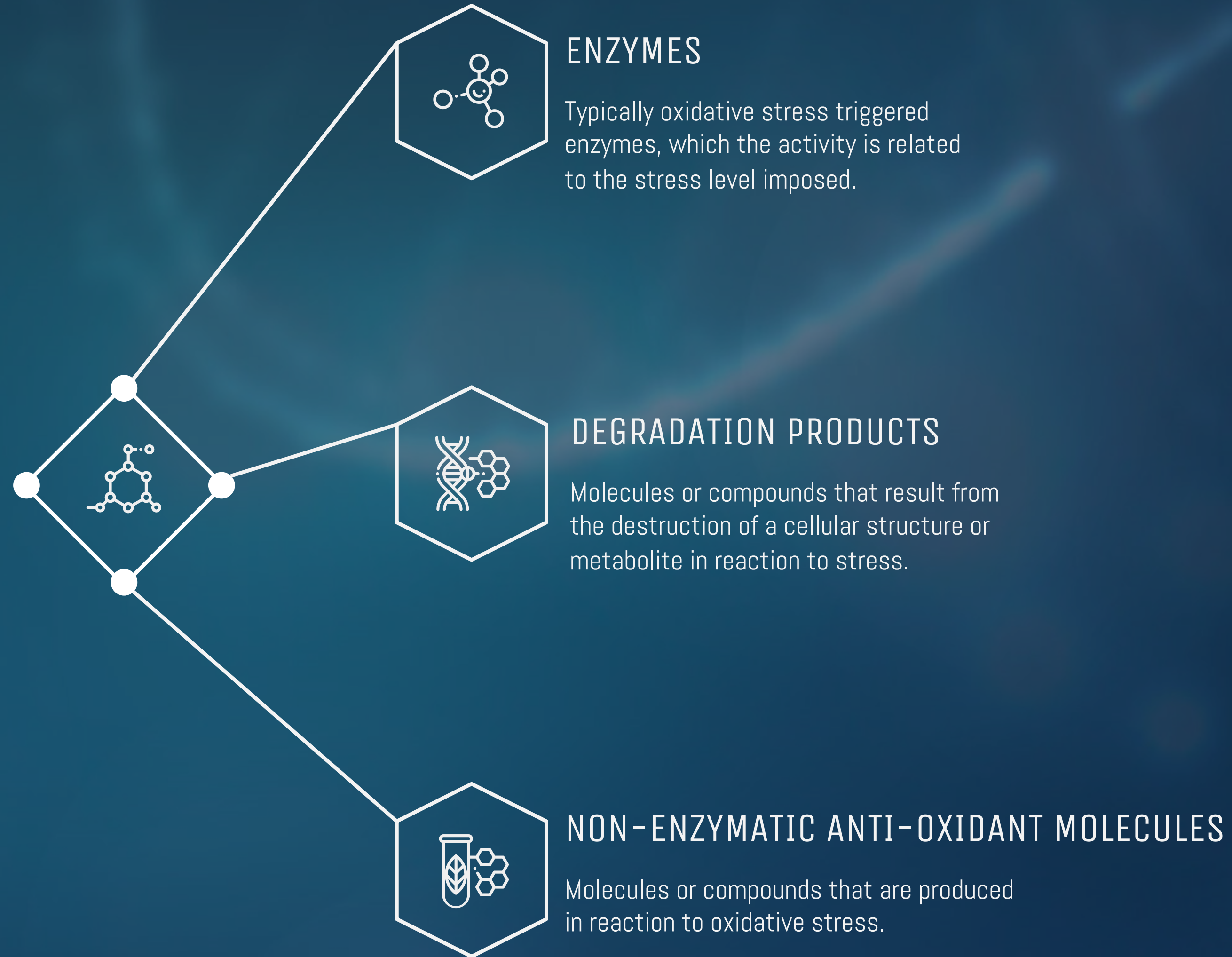
## MEMBRANE DAMAGE

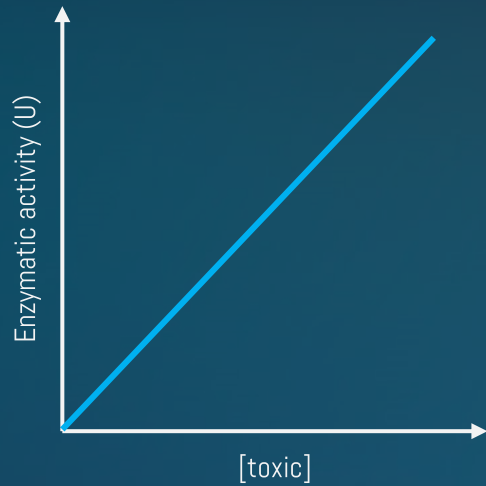
ROS interaction membrane fatty acids induces the formation of lipid hydroperoxides and lipid radicals inducing membrane disruption.

# 03 BIOMARKERS : TYPES

## BIOMARKER

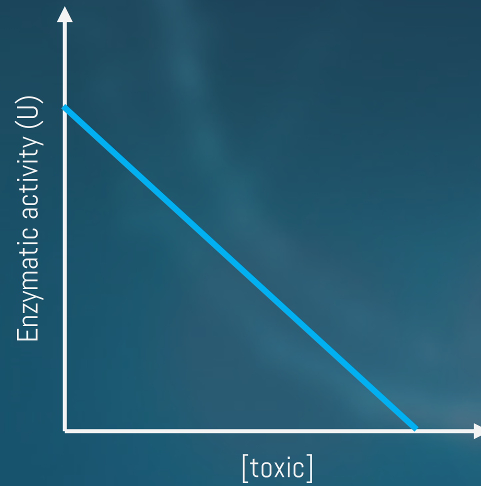
A trait or molecular entity that can be measured experimentally and indicate the occurrence of a certain function (normal or pathological) of a certain organisms towards a specific stressor. Ideally these biomarkers should produce a dose related response towards the stressor applied.





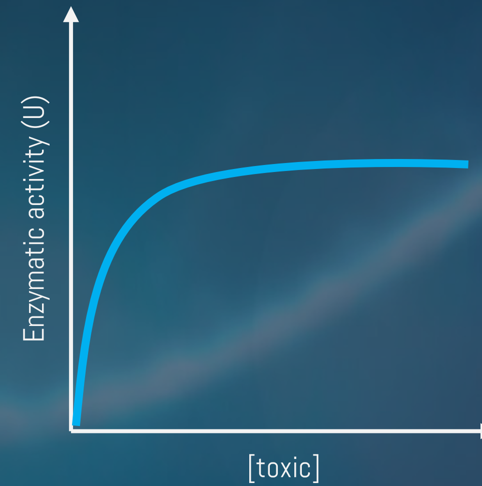

## DOSE-ACTIVITY PORPOTION

This would be the perfect biomarker. Its activity is proportional to the concentration of the toxic substance.



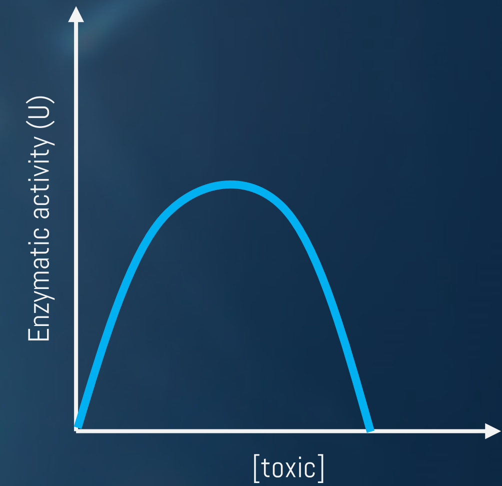

## INVERSE PORPOTION

The activity of some enzymes can be impaired by certain toxic substances.



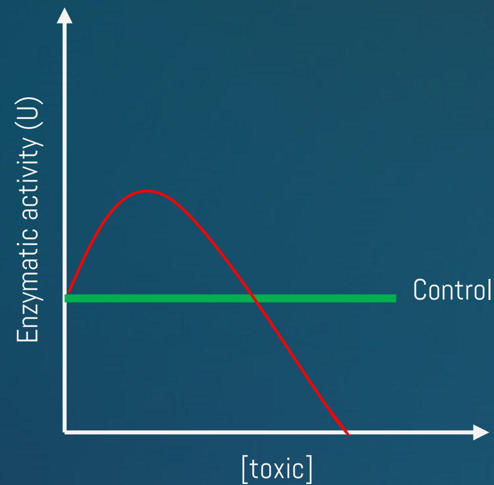

## TYPICAL ENZYME KINETICS

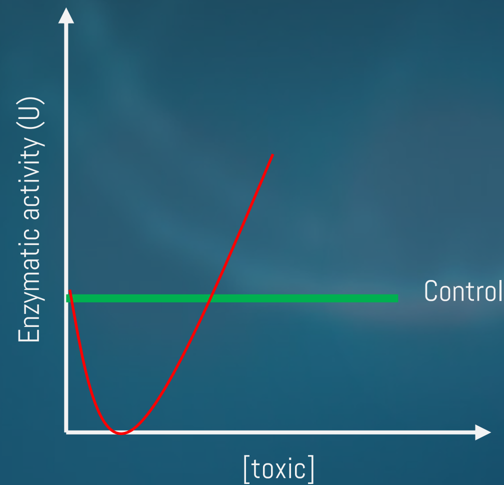
The enzyme increases its activity in response to stress until a maximum velocity after which increasing toxic concentrations do not produce any effect.




## PARABOLIC FUNCTION

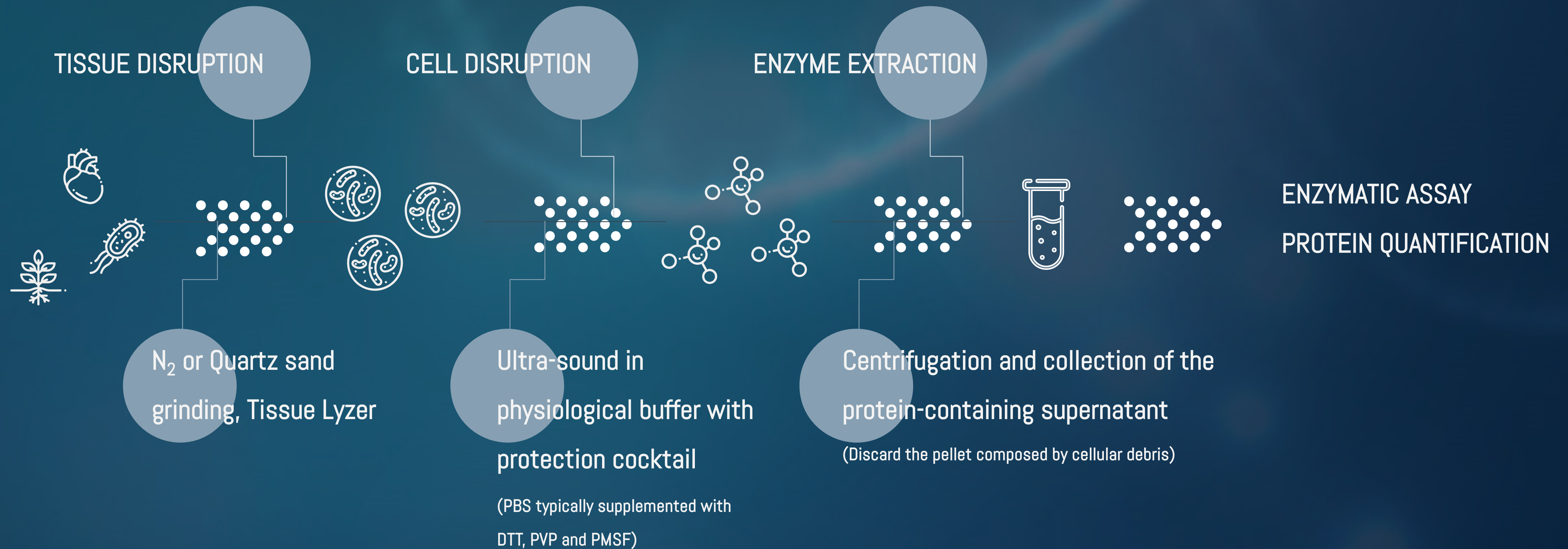
The enzyme exhibits an increase in its activity until a certain concentration after which it is inhibited. The activity should be only used as biomarker in the positive slope range of concentrations.



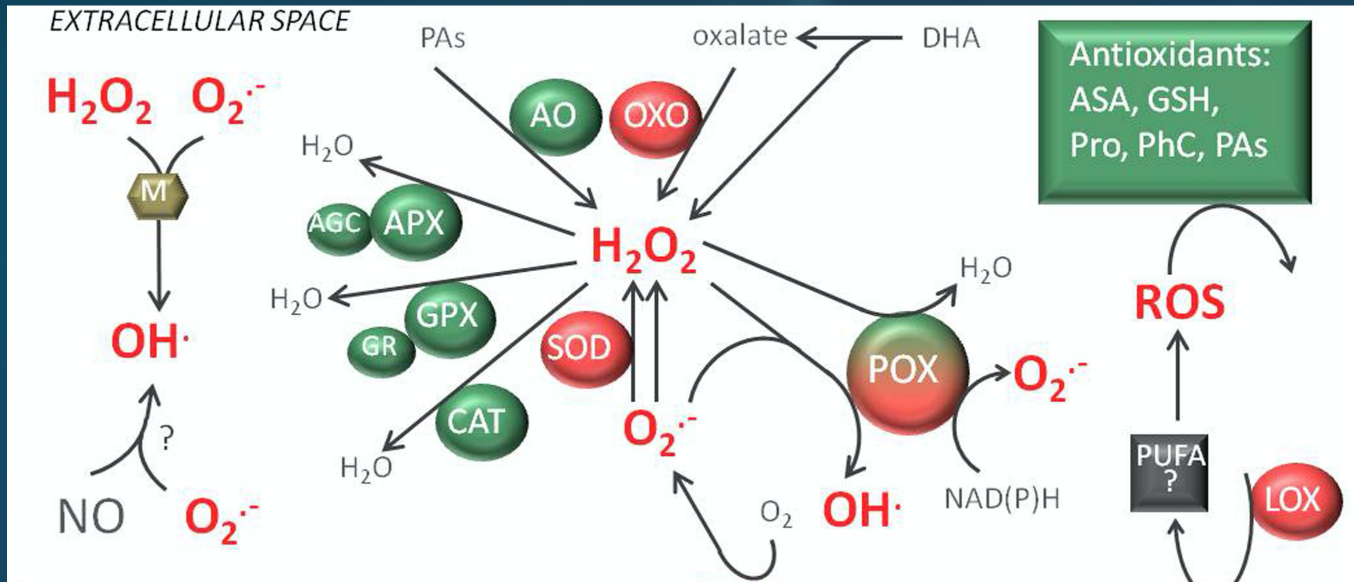




## HORMESIS EFFECT

- Hormesis is any process in a cell or organism that exhibits a biphasic response to exposure to increasing amounts of a substance or condition.
- Within the hormetic zone, there is generally a favorable biological response to low exposures to toxins and other stressors.
- In toxicology, hormesis is a dose response phenomenon characterized by a low dose stimulation, high dose inhibition, resulting in either a J-shaped or an inverted U-shaped dose response.
- Such environmental factors that would seem to produce positive responses have also been termed "eustress".



TISSUE DISRUPTION
CELL DISRUPTION
ENZYME EXTRACTION
ENZYMATIC ASSAY
PROTEIN QUANTIFICATION
$N_2$ 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)




- 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)

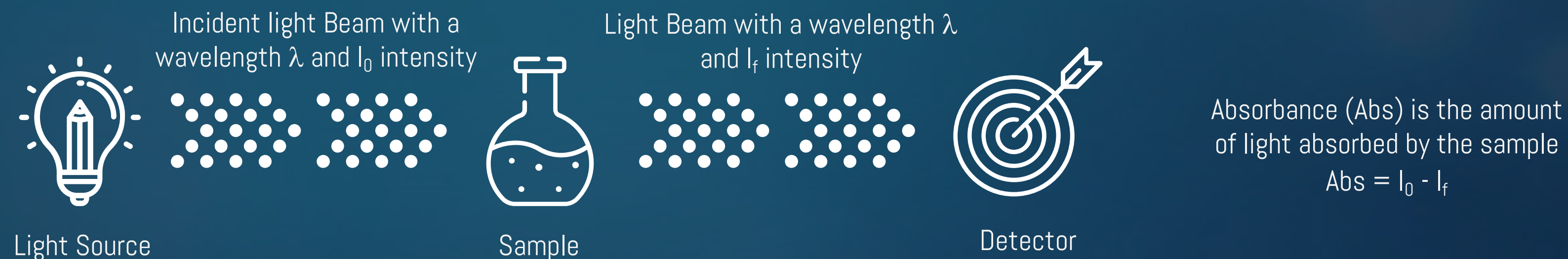
A class of several peroxidasic enzymes that convert $H_2O_2$ into $H_2O$ or $OH^-$ at the expense of NAD(P)H.

## SUPEROXIDE DISMUTASE (SOD)

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

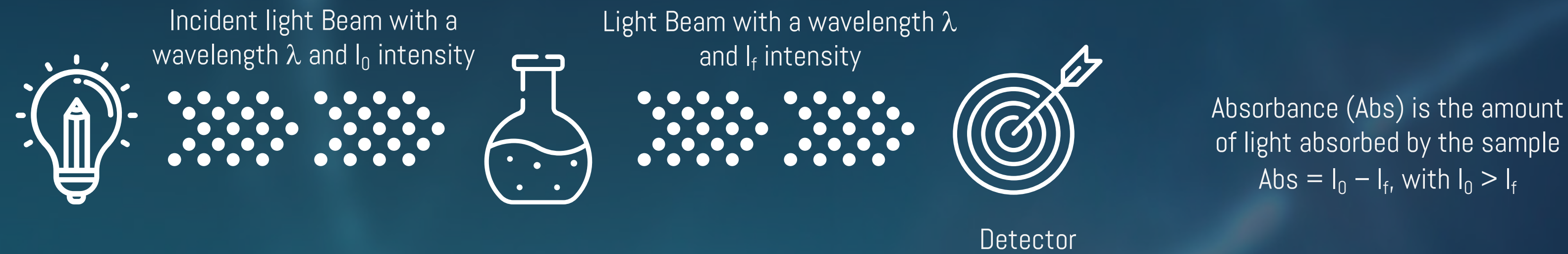
### SPECTROPHOTOMETRY APPLICATIONS IN ECOTOXICOLOGY:

- Spectrophotometry is a tool that hinges on the quantitative analysis of molecules depending on how much light is absorbed by colored compounds. Spectrophotometry uses photometers, known as spectrophotometers, that can measure a light beam's intensity as a function of its color (wavelength). Important features of spectrophotometers are spectral bandwidth (the range of colors it can transmit through the test sample), the percentage of sample-transmission, the logarithmic range of sample-absorption, and sometimes a percentage of reflectance measurement.
- Most biomarkers can be evaluated using spectrophotometric assays.




Absorbance (Abs) is the amount of light absorbed by the sample

$Abs = I_0 - I_f$




Absorbance (Abs) is the amount of light absorbed by the sample
$Abs = I_0 - I_f$, with $I_0 > I_f$

LAMBERT-BEER LAW:

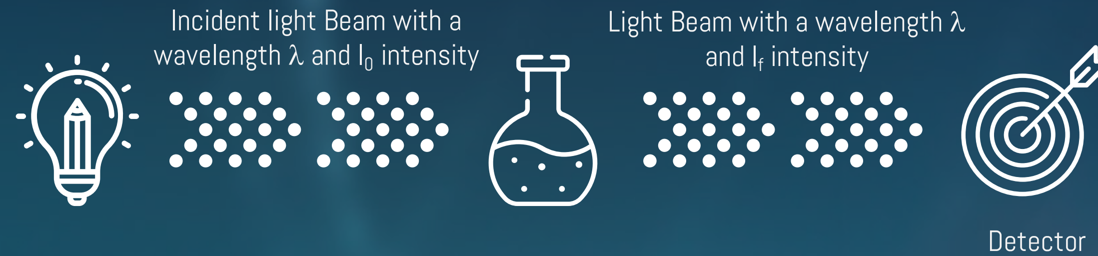
- All spectrophotometric analysis follow the Lambert-Beer Law.
- This law relates the attenuation of light to the properties of the material through which the light is travelling and describes the relationship between the absorbance of a certain compound with its concentration.

$Abs_\lambda = [\text{Compound A}] \times L \times \varepsilon_\lambda$

[Compound A] – molar concentration of the compound in analysis

L – light path through the sample (typically in most cuvettes is 1 cm so this term can be nullified from the equation)

$\varepsilon_\lambda$ – Molar extinction coefficient of the compound at a wavelength of λ nm

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$Abs_\lambda$ = [Compound A] x L x $\varepsilon_\lambda$

[Compound A] – molar concentration of the compound in analysis.

L – light path through the sample (typically in most cuvettes is 1 cm so this term can be nullified from the equation).

$\varepsilon_\lambda$ – Molar extinction coefficient of the compound at a wavelength of λ nm.

$Abs_\lambda$ = [Compound A] x L x $\varepsilon_\lambda$, if L = 1 cm ⇔ $Abs_\lambda$ = [Compound A] x $\varepsilon_\lambda$,

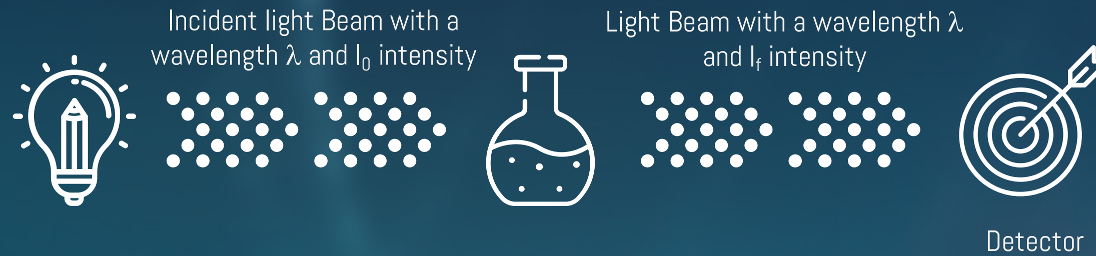
This equation resembles a linear correlation equation from the type of $y = mx + b$

$y = Abs_\lambda$ (dimensionless)

$m = \varepsilon_\lambda$ (units: $M^{-1}\ cm^{-1}$)

x = [Compound A] (units: M)

Considering that at [Compound A] = 0 then $Abs_\lambda$ = 0 then b = 0

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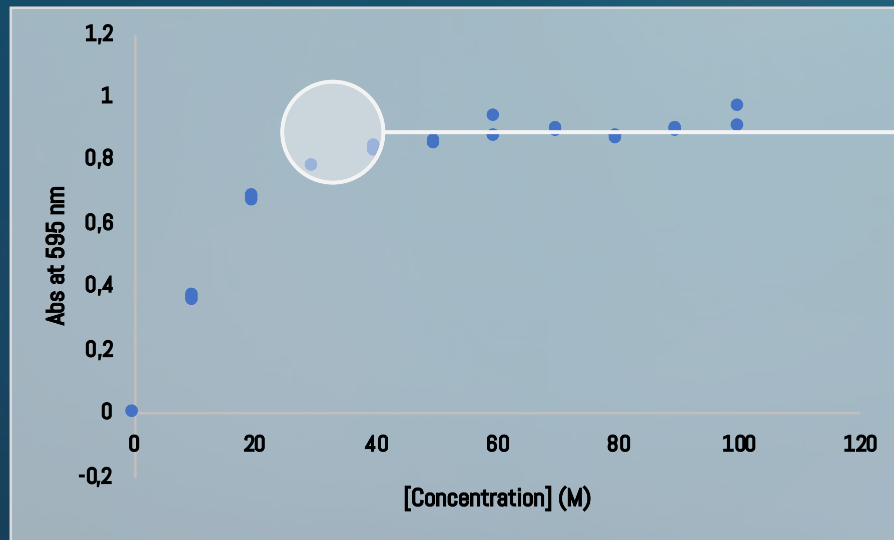
$x = [\text{Compound A}]$ (units: M)

Considering that at [Compound A] = 0 then $Abs_\lambda = 0$ then $b = 0$

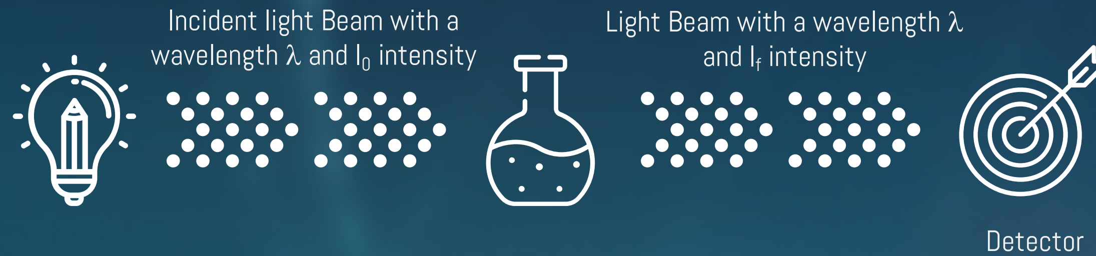
This can be attained with a calibration curve were several known concentration standards are analysed for its absorbance and a calibration curve is established.




## UPPER DETECTION LIMIT (ABS=0.781)

At this point the Lambert-Beer law does not apply any more, as the concentration continues to increase but the absorbance doesn't follow this trend

Samples with absorbance above this value should be diluted in order to have an absorbance within the linear range of the calibration curve

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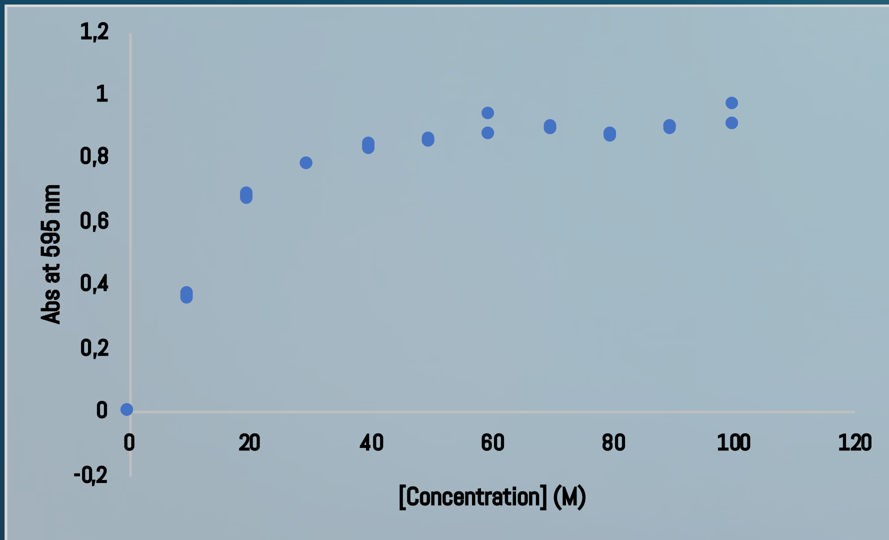
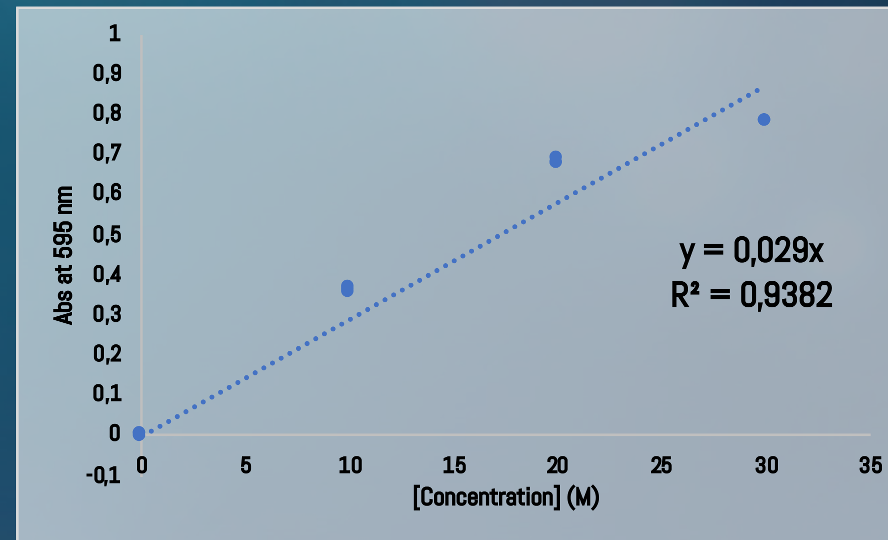





$Abs\ (y) = \varepsilon_\lambda \times [Concentration]$

$\varepsilon_\lambda = 0.029\ M^{-1}\ cm^{-1}$

Using this equation any sample can be analysed for its absorbance and its concentration derived from the equation.

PRINCIPLES OF THE METHOD:

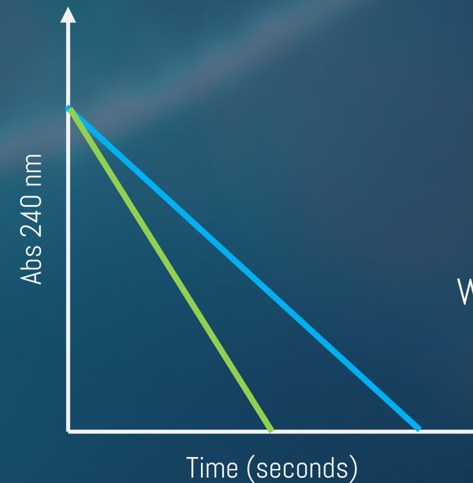
- $H_2O_2$ has a absorption peak at 240 nm ($\varepsilon$ = 394 $mM^{-1}\ cm^{-1}$).
- $H_2O_2$ has an auto-oxidation (degradation) rate.
- The method measures the consumption of $H_2O_2$ for 120 seconds at 240 nm.
- Catalase activity results from the difference between the autoxidation rate and the reaction promoted by Catalase.

$H_2O_2$ AUTO-OXIDATION ASSAY

1. 100 mM $H_2O_2$
2. Remaining volume with PBS buffer.

Catalase ASSAY

1. 100 µg protein (calculate the extract volume needed)
2. 100 mM $H_2O_2$
3. Remaining volume with PBS buffer.




CALCULATIONS

$$\Delta Abs_{240nm} = Abs_{t=120} - Abs_0$$

Using the formula: $Abs_\lambda = [\text{Compound A}] \times \varepsilon_\lambda$,

We have that the consumption of $H_2O_2$ during 120 sec is:

$$\Delta Abs_{240nm} = [H_2O_2] \times \varepsilon_{240\ nm}(H_2O_2)$$

Thus, Catalase activity is given from

$$CAT\ (U) = [H_2O_2] / \text{Reaction time}$$

# 03 BIOMARKERS : ASCORBATE PEROXIDASE ASSAY (PHASE II)

PRINCIPLES OF THE METHOD:

- Ascorbic acid (AscA) has a absorption peak at 290 nm ($\varepsilon$ = 2.8 $mM^{-1}$ $cm^{-1}$).
- Ascorbic acid doesn't has an auto-oxidation (degradation) rate.
- The method measures the oxidation of the ascorbic acid for 120 seconds at 290 nm.

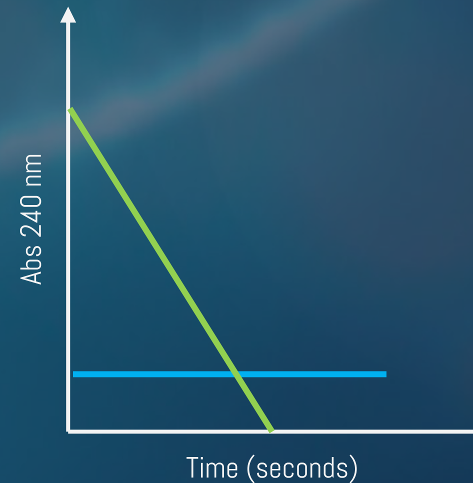
BLANK CELL

1. 0.25 μM AscA
2. 100 μg protein (calculate the extract volume needed).
3. Remaining volume with PBS buffer.

To zero the spectrophotomer.

APX ASSAY

1. 0.25 μM AscA
2. 100 μg protein (calculate the extract volume needed).
3. Remaining volume with PBS buffer.
4. 5 μM $H_2O_2$




CALCULATIONS

$$\Delta Abs_{290nm} = Abs_{t=120} - Abs_0$$

Using the formula: $Abs_\lambda$ = [Compound A] x $\varepsilon_\lambda$,

We have that the oxidation of the AscA during 120 sec is:

$$\Delta Abs_{240nm} = [AscA] \times \varepsilon_{290\ nm}\ (AscA)$$

Thus, Ascorbate peroxidase activity is given from

APX (U) = [AscA] / Reaction time

PRINCIPLES OF THE METHOD:

- Tetraguaiacol (TGu) has a absorption peak at 420 nm ($\varepsilon$ = 26,6 $mM^{-1}$ $cm^{-1}$).
- Guaiacol doesn't has an auto-oxidation (degradation) rate.
- The method measures the formation of TGu for 120 seconds at 470 nm.

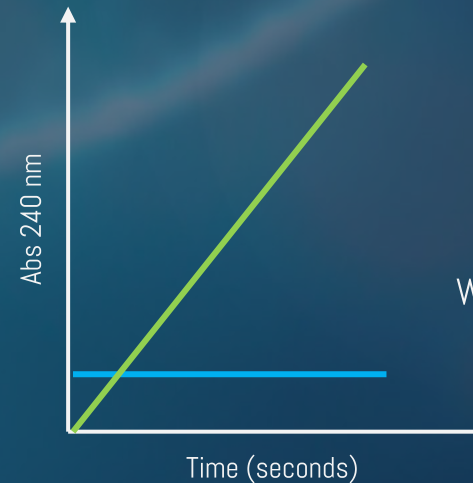
BLANK CELL

1. 2 mM $H_2O_2$
2. 100 µg protein (calculate the extract volume needed).
3. Remaining volume with PBS buffer.

To zero the spectrophotomer.

POD ASSAY

1. 2 mM $H_2O_2$
2. 100 µg protein (calculate the extract volume needed).
3. Remaining volume with PBS buffer.
4. 4 mM Guaiacol




CALCULATIONS

$$\Delta Abs_{420nm} = Abs_{t=120} - Abs_0$$

Using the formula: $Abs_\lambda$ = [Compound A] x $\varepsilon_\lambda$,

We have that the formation of TGu during 120 sec is given by:

$$\Delta Abs_{240nm} = [TGu] \times \varepsilon_{470\ nm}(TGu)$$

Thus, Peroxidase activity is given from

POD (U) = [TGu] / Reaction time

# 03 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.

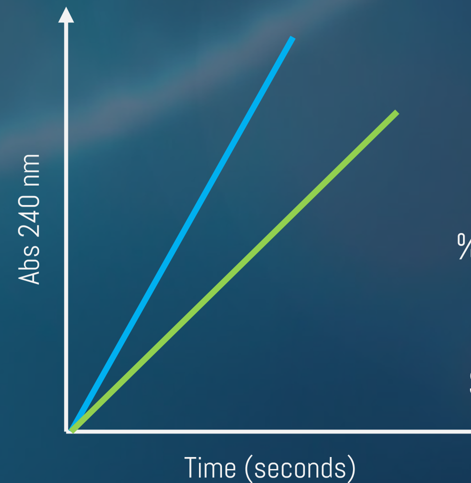
BLANK CELL

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

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

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

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

PRINCIPLES OF THE METHOD:

- EROD catalyses the conversion of of Ethoxyresorufin into Resorufin.
- EROD activity is a measure of the cytochrome P450 1A (CYP1A), essential for the detoxification in vertebrates (and some invertebrates)
- Fluorometric assay: substrate emits fluorescence (UF) when excited at a specific wavelength.
- Resorufin when excited at 537 nm ($Ex_{WL}$) emits fluorescence ($Em_{WL}$) at 583 nm.

BLANK CELL

1. Reaction mix (100mM PBS, 0,25mM NADPH, 4,15mM 7-ethoxyresorufin)
2. Remaining volume with PBS buffer.
3. Fluorescence read for 30 minutes.

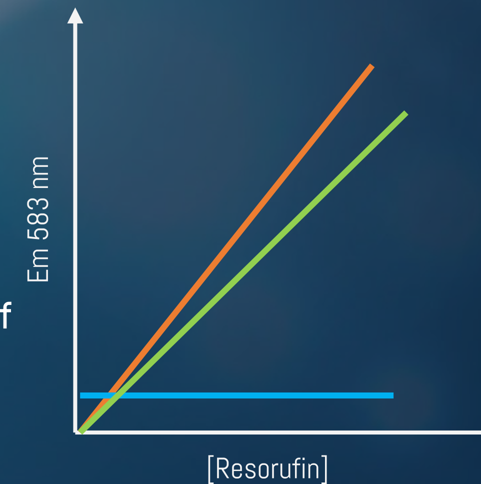
To determine baseline fluorescence.

EROD ASSAY

1. Reaction mix (100mM PBS, 0,25mM NADPH, 4,15mM 7-ethoxyresorufin)
2. 100 µg protein (calculate the extract volume needed).
3. Fluorescence read for 30 minutes.

CALIBRATION CURVE

1. Prepare Resorufin standard calibration curve (dilutions of stock solution 0-300nM)




CALCULATIONS

$\Delta UF = (UF_{final} - UF_0) - (UF_{initial} - UF_0)$

Determine standard calibration curve for [Resorufin]

$\Delta UF = m*[Res.sample]+b$

EROD (U) = [Res.sample]/ Reaction Time

PRINCIPLES OF THE METHOD:

- Peroxidation of PUFA fatty acids results in the formation of lipid aldehydes (such as Malondialdehyde, MDA).
- These can be extracted in acid medium (commonly Trichloroacetic Acid).
- The reaction of MDA in acid medium with Thiobarbituric Acid (TBA) results in a coloured compound.
- MDA-TBA commonly known as Thiobarbituric Reactive Substances (TBARS) has a absorption peak at 531 nm ($\varepsilon$ = 155 $mM^{-1}$ $cm^{-1}$).

BLANK CELL

1. Extraction solution TCA (20%, w/v) + TBA (0.5%, w/v)

To zero the spectrophotomer.

TBARS ASSAY

1. Pre-weighted fresh sample of buffered protein extract.
2. Extraction solution TCA (20%, w/v) + TBA (0.5%, w/v)

30 min at 95 ºC

CALCULATIONS

$\Delta Abs_{420nm} = Abs_{t=120} - Abs_0$

$[MDA] = Abs_{531nm} / \varepsilon_{MDA}$

# 04
# STATISTICS IN ECOTOXICOLOGY

Univariate and Multivariate approaches for data analysis and interpretation in Ecotoxicology