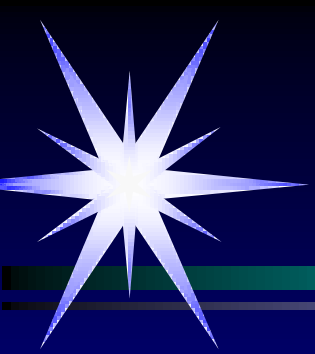




INVESTIGATIONS ON THE PHOTOLYSIS OF
IMAZETHAPYR AND PRETILACHLOR IN AQUEOUS
SOLUTIONS UNDER DIRECT SUNLIGHT BY LC-
MS/MS-ESI – IDENTIFICATION OF POTENTIAL
BIOMARKERS
IN DETECTION OF PESTICIDE TOXICITY

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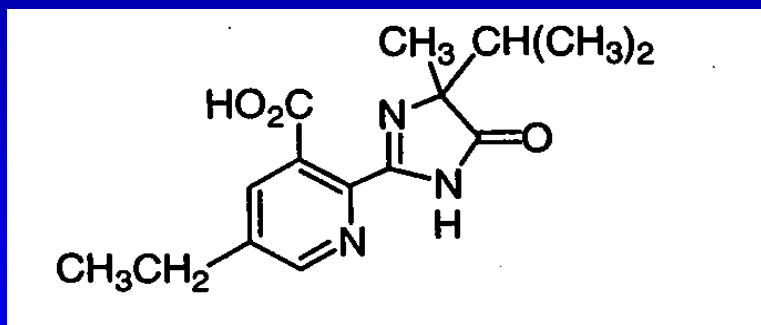
OBJECTIVES

- ❖ Investigation on the photolytic and hydrolytic behavior of Imazethapyr and Pretilachlor in water.
- ❖ Factors influencing the dissipation kinetics.
- ❖ Identification of metabolites using LC-MS/MS.
- ❖ Impact of residues on the growth of aquatic species (Green alga).

CHEMICAL DETAILS

Imazethapyr

Imidazolinone - pre and post-emergence herbicide - to control grasses and broad leaved weeds in pulses and oil seeds.



(RS)-5-ethyl-2-(4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl)nicotinic acid



HPLC UV Conditions

| | |
|---------------------------|--|
| Instrument | Shimadzu HPLC equipped with LC-10 ATvp pump & UV-VIS detector |
| Column | Phenomenex C ₁₈ (25 cm length x 4.6 mm i.d x 5 μ particle size) |
| Mobile phase | Acetonitrile: pH 3.0 phosphoric acid buffer (40:60) |
| Volume injected | 25 μL |
| Flow rate | 1.0 mL/min |
| Wavelength | 230 nm |
| Retention time in minutes | 6.2 |



PHOTOLYTIC DEGRADATION STUDY

- Fortified concentrations : 1 $\mu\text{g/ml}$ and 2 $\mu\text{g/ml}$.
- Exposure : Direct sunlight
- Substrates : Milli-Q water,
acidic, neutral and basic buffers
- Buffer Condition : Non - Sterile
- Sampling occasions : 0, 1, 3, 5, 7, 10 and 15 days.
- Temperature : 27 to 43°C



HYDROLYSIS

- Fortified concentrations : 1 $\mu\text{g/ml}$ and 2 $\mu\text{g/ml}$.
- Exposure : Dark
- Substrates : Milli-Q water,
acidic, neutral and basic buffers
- Sampling occasions : 0, 1, 3, 7, 15, 20, 30, 40, 50, 60, 80,
100 and 120 days
- Buffer Condition : Sterile
- Temperature : 25°C



INFLUENCE OF CATIONS, ANIONS & AERATION

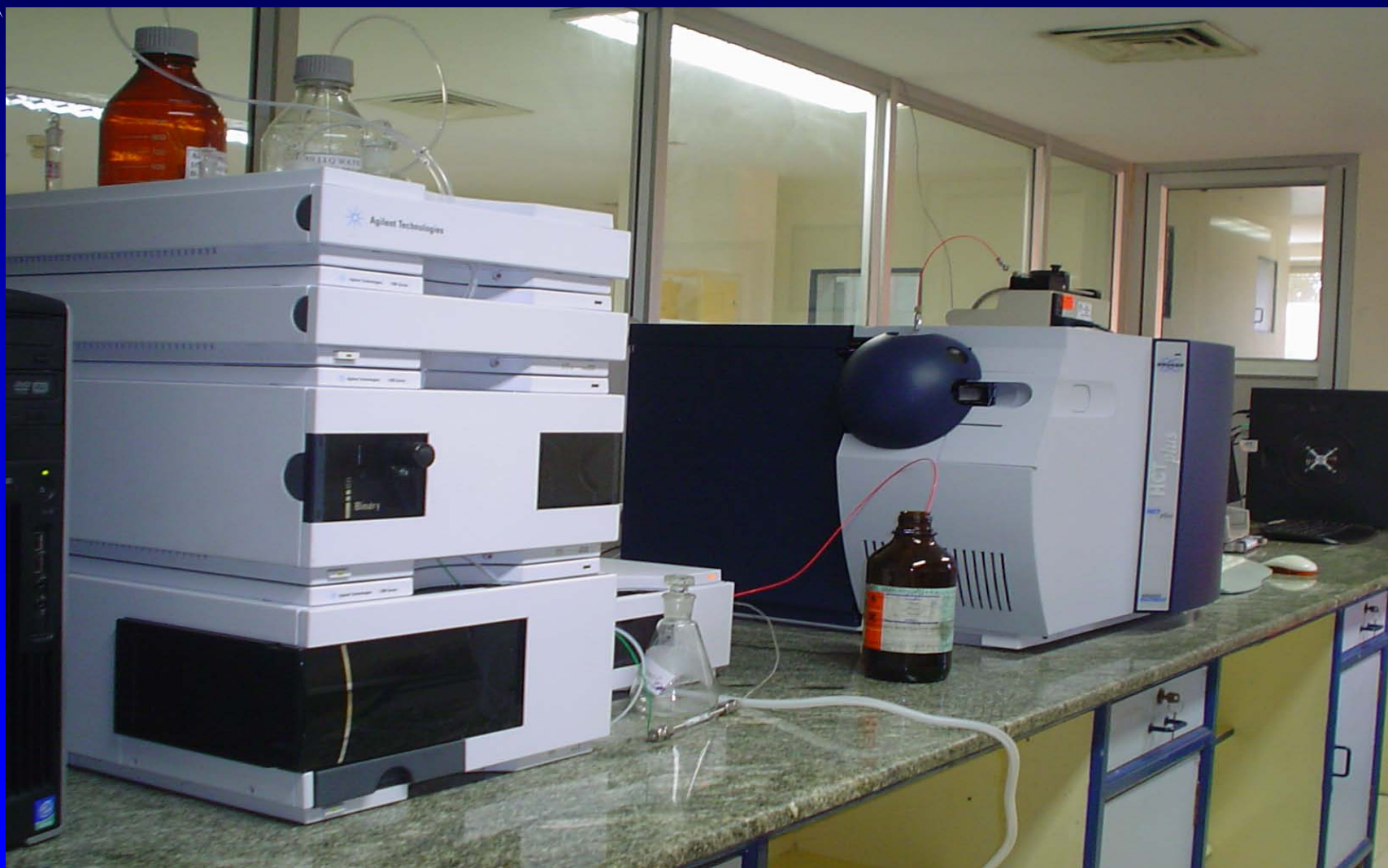
- Cations and anions used : Ferrous sulfate (Fe^{2+}), Copper sulfate (Cu^{2+}), Cobalt nitrate (Co^{2+}), Nickelsulfate(Ni^{2+}), Manganese sulfate (Mn^{2+}), Zinc sulfate (Zn^{2+}), Sodium sulfate (SO_4^{2-}), sodium chloride (Cl^-), Sodium perchlorate (ClO_4^-), Sodium carbonate (CO_3^-) Sodium bicarbonate (HCO_3^-).
- Concentration of ions : 10^{-1}M , 10^{-2}M and 10^{-3}M

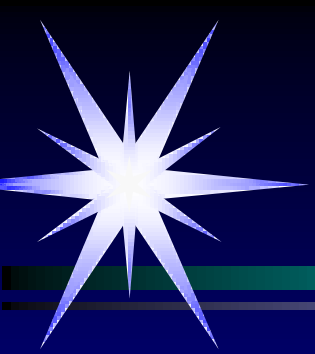


LC-MS/MS CONDITIONS

- ∅ Mobile phase A : 0.1 % formic acid in acetonitrile
- ∅ Mobile phase B : 0.1 % formic acid in milli Q water
- ∅ Column : Zorbax SB C18 column (5 μ m, 4.6 mm, 75 mm)
- ∅ Gradient Flow : 0.25 mL per minute
- ∅ Mobile phase ratio : 5% to 95 % A
- ∅ Source : Electro spray ionisation
- ∅ Mode : +ve
- ∅ Scan range : 50 - 400 m/Z

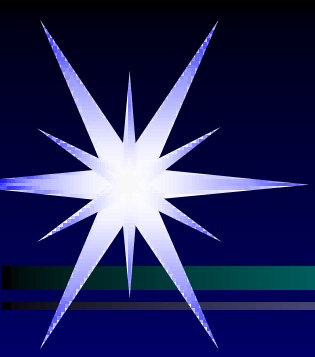
ELECTROSPRAY TANDEM MASS SPECTROMETRY





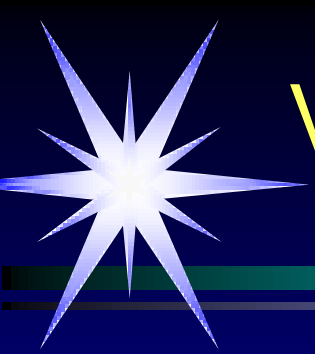
BIOLOGICAL ANALYSIS

- ❖ Effect of residues of Imazethapyr on the growth of green alga, *Pseudokirchneriella subcapitata* in OECD TG 201 medium was investigated.
- ❖ Intensity of light in the growth chamber was 6000-8000 Lux. Under continuous illumination, the control and treatment flasks were kept in the shaker incubator.
- ❖ Shaken continuously at the speed of 110-120 rotations per minute.



PRE-CULTURE

- Pre-culture of *Pseudokirchneriella subcapitata* prepared three days before initiation of the study, inoculated flasks in the shaker incubator with rpm of 112 - 116 and maintained with continuous illumination of 6837 - 6956 lux light intensity at 22.6 to 23.1°C for three days.
- After three days, the culture was examined under a microscope and checked for any abnormality or microbial contamination.



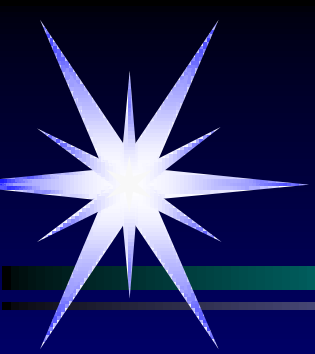
VALIDATION OF THE ALGA STUDY

- Biomass in the control flasks should be increased exponentially by a factor of at least 16 times (**OECD 201 guideline**) within the 72 hours test period.
- Mean coefficient of variation for section by section specific growth rates in the control must not exceed 35%.
- Coefficient of variation of average specific growth rate in control replicates must not exceed 7%.



STUDY DETAILS

| | | |
|------------------|---|---|
| ॐ Test Species | : | <i>Pseudokirchneriella subcapitata</i> |
| ॐ Strain No. | : | SAG 61.81 (Primary culture from University of Gottingen, Germany) |
| ॐ Test Duration | : | 72 hours |
| ॐ Culture Medium | : | OECD TG 201 medium |
| ॐ Test Type | : | Static |
| ॐ Illumination | : | 6000 - 8000 Lux light intensity |
| ॐ Temperature | : | 22 ± 2°C |
| ॐ pH | : | 8.1±0.1 |
| ॐ Test Chamber | : | shaker incubator under continuous illumination |
| ॐ Dosage | : | 1.0 µg/mL. |



GREEN ALGA GROWTH





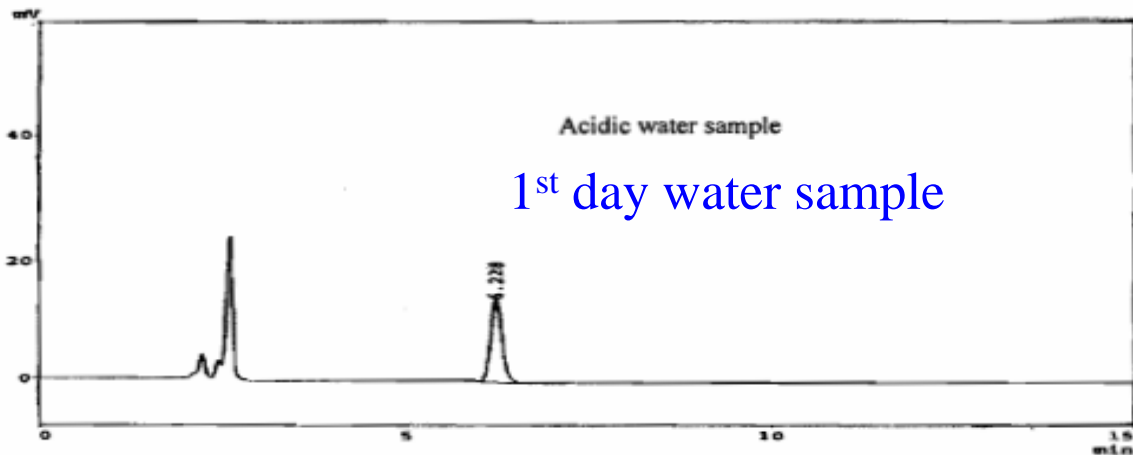
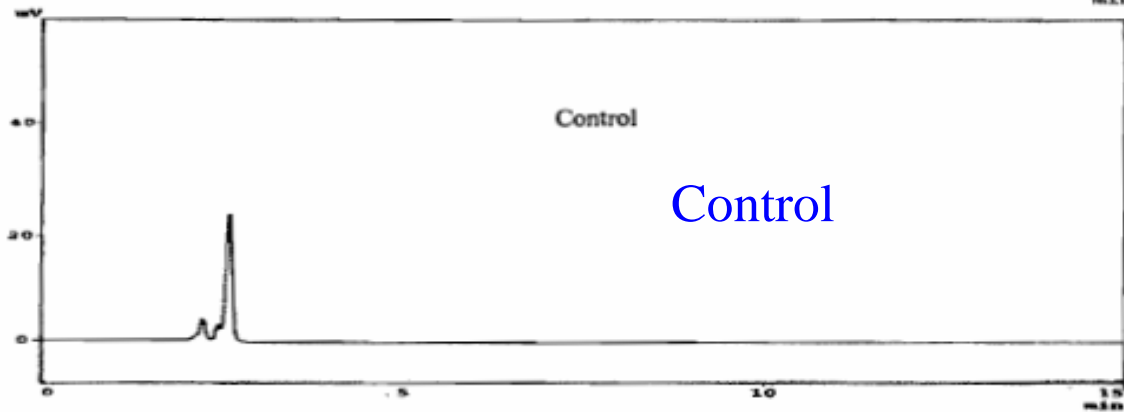
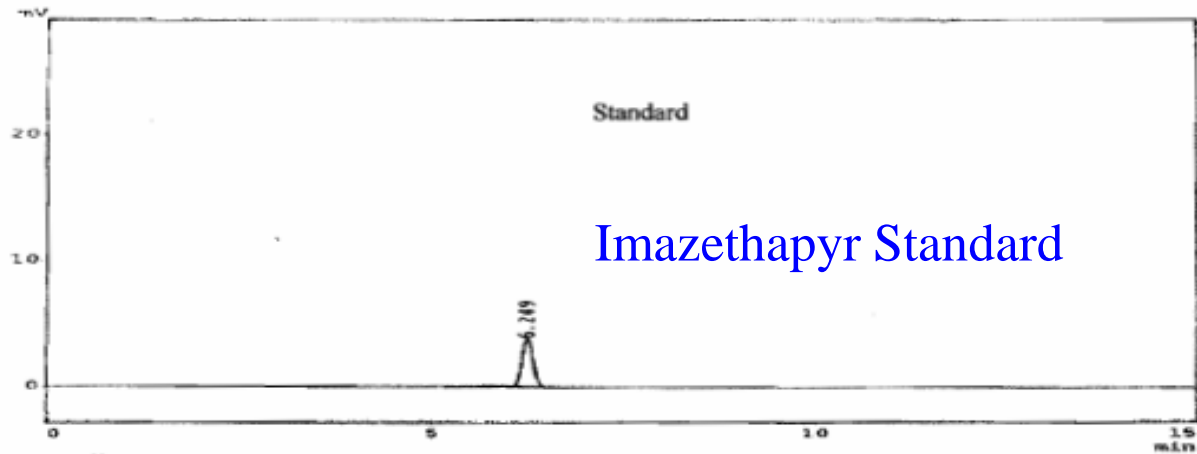
RESULTS

- ∞ Linearity : 2.0 - 0.001 $\mu\text{g/mL}$.
- ∞ Limit of Detection (LOD) : 0.001 $\mu\text{g/mL}$
- ∞ Recovery : 90% in Milli-Q water,
91% in acidic,
89% in neutral
88% in basic buffer
- ∞ Limit of Quantification (LOQ): 0.01 $\mu\text{g/L}$.

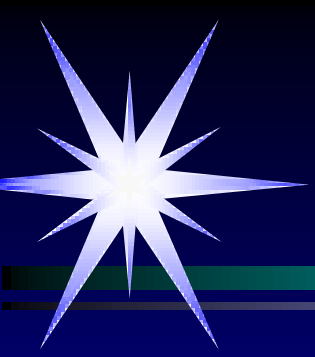
DT₅₀ AND DT₉₀ - UNDER DIRECT SUNLIGHT AND IN DARK



| Pesticide | DT ₅₀ and DT ₉₀ in days (Under Direct Sunlight) | | | | | | | |
|-------------|---|------------------|------------------|------------------|------------------|------------------|------------------|------------------|
| | Milli Q water | | pH 5.0 | | pH 7.0 | | pH 9.0 | |
| | DT ₅₀ | DT ₉₀ | DT ₅₀ | DT ₉₀ | DT ₅₀ | DT ₉₀ | DT ₅₀ | DT ₉₀ |
| | 2.1 | 6.9 | 2.2 | 7.2 | 2.4 | 8.0 | 2.6 | 8.6 |
| Imazethapyr | DT ₅₀ and DT ₉₀ in days (Under Dark) | | | | | | | |
| | Milli Q water | | pH 5.0 | | pH 7.0 | | pH 9.0 | |
| | DT ₅₀ | DT ₉₀ | DT ₅₀ | DT ₉₀ | DT ₅₀ | DT ₉₀ | DT ₅₀ | DT ₉₀ |
| | 21.5 | 71.4 | 22.7 | 75.4 | 24.5 | 81.2 | 26.4 | 87.8 |



CHROMATOGRAM S - IMAZETHAPYR



EFFECTS OF CATIONS AND ANIONS

- Anions (SO_4^{2-} , Cl^- , ClO_4^- , CO_3^- and HCO_3^-) positively enhanced the degradation of residues.
- Presence of iron, copper and zinc enhanced the degradation rate of Imazethapyr.



DISSIPATION KINETICS

| Rate constant of Imazethapyr under direct sunlight in two spiked concentrations | Rate constant values for hydrolysis |
|---|-------------------------------------|
| 0.489, 0.464 in Milli-Q water | 0.312, 0.340 in Milli-Q |
| 0.545, 0.523 in acidic water | 0.287, 0.272 in acidic water |
| 0.599, 0.596 in neutral water | 0.322, 0.349 in neutral water |
| 0.588, 0.589 in basic water | 0.362, 0.345 in basic water |

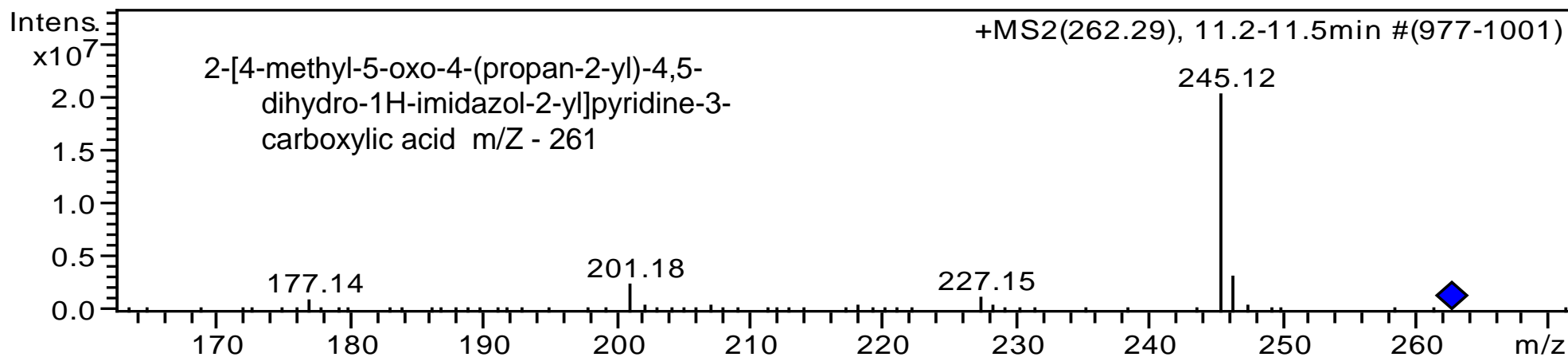
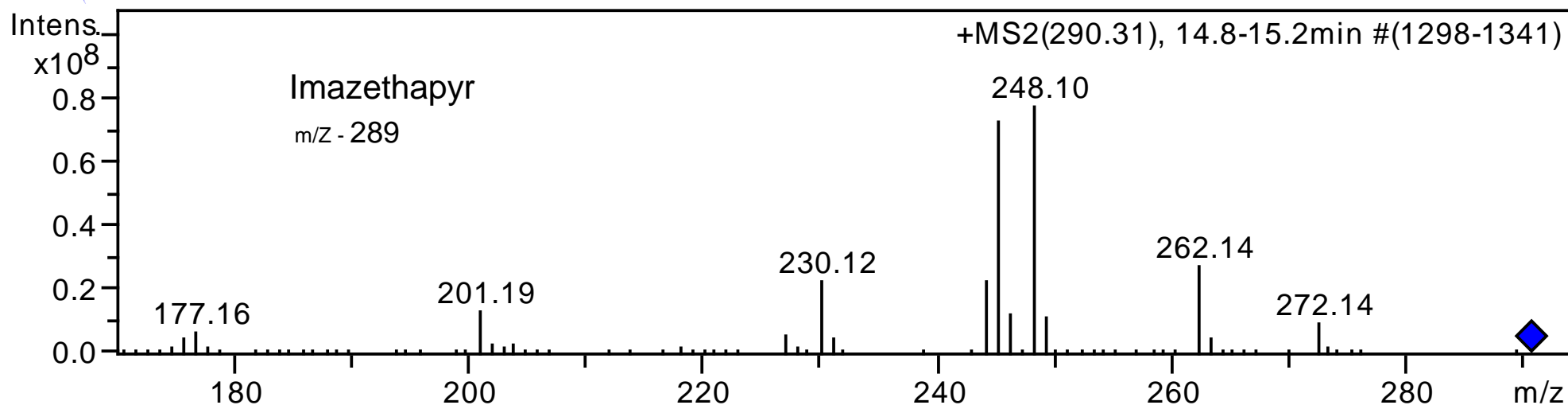
Clearly indicates that sunlight enhanced the degradation of residues when compared to analysis in dark



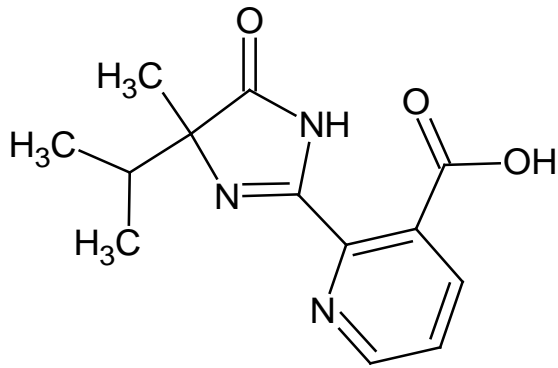
CONFIRMATION OF RESIDUES BY LC-ESI-MS/MS - IMAZETHAPYR

| Name | Molecular mass (m/z) | Fragment ions (m/z) |
|---|----------------------|---------------------|
| Imazethapyr | 289 | 262, 248, 177 |
| 2-[4-methyl-5-oxo-4-(propan-2-yl)-4,5-dihydro-1H-imidazol-2-yl]pyridine-3-carboxylic acid | 261 | 245, 227, 201 |

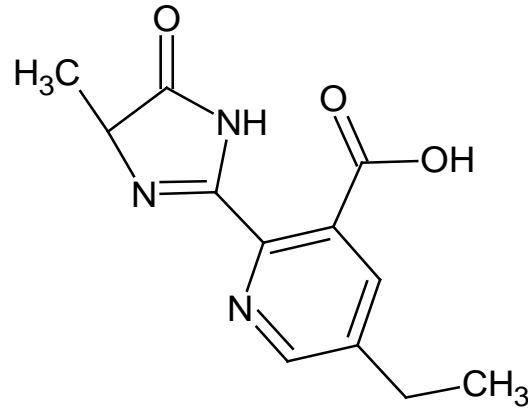
CONFIRMATION OF RESIDUES BY LC-ESI-MS/MS



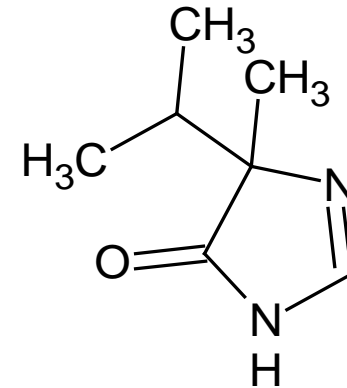
DEGRADATION PATHWAY OF IMAZETHAPYR



2-[4-methyl-5-oxo-4-(propan-2-yl)-4,5-dihydro-1H-imidazol-2-yl]pyridine-3-carboxylic acid
m/Z - 261

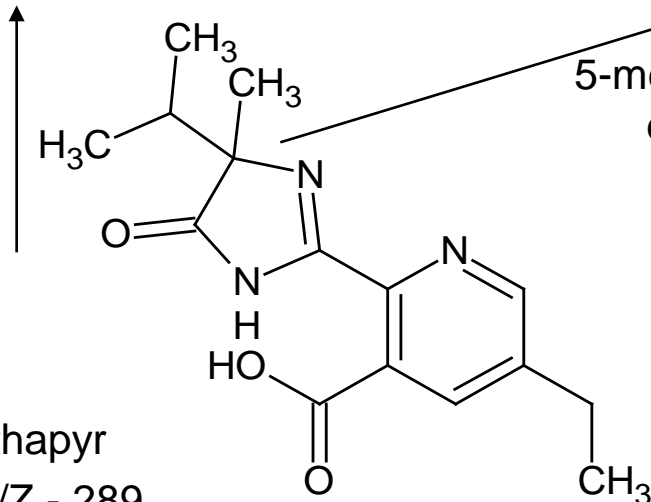


5-ethyl-2-(4-methyl-5-oxo-4,5-dihydro-1H-imidazol-2-yl)pyridine-3-carboxylic acid
m/Z - 247

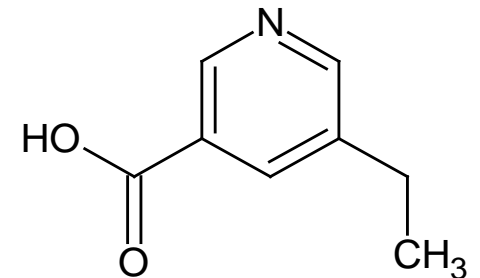


5-methyl-5-(propan-2-yl)-3,5-dihydro-4H-imidazol-4-one
m/Z - 140

Imazethapyr
m/Z - 289



5-ethylpyridine-3-carboxylic acid
m/Z - 151



IMPACT OF IMAZETHAPYR ON GROWTH OF GREEN ALGA

| Sampling Occasions (Days) | Sample Description | Cell count - No. of cells / mL (x 10 ⁴ cells/mL) | | | | | | |
|--------------------------------|--------------------|--|----------------|----------------|----------------|----------------|----------------|------|
| | | R ₁ | R ₂ | R ₃ | R ₄ | R ₅ | R ₆ | Mean |
| 0 (Before exposed to sunlight) | Control | 151 | 158 | 168 | 161 | 160 | 155 | 159 |
| | Treatment | 29 | 23 | 23 | - | - | - | 25 |
| 15 | Control | 65 | 52 | 53 | 51 | 57 | 62 | 57 |
| | Treatment | 26 | 17 | 17 | - | - | - | 20 |
| 25 | Control | 58 | 68 | 49 | 60 | 51 | 59 | 58 |
| | Treatment | 50 | 58 | 37 | - | - | - | 48 |
| 40 | Control | 59 | 64 | 48 | 60 | 58 | 53 | 57 |
| | Treatment | 59 | 61 | 55 | - | - | - | 58 |



IMPACT OF IMAZETHAPYR ON GROWTH OF GREEN ALGA

♣ Before sunlight exposure - 84% inhibition at 1.0 $\mu\text{g}/\text{mL}$ concentration level.

♣ 15th day - 65% inhibition

♣ 25th day - 16%.

♣ The inhibition may be due to the presence of persistent metabolites/breakdown products of Imazethapyr in water samples, which was confirmed by the LC-MS/MS analysis.

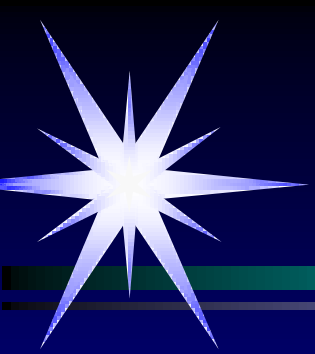
♣ The breakdown product identified on this occasion was 2-[4-methyl-5-oxo-4-(propan-2-yl)-4,5-dihydro-1H-imidazol-2-yl]pyridine-3-carboxylic acid has the molecular ion peak at $m/Z - 262$.

♣ Analysis of 40th day samples showed no sign of inhibition in the growth of green alga and the growth was on par with the control.



CONCLUSIONS

- Under direct sunlight, the photolysis of Imazethapyr in water was very rapid when compared
- The dissipation of Imazethapyr in water followed first order kinetics.
- Metabolite of Imazethapyr 2-[4-methyl-5-oxo-4-(propan-2-yl)-4,5-dihydro-1H-imidazol-2-yl]pyridine-3-carboxylic acid was identified using the LC-MS/MS-ESI.
- The rate of degradation was influenced due to presence of cations and anions.
- The degradation of Imazethapyr was rapid while aerating the water.



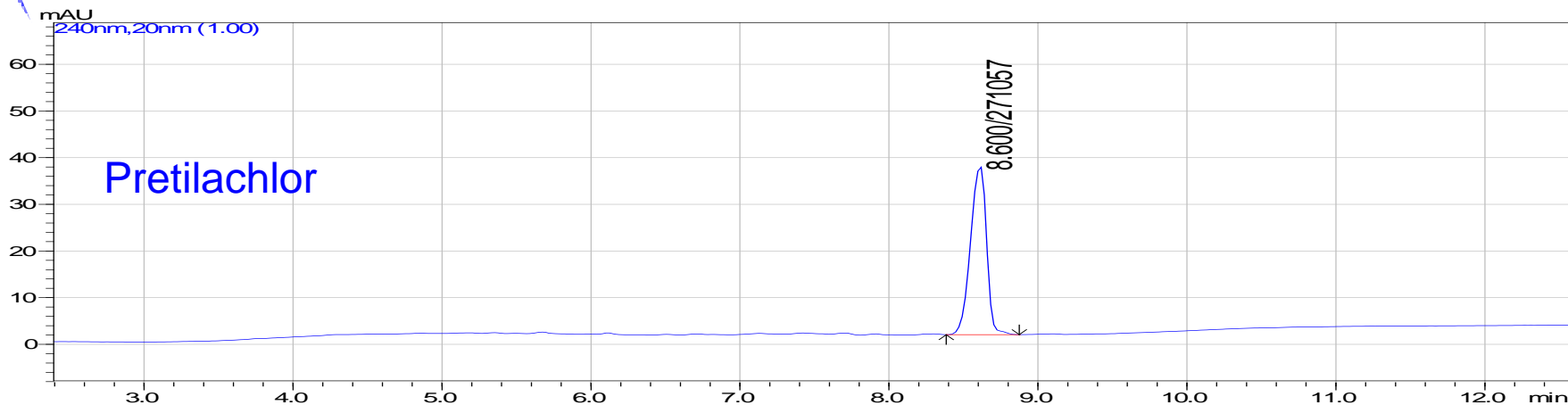
Investigations on the Photolysis of Pretilachlor



EXPERIMENTAL PROCEDURE

- *Lemna gibba* exposed to the paddy water at different occasions (both treatment and control) with known number of lemna fronds to calculate the percentage of inhibition of growth rate and yield till harvesting.
- Analysis : Residue analysis and lemna toxicity
- Sampling occasions : 0, 5, 10, 15, 30, 50, 70 and 90 days.

REPRESENTATIVE HPLC CHROMATOGRAM



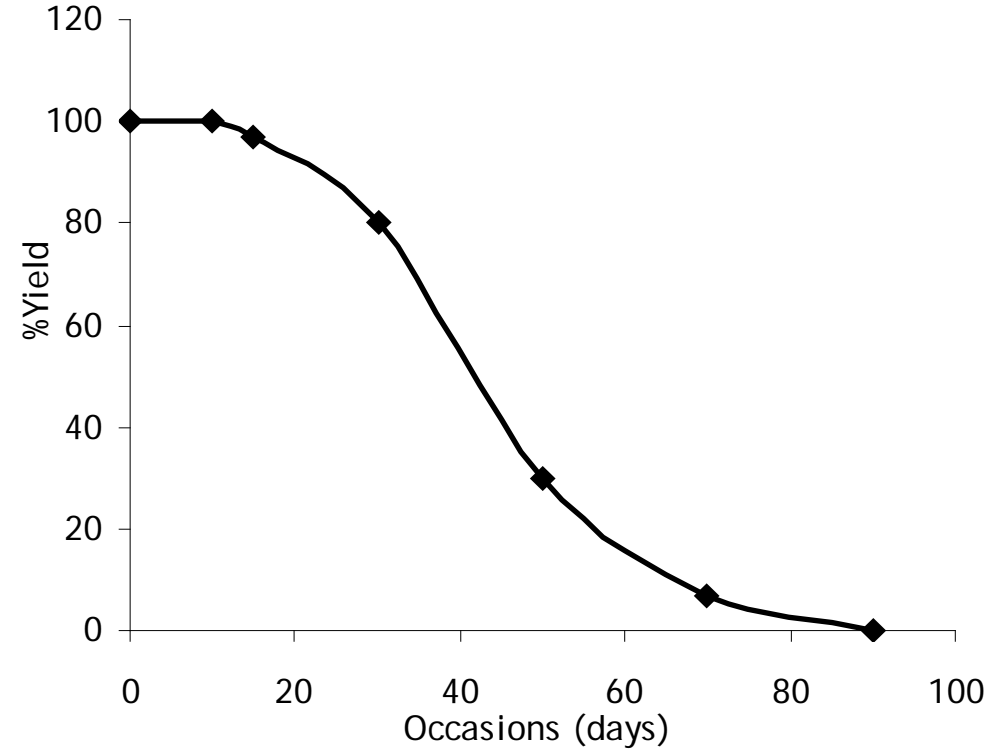
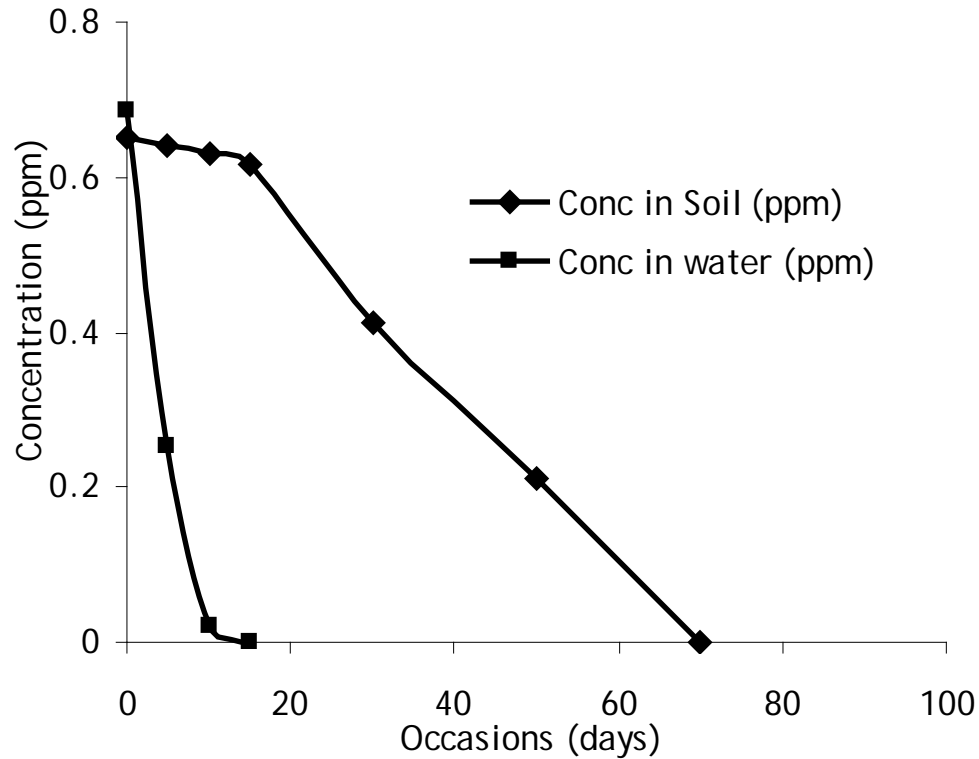
HPLC Condition

Chromatorgraph : Agilent 1200 series HPLC
Column : 150 x 2.1 mm i.d, 5.0 micron Zorbax SB-C18
Detector : UV detection, 210 nm
Injection Volume : 20 μ L
Flow Rate : 1 mL min⁻¹
Mobile phase : Acetonitrile:H₂O (90:10, v/v)

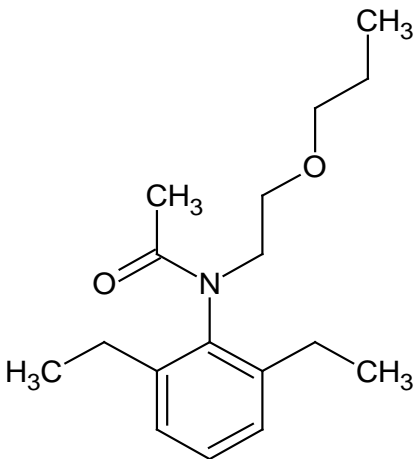
LC-ESI-MS/MS FRAGMENTATION IONS OF PRETILACHLOR AND ITS BREAKDOWN PRODUCTS

| Compound name | Molecular mass | Molecular ion | Fragment ions |
|---|----------------|---------------|---------------|
| Pretilachlor | 311 | 312 | 252, 176 |
| 2-acetyl-6-ethyl-N-(propyloxyethyl)acetanilide | 293 | 294 | 234, 176 |
| Metabolite – Hydroxylalachlor | 327 | 328 | 268, 252 |
| Metabolite-2-chloro-1-(9-ethyl-3-hydroxy-2,3,4,5-tetrahydro-1H-1-benzazepin-1-yl)ethanone | 267 | 268 | 220, 137 |
| 2,6-diethyl-N-(propyloxyethyl)aniline | 235 | 236 | 176, 148, 120 |
| 2,6-diethyl-N-(propyloxyethyl)acetanilide | 277 | 278 | 218, 176 |

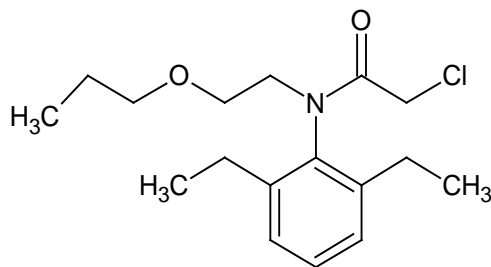
DISSIPATION CURVE OF PRETILACHLOR AND PERCENTAGE YIELD OF *LEMNA GIBBA*



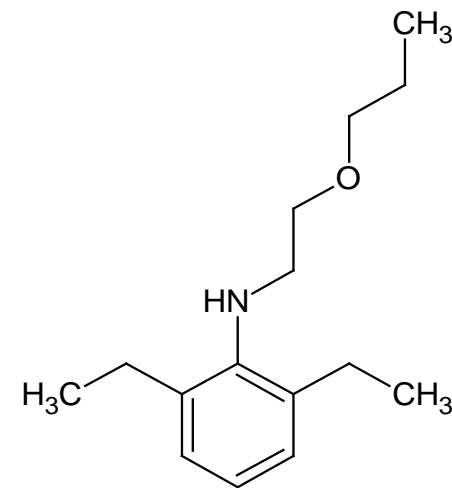
DEGRADATION PATHWAY OF PRETILACHLOR IN WATER



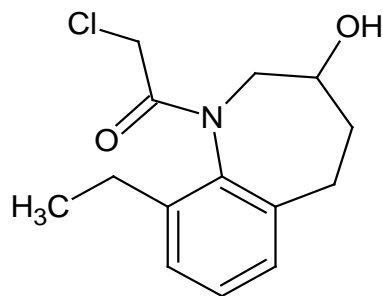
2,6-diethyl-N-(propoxyethyl)acetanilide
m/Z - 277



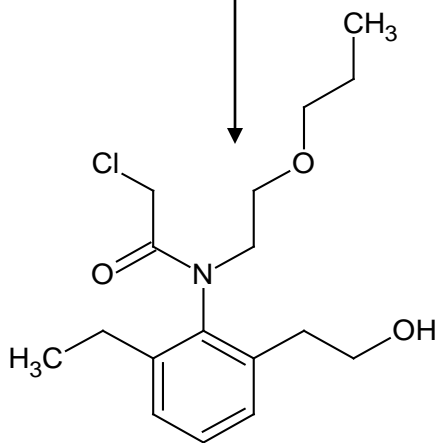
Pretilachlor -
m/Z - 311



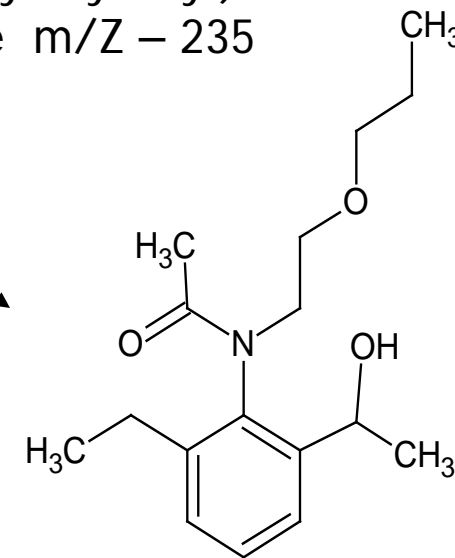
2,6-diethyl-N-(propoxyethyl)aniline
m/Z - 235



Metabolite -2-chloro-1-(9-ethyl-3-hydroxy-2,3,4,5-tetrahydro-1H-1-benzazepin-1-yl)ethanone
m/Z - 267

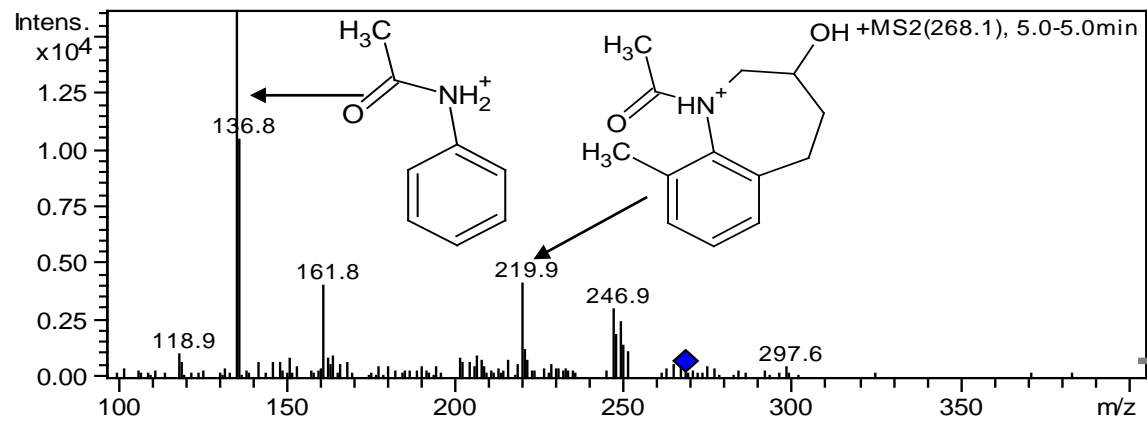
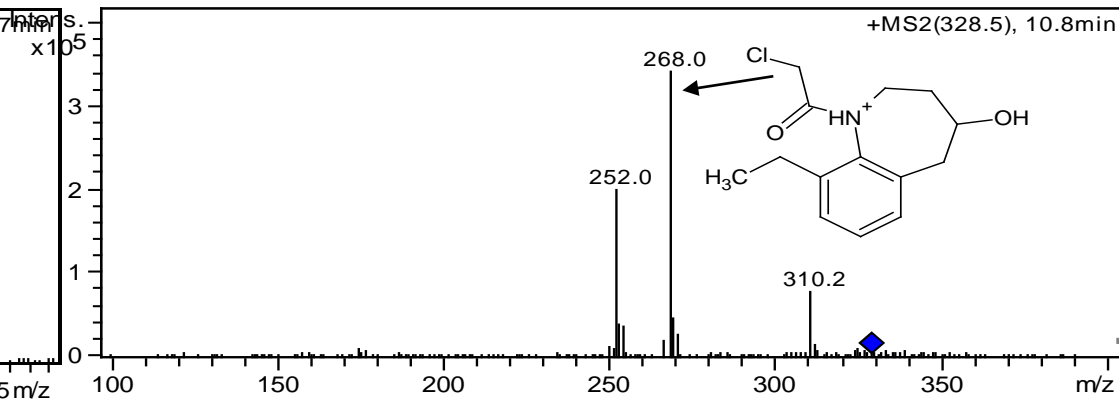
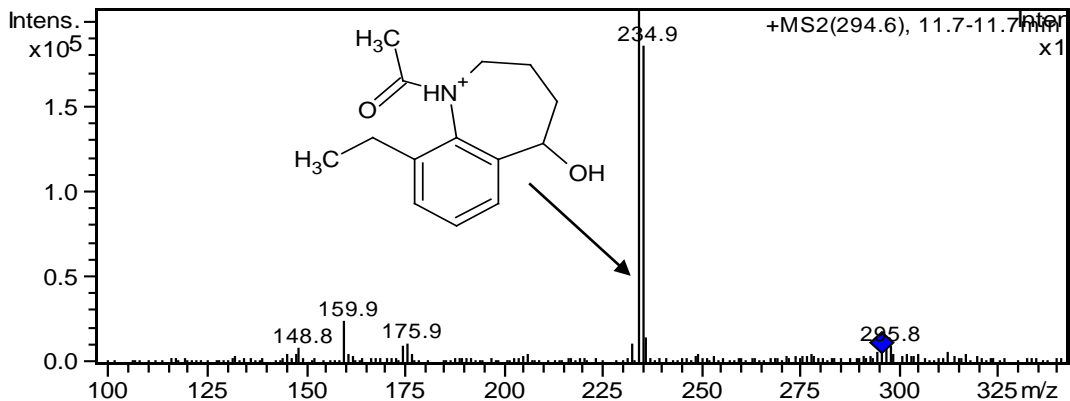
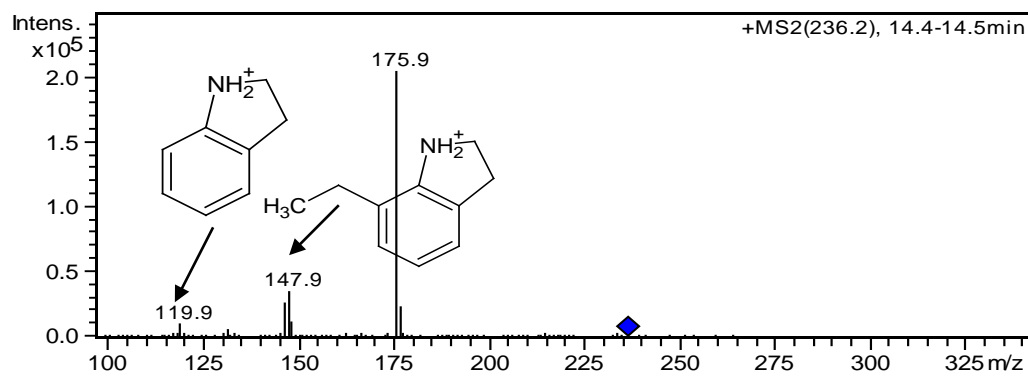
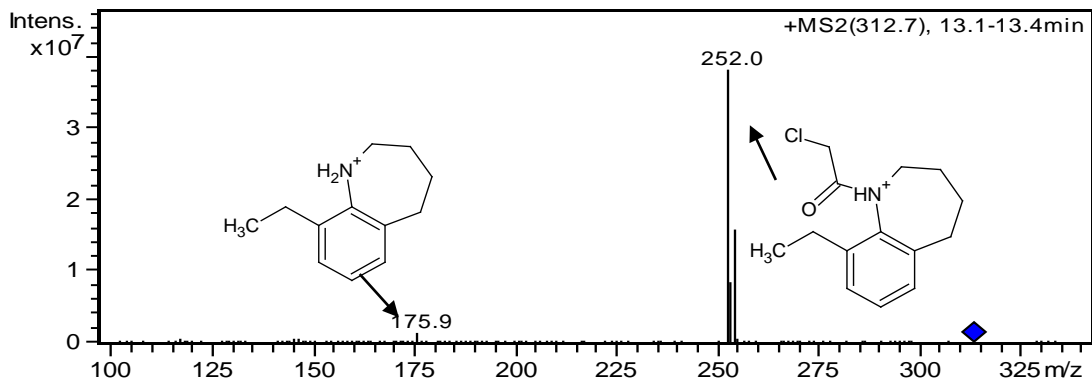


Hydroxylalachlor m/Z - 327



2-acetyl-6-ethyl-N-(propoxyethyl)acetanilide
m/Z - 293

SPECTRA OF PRETILACHLOR AND ITS BREAKDOWN PRODUCTS IN WATER





DISSIPATION DATA OF PRETILACHLOR AND % INHIBITION OF GROWTH RATE AND YIELD OF *LEMNA GIBBA* IN PADDY FIELD CONDITION

| Occasions (days) | 0 | 5 | 10 | 15 | 30 | 50 | 70 | 90 | DT ₅₀ |
|-----------------------------------|-------|-------|-------|-----|----|----|----|----|------------------|
| Conc (ppm) in water | 0.685 | 0.253 | 0.021 | BDL | - | - | - | - | 2.0 |
| Percentage inhibition yield | 100 | 100 | 100 | 97 | 80 | 30 | 7 | 0 | - |



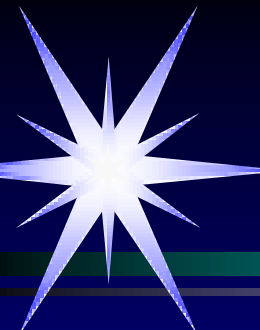
EVALUATION OF PERCENTAGE OF INHIBITION ON GROWTH AND YIELD OF *LEMNA GIBBA*

- Plants of the *Lemna gibba* were allowed to grow as monocultures in recommended test dosage over a period of seven days intervals till harvesting.
- The substance-related effect on vegetative growth over this period was evaluated based on assessments of frond number.
- To quantify substance-related effects, growth in the test solutions is compared with controls and the concentration bringing about a specified x % inhibition of growth and y % inhibition of yield based on frond numbers.
- Toxicity index values - concentration of the Herbicides in the sample occasionally divided by the EC50 or LC50 of an aquatic organism.



CONCLUSIONS

- The huge difference in dissipation of pretilachlor in water under direct sunlight and the DT_{50} value was 2.0. The value indicated that pretilachlor disappeared rapidly in water due to strong soil adsorption.
- Residues of Pretilachlor get dissipated to below detectable level on 15th day in water and at this occasion the growth inhibition was 97% due to the presence of the primary degradation products of pretilachlor in paddy water.
- The toxicity index (TI) value on 15th day sample (> 1.0) indicated that highly toxicity to *Lemna gibba*.
- The metabolite concentrations of degradable paddy pesticides were higher than the concentrations of the parent compounds (Iwafune et.al 2010).



Conclusions

- The residues are likely to occur either in the form of a metabolite/breakdown product in the environment – Their presence and absence is a critical task and is limited to analytical sensitivity
- Plant Biomarks can be used as an indicators of these environmental pollutants